Epidemiol. Infect. (2011), **139**, 1587–1593. © Cambridge University Press 2011
doi:10.1017/S0950268811000173

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

F. AHMADNEJAD[1,2]*, V. OTAROD[3], M. H. FALLAH[4], S. LOWENSKI[5], R. SEDIGHI-MOGHADDAM[6], A. ZAVAREH[1], B. DURAND[5], S. LECOLLINET[5] AND P. SABATIER[2]

[1] *Viral Vaccines Production Department, Pasteur Institute, Tehran, Iran*
[2] *TIMC-IMAG Team EPSP, VetAgroSup, Campus Vétérinaire de Lyon, France*
[3] *Directorate Investigation Control of Animal Diseases, Iran Veterinary Organization, Tehran, Iran*
[4] *Engineering Research Institute, Tehran, Iran*
[5] *ANSES Laboratoire de Santé Animale, ANSES, Maisons-Alfort, France*
[6] *Central Veterinary Laboratory, Iran Veterinary Organization, Tehran, Iran*

(*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].

* Author for correspondence: Miss F. Ahmadnejad, TIMC-IMAG Vet Agro Sup, 1 avenue Bourgelat, 69280 Marcy l'Etoile, Lyon, France.
(Email: f_ahmadnejad@yahoo.com)

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

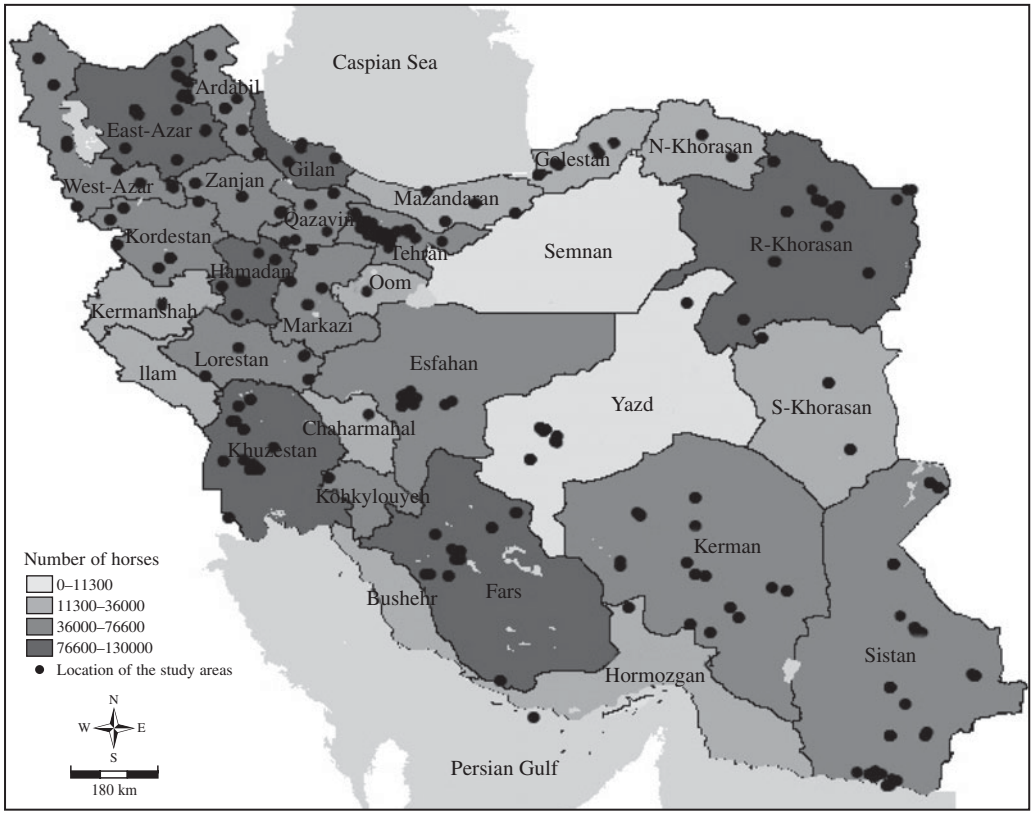

Fig. 1. Geographic distribution of equine population by province and geographic location of collected samples in 27 out of 30 provinces, Iran, 2008–2009.

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

https://doi.org/10.1017/S0950268811000173 Published online by Cambridge University Press

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:

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

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

PRNT

$PRNT_{90}$ was performed to confirm the presence of WNV-specific antibodies and was derived from the OIE $PRNT_{90}$ 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 ($PRNT_{90}$).

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 (ESRI1, 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

https://doi.org/10.1017/S0950268811000173 Published online by Cambridge University Press

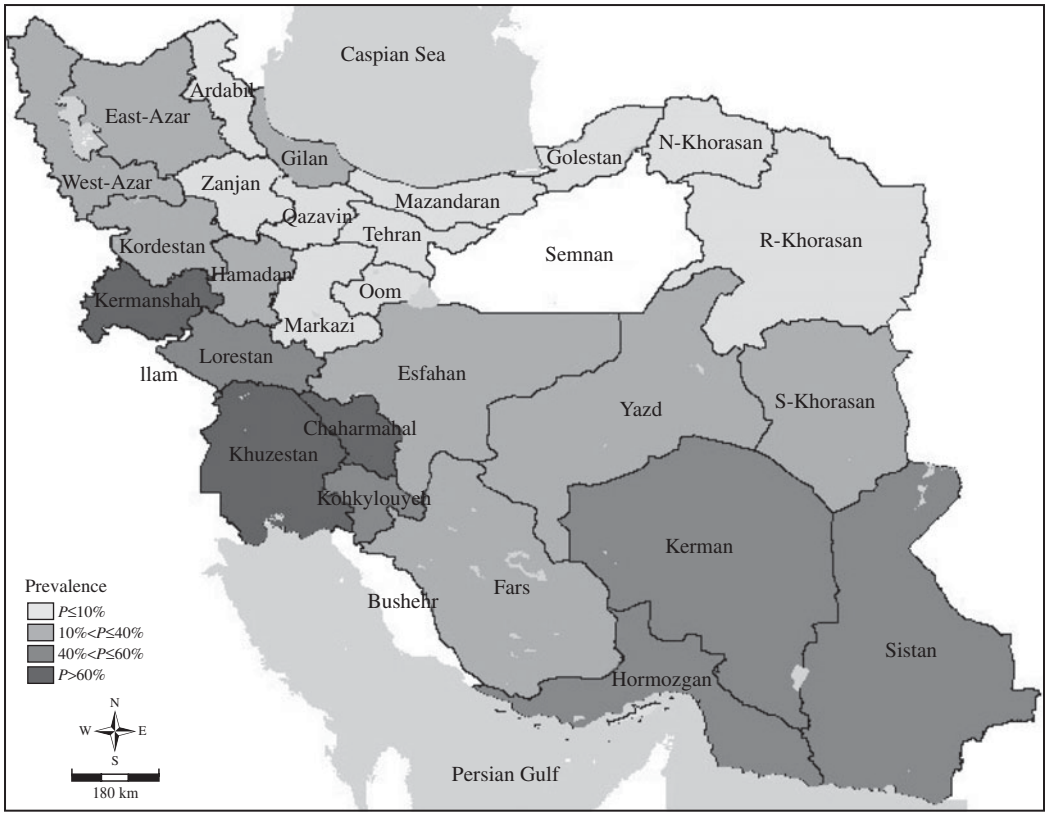

Fig. 2. Anti-WNV seroprevalence determined by plaque reduction neutralization test in equines in 27 out of 30 provinces, Iran, 2008–2009.

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 $\geqslant 30\%$, five provinces a prevalence $\geqslant 50\%$, and two provinces a prevalence $\geqslant 80\%$. 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

https://doi.org/10.1017/S0950268811000173 Published online by Cambridge University Press

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	Δ = 0·5 degree	0·37 (0·17–0·80)	0·005*, 0·02†
Easting	Δ = 0·5 degree	0·54 (0·32–0·90)	0·008*, 0·001†
Northing–Easting	Δ = 0·5 degree	1·22 (1·06–1·39)	0·02
Elevation	Δ = 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	Δ = 5 years	1·34 (1·04–1·71)	0·01
Group size	Δ = 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

https://doi.org/10.1017/S0950268811000173 Published online by Cambridge University Press

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.

REFERENCES

1. **Gubler DJ, Kuno G, Markoff L.** Flaviviruses. In: Knipe DM, *et al.*, eds. *Fields Virology*. Philidelphia, PA: Lippincott Williams & Wilkins, 2007, pp. 1154–1252.
2. **Murgue B, *et al.*** West Nile in the Mediterranean basin: 1950–2000. *Annals of the New York Academy of Sciences* 2001; **951**: 117–126.
3. **Trock SC, *et al.*** West Nile virus outbreak among horses in New York State, 1999 and 2000. *Emerging Infectious Diseases* 2001; **7**: 745–747.
4. **Lanciotti RS, *et al.*** Complete genome sequences and phylogenetic analysis of West Nile virus strains isolated from the United States, Europe, and the Middle East. *Virology* 2002; **298**: 96–105.
5. **Bondre VP, *et al.*** West Nile virus isolates from India: evidence for a distinct genetic lineage. *Journal of General Virology* 2007; **88**: 875–884.
6. **Bernkopf H, Levine S, Nerson R.** Isolation of West Nile virus in Israel. *Journal of Infectious Diseases* 1953; **93**: 207–218.
7. **Melnick JL, *et al.*** Isolation from human sera in Egypt of a virus apparently identical to West Nile virus. *Proceedings of the Society for Experimental Biology and Medicine* 1951; **77**: 661–665.
8. **Work TH, Hurlbut HS, Taylor RM.** Indigenous wild birds of the Nile Delta as potential West Nile virus circulating reservoirs. *American Journal of Tropical Medicine and Hygiene* 1955; **4**: 872–888.
9. **Dauphin G, *et al.*** West Nile: worldwide current situation in animals and humans. *Comparative Immunology, Microbiology and Infectious Diseases* 2004; **27**: 343–355.
10. **Zeller HG, Schuffenecker I.** West Nile virus: an overview of its spread in Europe and the Mediterranean basin in contrast to its spread in the Americas. *European Journal of Clinical Microbiology & Infectious Diseases* 2004; **23**: 147–156.
11. **Pollock CG.** West Nile virus in the Americas. *Journal of Avian Medicine and Surgery* 2008; **22**: 151–157.
12. **Charrel RN, *et al.*** Evolutionary relationship between Old World West Nile virus strains. Evidence for viral gene flow between Africa, the Middle East, and Europe. *Virology* 2003; **315**: 381–388.
13. **Naficy K, Saidi S.** Serological survey on viral antibodies in Iran. *Tropical and Geographical Medicine* 1970; **22**: 183–188.
14. **Sharifi Z, Mahmoodian Shooshtari M, Talebian A.** A study of West Nile virus infection in Iranian blood donors. *Archives of Iranian Medicine* 2010; **13**: 1–4.
15. **World Organization for Animal Health (OIE).** Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees) (http://www.oie.int/eng/normes/mmanual/2008/pdf/2.01.20_WEST_NILE.pdf) 2008. **2**: 377–385.
16. **Murgue B, *et al.*** West Nile outbreak in horses in southern France, 2000: the return after 35 years. *Emerging Infectious Diseases* 2001; **7**: 692–696.
17. **Lucas M, *et al.*** The Israeli strain IS-98-ST1 of West Nile virus as viral model for West Nile encephalitis in the Old World. *Virology Journal* 2004; **1**: 9.
18. **R Development Core Team.** *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, 2009.

19. **Rubin DB.** *Multiple Imputation for Nonresponse in Surveys*. New York: John Wiley and Sons, 1987.
20. **Horton NJ, Kleinman KP.** Much ado about nothing: a comparison of missing data methods and software to fit incomplete data regression models. *American Statistician* 2007; **61**: 79–90.
21. **Stone M.** Cross-validatory choice and assessment of statistical predictions. *Journal of the Royal Statistical Society. Series B, Statistical Methodology* 1974; **36**: 111–147.
22. **Chevalier V, *et al.*** Serological assessment of West Nile fever virus activity in the pastoral system of Ferlo, Senegal. *Annals of the New York Academy of Sciences* 2006; **1081**: 216–225.
23. **Cohen D, *et al.*** Prevalence of antibodies to West Nile fever, sandfly fever Sicilian, and sandfly fever Naples viruses in healthy adults in Israel. *Public Health Reviews* 1999; **27**: 217–230.
24. **Castillo-Olivares J, Wood J.** West Nile virus infection of horses. *Veterinary Research* 2004; **35**: 467–483.
25. **Bunning ML, *et al.*** Experimental infection of horses with West Nile virus. *Emerging Infectious Diseases* 2002; **8**: 380–386.
26. **Tsai TF, *et al.*** West Nile encephalitis epidemic in southeastern Romania. *Lancet* 1998; **352**: 767–771.
27. **Komar N.** West Nile virus: epidemiology and ecology in North America. *Advances in Virus Research* 2005; **61**: 185–234.
28. **Balenghien T, *et al.*** Horse-, bird-, and human-seeking behavior and seasonal abundance of mosquitoes in a West Nile virus focus of southern France. *Journal of Medical Entomology* 2006; **43**: 936–946.
29. **Jourdain E, *et al.*** Bird migration routes and risk for pathogen dispersion into western Mediterranean wetlands. *Emerging Infectious Diseases* 2007; **13**: 365–372.
30. **Hubalek Z.** An annotated checklist of pathogenic microorganisms associated with migratory birds. *Journal of Wildlife Diseases* 2004; **40**: 639–659.
31. **Reed KD, *et al.*** Birds, migration and emerging zoonoses: West Nile virus, Lyme disease, influenza A and enteropathogens. *Clinical Medicine & Research* 2003; **1**: 5–12.
32. **Briese T, *et al.*** Phylogenetic analysis of a human isolate from the 2000 Israel West Nile virus epidemic. *Emerging Infectious Diseases* 2002; **8**: 528–531.