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Summary

The circulation of West Nile virus (WNV) in horses has been investigated in the South West Indian Ocean. In 2010, a total of 311 horses originating from Madagascar, Mauritius, Reunion and Seychelles have been blood sampled and tested for WNV specific antibodies. An overall seroprevalence of 27.0% was detected with the highest WNV antibody prevalence of 46.3% (95% CI: 37.4-55.2%) detected in Madagascar. Age and origin of the horses were found to be associated with WNV infection risk. This paper presents the first seroprevalence study investigating WN fever in horses in the South West Indian Ocean area and indicates a potential risk of infection to humans and animals. In order to better understand WN transmission cycles, WNV surveillance need to be implemented in each of the countries.

Keywords: West Nile Virus, Horses, antibody prevalence, Indian Ocean

1. Introduction

Several flaviviruses such as West Nile virus (WNV), tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV) are considered as emerging diseases threatening humans and/or animals (Hubalek & Halouzka, 1999). WN is one of the two major encephalitic viruses in horses and humans belonging to the *Flaviriridae* family within the Japanese encephalitis serocomplex along with JEV (Gould, 2002).

WNV was firstly isolated in 1937, from a febrile woman in Omogo in the West Nile district of Uganda during an epidemiological study defining the endemic zone of yellow fever (2). Up to the mid-1990s, the dispersal of human WNV infections in Europe was mainly associated with sporadic cases. Serious outbreaks with neurological symptoms were reported in 1994 in Algeria and, in 1996 in Romania (Reiter, 2010). The introduction of WNV in the New York district of the USA in 1999, disseminating outwards in a wave across the entire country over subsequent years was an historical epidemiological event (Lanciotti et al., 1999). Therefore WNV is now considered as an emerging pathogen, with the most widespread geographical distribution among flaviviruses ever described, Africa, India, Europe, Asia and America (David & Abraham, 2016).

As far as the Indian Ocean area is concerned, an important WN fever epidemic was reported in South Africa in 1974 (3). Since, the virus has been shown to circulate in several East-African and Asian countries (Kenya, South Africa, Madagascar, Thailand, India) (4), although no human epidemics have been ever reported.

This virus was isolated several times in Madagascar mainly from birds in close contacts with humans such as parrots (5). Previous studies starting from 1975 in Madagascar showed evidence of past WN infections leading to an endemic circulation of WN (6,7). In Reunion Island, a seroprevalence study suggested that WNV might have been circulated a few decades ago (8), but no human case has been confirmed until 2011 (9). In Mauritius, a serological

survey aiming to hepatitis C virus (HCV), dengue virus (DEN), West Nile virus (WN), and sindbis virus (SIN) specific antibodies detection was carried out in 1993. Evidence of WNV specific antibodies was stated (10). No scientific information about the situation of WNV in Seychelles has been published.

WNV has a natural transmission cycle involving mosquitoes, particularly Culex spp. and birds (wild and captive).

While over 60 species of mosquitoes have been involved in the transmission of WN, the key species most likely ornithophilic that are considered as vector competent and involved in the bird-to-mosquito-to-bird-transmission are those belonging to the genus *Culex* (11). However, the abundance of mosquitoes closely associated to urban environments, the presence of immature stages in aquatic habitats within human-made structures, the close contact of mosquitoes with human beings result in an increased risk of mosquitoes acting as epizootic vectors (ie, bird-to-mosquito-to human transmission). A shift to improve water storage conditions in urban areas (ie, the construction of waste water systems and wildlife conservation wetlands) may further influence WN infections risk, with changes to vector and reservoir host populations (12-14). Wild birds are known to be the main reservoir hosts in endemic areas since they develop transient high-titer viraemia allowing transmission of the virus to feeding female mosquitoes (15). Humans and horses having low-titer viraemia are considered as incidental dead-end hosts (16). In humans, the virus can be transmitted via blood transfusions and organ transplants, congenital infection, bite of an infected mosquito, direct bird to human contact, as laboratory acquired infections and from mother-to-child through intrauterine transmission or breast-feeding (17-20).

Horses do not exhibit any pathognomonic signs of WN fever. In South Africa, Venter et al. (21) communicated on a transplacental transmission of WNV in a mare. Depending on geographic location and season, several pathogens can cause similar clinical signs. Therefore,

laboratory based diagnostic techniques such as the detection of the viral genome by real-time RT-PCR (Reverse –Transcriptase- Polymerase Chain Reaction) or the detection of WNV specific IgM or IgG antibodies by ELISA are useful to detect any acute WN infections in equids. The detection of a high level of IgM antibodies is the best serological indicator to conclude for recent infections, as in horses their lifespan is considered to be less than three months (22) in contrast to humans where IgM antibodies may persist longer (23,24). IgM antibodies may also be detected in vaccinated horses as well as IgG antibodies as a consequence of an earlier subclinical WNV infection (25).

In the South West Indian Ocean (SWIO) area, no data on the circulation of WNV in horses were available until 2010. We therefore investigated whether the Indian Ocean horse populations could be involved in the WN epidemiological cycle as healthy/asymptomatic hosts. To our knowledge, this paper presents the first seroprevalence study of WNV specific antibodies conducted in horses in the SWIO.

2. Material and methods

2.1. Ethic statement

This study was approved by the Animal Care and Use Research Ethics Committee from the Malagasy livestock ministry (number 2010/WN/MinEl/3). Horses were randomly chosen after agreement of their owners. Collaborations were established with state veterinarians in affiliations with their respective Ministries of Agriculture.

2.2. Study area, design and sample collection

A total of 311 horses originating from four different islands of the SWIO area: Madagascar, Mauritius, Reunion and Seychelles have been blood-sampled in 2010 (Figure 1, Table 1). In Madagascar, a total of 121 horses originating from the 19 existing horse stables of Antananarivo (Capital city) out of a country estimated population of 300 were sampled. In

Mauritius, the 77 sampled horses out of a country population of 150 originated either from the Mauritius Turf Club or from the two main horse stables of the island. In Reunion, the 104 sampled horses out of a horse global population of 300 originated from the 13 existing horse stables. In Seychelles, since the horse population is very low, the entire horse population consisting of 9 horses located at la Digue has been sampled (attention, il y a des chevaux à Mahé et à Praslin je crois). Each blood sample was linked to a questionnaire in order to identify the name of the horse/owner, the location of the stable and its origin. Each of the animals was privately owned without any WN infection ever-recorded. Some of the Mauritius and Reunionese horses had records of equine influenza vaccination but none of the horses were vaccinated against WNV.

Blood samples aseptically withdrawn by jugular venipuncture were allowed to clot and were centrifuged, separated and stored at -20°C until analysis.

2.3. Detection of WNV specific antibodies

Sera were screened for the presence of anti-WNV IgG antibodies using the ID Screen® West Nile Competition ELISA kit (IDVet, Grabels, France) that detects WNV anti-Pr-E antibodies according to the manufacturer's instructions. To confirm their status, samples that were detected positive for WNV antibodies by ELISA were tested using plaque reduction neutralization tests (PRNTs) considered as the gold standard method by OIE (Office International des Epizooties) as previously described (Lefrancois *et al.*, 2006). A positive control was included. A serum sample with a titer of 1/10 or higher was considered seropositive. The detection of antibodies against Usutu and Japanese Encephalitis was also tested using the same PRNT.

2.3 Data analysis and statistics

Data originating from the four locations, Madagascar, Mauritius, Reunion and Seychelles were pooled to perform the statistical analysis. Multivariate logistic regression analyses to test the associations between horse characteristics with the seropositivity to WNV as outcome variable were performed. The horse characteristics were: the age (4 categories: 1-5, 6-10, 11-15, more than 15), the sex (male or female) and the origin of the horses (Australia, France, Madagascar, Mauritius, Reunion, Seychelles, South Africa and USA). All statistical analysis were performed using R.3.0.1 ((26), with $\alpha = 0.05$. A seropositive animal was defined as an animal found with a positive IgG ELISA result. The apparent prevalence of WNV by region in the Indian Ocean observed in 2010 was mapped to evaluate the spatial distribution of this prevalence using ArcGIS software (QGIS Development team, 2012) (Figure 1).

3. Results

3.1 Horse population characteristics

The horses were mostly male, 87.4% of the horses were more than 6 years old and only 63.8% of the horses that were sampled in a given country were born in this same country (Table 2).

3.2 WNV specific antibodies detection

The cross-sectional study includes a total of 311 horses. Anti-WNV IgG antibodies were detected in 84 horses (27.0%, 95% CI: 22.1-31.9%). Anti-WNV IgG seropositivity was 46.3% (n=121), 22.1% (n=77), 6.7% (n=104) and 44.4% (n=9) for Madagascar, Mauritius, Reunion and Seychelles, respectively (table 3). The highest seroprevalence was observed in Madagascar whereas the lowest was in Reunion.

The older the horses were (age between 1 to 15 years old), the higher the percentage of WNV antibodies was (table 4).

No antibodies against Usutu and Japanese Encephalitis viruses were detected.

155 3.3 Data Analysis

156 Two out of the three variables tested in the model were significantly correlated: the age
157 and the origin of the horses (Table 5). For example, the Odds Ratio (OR) of being infected
158 by WNV associated with horses aged from 11 to 15 years was 2.98 (95% CI, 1.16-7.70)
159 compared to horses aged from 1 to 5 years old. The OR of being infected by WNV
160 associated with horses originating from South Africa or Madagascar was higher than 2
161 compared to horses originating from France.

162

163 **4. Discussion**

164 West Nile fever was diagnosed in Madagascar in the human population as early as 1990
165 [Morvan et al 1991] with a neuroinvasive fatal case in 2011 [Larrieu et al 2013]. Our study
166 demonstrates that WNV specific antibodies are circulating in horses in SWIO islands with the
167 highest level for Madagascar despite of the absence of apparent clinical signs in the horse
168 population. An overall WNV antibody prevalence of 27.0% was detected in horses of the
169 Indian Ocean area by ELISA tests commonly used for the detection of WNV specific IgG in
170 serosurveillance studies (30, 31). To detect any false positive results because of cross-
171 reactions with other flaviviruses, the positive samples were confirmed by plaque reduction
172 neutralization tests (PRNTs) as recommended by the OIE.

173 WNV specific antibodies prevalence ranged from 6.7% for Reunion to 46.3% for
174 Madagascar. These values seemed to be very low compared to those obtained in horses in
175 Chad (97%) and in Senegal (92%) (28); but in other parts of Africa such as Gabon, the
176 recorded horse WNV antibody prevalence was only of 3% (28). To our knowledge, no recent
177 information on livestock WNV antibody prevalence are available in Madagascar, Mauritius,
178 Reunion or Seychelles, so far only human cases were reported (9,10,29).

179 The highest level of 46.3% of WNV specific antibodies detected in Madagascar was not
180 surprising since several studies already stressed that WNV was endemic in Madagascar
181 (Rousset *et al.* , 2003) confirming an antibody prevalence around 20% in the human
182 population, mainly in and around Antananarivo, the capital city (Morvan et al., 1989). These
183 findings are also supported by the presence of potential WNV competent vectors on the
184 territory such as Culex spp. So far, twenty-six potential mosquito-vector species mainly
185 ornithophilic have been described in Madagascar (Boyer et al., 2013). Several studies have
186 been performed in wild and domestic birds. Fontenille et al. (1989) isolated the WNV from
187 wild birds (parrots and egrets). WNV was also shown to be infecting domestic birds (goose,
188 duck, chicken and wild turkey) in 2009 with 28.7% of WNV antibody prevalence (95%
189 confidence interval [CI] = 21.1–36.3) Madagascar offers all the stakeholders committed in the
190 epidemiological cycle of WNV: WNV competent mosquitoes, wild and domestic birds
191 vectors (Larrieu et al., 2013), and humans and horses "dead-end" incidental hosts (Pradier *et*
192 *al.*, 2012).
193 The expected prevalence of WNV specific antibodies of 22.1 % (95% [CI] = 12.8-31.3) in
194 Mauritius horses can be explained either by the importation of horses from South Africa or by
195 the fact that Mauritius is considered as a transit platform before exportation to Europe.
196 Indeed, WNV is endemic in South Africa at least since 1970 (Angenvoort *et al.*, 2013) with
197 two lineages 1 and 2 being reported and affecting severely horses (Venter & Swanepoel,
198 2010). WNV is maintained in South Africa through an endemic transmission cycle involving
199 several species of wild birds and the predominant ornithophilic mosquito, *Culex (Cx.)*
200 *univittatus* with a wide distribution throughout South Africa with 11% of WNV positive
201 yearlings and up to 75% of their dams being exposed (Guthrie et al. 2003). However, WN
202 clinical signs have only been reported in few horses in the country (Jupp 2001; (Burt *et al.*,
203 2002). Additionally, horses may have been vaccinated prior to their importation by their

owners to ensure protection against WNV infection (Pearce *et al.*, 2013). Finally, anti-WN IgG antibodies were also detected in the Mauritius human population at a low level (2%) in 1994 also suggesting the presence of the virus but to date, no endemic clinical case has been identified. (Zeller et al., 1998);

The unexpected high level of WNV specific antibodies prevalence of 44.4 % (95% [CI] = 12.-76.9) from Seychelles horses could be explained by the low number of sampled horses. Moreover, no scientific information highlighting a WNV circulation neither in humans nor in animals has been gathered (Zeller et al., 1998; Bovet et al., 2013). Two out of the four horses detected positive that have been imported from South Africa may have been responsible for the transmission of WNV among the Seychelles horse population since their introduction in 2000; and that would also explain why the 11-15 years of age horses only were positive.

Finally, even in Reunion where the importation of live animals is forbidden since 2004, several horses have exhibited WNV specific antibodies, suggesting that they have been in contact with the virus long ago, since only the horses older than 11 years of age were found positive. On one hand, sanitary records indicated that some of these horses were imported from South Africa or Mauritius. On the other hand, Kles et al., 1994 demonstrated, in 1987, a WNV antibody prevalence of 24,2% in the Reunion human population, suggesting that the flavivirus may have circulated several decades ago.

Most horses seroconvert after exposure to WNV without any reported clinical disease, similar to what has been described in humans. However Gardner et al., 2007 (Gardner *et al.*, 2007) showed that, in approximately 8% of exposed naive horses, severe WNV disease with neurological symptoms develops. In humans, although the importance of the West Nile virus and its real impact on human health remain unknown, the occurrence of encephalitis cases hospitalized in the Antananarivo hospitals and observed sporadic cases confirmed its

pathogenic virulence leading to the awareness of the medical doctors in the SWIO region (Longchampt et al, 2003; Larrieu et al; 2013).

Finally, these serological data should be considered as evidence of present and past WNV activity in the different islands. Consequently, such results confirm that there is a potential risk of exposure for the human population. In this context, and particularly because of the favorable tropical conditions (temperature and hygrometry) for the development of insect vectors and for the virus survival, a surveillance network including identification of mosquito vectors and vertebrate hosts (mainly bats) involved in WNV transmission to birds, horses, humans, and possibly other mammals living in these islands as well as environmental factors must be set up to ensure potential preventive strategies. These findings raise the need of collaboration between neighboring countries, especially when exposed to similar risks of emergence to limit the risk of spread of infectious diseases.

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Conflict of interests

The authors have no conflict of interests to declare.

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358 **Figure legends**

359

360 Figure 1: Location of the sampling sites and distribution of the samples detected positive for

361 WNV specific antibodies

362 **Tables**

363 Table 1: Study sites and number of sampled horses per site (West Nile, 2010, 311 horses). No

364 stands for Number.

Study location	No. (%) of sampled horses
Madagascar	121 (38.9)
Mauritius	77 (24.8)
Reunion Island	104 (33.4)
Seychelles	9 (2.9)
Total	**311 (100)**

365

366 Table 2: Horse population characteristics (West Nile, 2010, 311 horses). No stands for

367 Number.

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	N=311	
Characteristics	**No of Animals.**	**(%)**
Age group (yrs)		
1-5	38	(12.6)
6-10	94	(31.2)
11-15	90	(29.9)
More than 15	79	(26.2)
Sex		
Male	218	(71.5)
Female	87	(28.5)
Origin		
Born locally	194	(63.8)
Others	110	(36.2)

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375 Table 3: WNV antibody prevalence, Indian Ocean (West Nile, 2010, 311 horses). No stands

376 for Number, CI for Confidence Interval**.**

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Samples origin	**No of seropositive horses**	**WNV antibody prevalence (%)**	**CI (95%)**
Madagascar	56	46.3	(37.4-55.2)
Mauritius	17	22.1	(12.8-31.3)
Reunion	7	6.7	(1.9-11.5)
Seychelles	4	44.4	(12.0-76.9)
Total	**84**	**27.0**	**(22.1-31.9)**

378

379 Table 4 Descriptive statistics of WNV antibody positive samples per study site

	Madagascar		Reunion Island		Mauritius Island		Seychelles Island		Total	
	N=121		N=104		N=77		N=9		N=311	
Variables	**No. Of Pos.**	**(% of Pos.)**	**No. Of Pos.**	**(% of Pos.)**	**No. Of Pos.**	**(% of Pos.)**	**No. Of Pos.**	**(% of Pos.)**	**No. Of Pos.**	**(% of Pos.)**
Age group (yrs)										
1-5	9	(32.1)	—	—	0	(0)	—	—	9	(23.7)
6-10	16	(39.0)	0	(0)	10	(26.3)	0	(0)	26	(27.7)
11-15	14	(58.3)	4	(10.5)	7	(31.8)	4	(66.6)	29	(32.2)
More than 15	14	(58.3)	3	(6.25)	0	(0)	—	—	17	(21.5)
Sex										
Male	39	(45.3)	3	(5.5)	17	(24.6)	4	(44.4)	63	(28.9)
Female	17	(48.5)	4	(9.0)	0	(0)	0	(0)	21	(24.1)
Origin										
Born locally	50	(47.1)	4	(6.77)	0	(0)	2	(28.5)	56	(28.9)
Others	6	(40)	42	(93.3)	17	(30.9)	2	(100)	28	(25.5)

Table 5 Multiple regression model for l risk factors associated with WN antibody prevalence in horses,Indian Ocean. CI stands for Confidence Interval

Variables	Categories	Adjusted odds ratio (95% CI)	*p*-value
Age (yrs)	1-5	Reference	
	6-10	1.44 (0.59-3.58)	0.423
	11-15	**2.98 (1.16-7.70)**	**< 0.05**
	More than 15	1.72 (0.64-4.62)	0.281
Sex	Male	0.85 (0.41-1.73)	0.647
	Female	Reference	
Horse Origin	Australia	0.66 (0.01-42.07)	0.842
	France	Reference	
	Madagascar	**7.92 (2.57-24.42)**	**< 0.05**
	Mauritius	0.37 (0.05-2.71)	0.332
	Reunion	0.47 (0.11-1.94)	0.297
	Seychelles	2.49 (0.37-16.52)	0.346
	South Africa	**3.72 (1.14-12.14)**	**< 0.05**
	USA	0.55 (0.01-29.16)	0.768