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► To cite this version:

E. Cardinale, C. Bernard, S. Lecollinet, V M Rakotoharinome, J. Ravaomanana, et al.. West Nile virus infection in horses, Indian ocean. *Comparative Immunology, Microbiology and Infectious Diseases*, 2017, 53, pp.45 - 49. 10.1016/j.cimid.2017.06.006 . pasteur-01675153

HAL Id: pasteur-01675153

<https://riip.hal.science/pasteur-01675153v1>

Submitted on 28 Feb 2018

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1 **West Nile Virus Infection in Horses, Indian Ocean**

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16

17 **Summary**

18 The circulation of West Nile virus (WNV) in horses has been investigated in the South West
19 Indian Ocean. In 2010, a total of 311 horses originating from Madagascar, Mauritius, Reunion
20 and Seychelles have been blood sampled and tested for WNV specific antibodies. An overall
21 seroprevalence of 27.0% was detected with the highest WNV antibody prevalence of 46.3%
22 (95% CI: 37.4-55.2%) detected in Madagascar. Age and origin of the horses were found to be
23 associated with WNV infection risk. This paper presents the first seroprevalence study
24 investigating WN fever in horses in the South West Indian Ocean area and indicates a
25 potential risk of infection to humans and animals. In order to better understand WN
26 transmission cycles, WNV surveillance need to be implemented in each of the countries.

27

28

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

30 **1. Introduction**

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

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

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

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

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

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

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

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

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

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

93

94 **2. Material and methods**

95 2.1. Ethic statement

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

100 2.2. Study area, design and sample collection

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

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

115
116 Blood samples aseptically withdrawn by jugular venipuncture were allowed to clot and were
117 centrifuged, separated and stored at -20°C until analysis.

118

119 2.3. Detection of WNV specific antibodies

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

129 2.3 Data analysis and statistics

130 Data originating from the four locations, Madagascar, Mauritius, Reunion and Seychelles
131 were pooled to perform the statistical analysis. Multivariate logistic regression analyses to test
132 the associations between horse characteristics with the seropositivity to WNV as outcome
133 variable were performed. The horse characteristics were: the age (4 categories: 1-5, 6-10, 11-
134 15, more than 15), the sex (male or female) and the origin of the horses (Australia, France,
135 Madagascar, Mauritius, Reunion, Seychelles, South Africa and USA). All statistical analysis
136 were performed using R.3.0.1 ((26), with $\alpha = 0.05$. A seropositive animal was defined as an
137 animal found with a positive IgG ELISA result. The apparent prevalence of WNV by region
138 in the Indian Ocean observed in 2010 was mapped to evaluate the spatial distribution of this
139 prevalence using ArcGIS software (QGIS Development team, 2012) (Figure 1).

140

141 **3. Results**

142 3.1 Horse population characteristics

143 The horses were mostly male, 87.4% of the horses were more than 6 years old and only
144 63.8% of the horses that were sampled in a given country were born in this same country
145 (Table 2).

146 3.2 WNV specific antibodies detection

147 The cross-sectional study includes a total of 311 horses. Anti-WNV IgG antibodies were
148 detected in 84 horses (27.0%, 95% CI: 22.1-31.9%). Anti-WNV IgG seropositivity was
149 46.3% (n=121), 22.1% (n=77), 6.7% (n=104) and 44.4% (n=9) for Madagascar, Mauritius,
150 Reunion and Seychelles, respectively (table 3). The highest seroprevalence was observed in
151 Madagascar whereas the lowest was in Reunion.

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

154 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

204 owners to ensure protection against WNV infection (Pearce *et al.*, 2013). Finally, anti-WN
205 IgG antibodies were also detected in the Mauritius human population at a low level (2%) in
206 1994 also suggesting the presence of the virus but to date, no endemic clinical case has been
207 identified. (Zeller *et al.*, 1998);

208 The unexpected high level of WNV specific antibodies prevalence of 44.4 % (95% [CI] =
209 12.-76.9) from Seychelles horses could be explained by the low number of sampled horses.
210 Moreover, no scientific information highlighting a WNV circulation neither in humans nor in
211 animals has been gathered (Zeller *et al.*, 1998; Bovet *et al.*, 2013). Two out of the four horses
212 detected positive that have been imported from South Africa may have been responsible for
213 the transmission of WNV among the Seychelles horse population since their introduction in
214 2000; and that would also explain why the 11-15 years of age horses only were positive.

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

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

228 pathogenic virulence leading to the awareness of the medical doctors in the SWIO region
229 (Longchamp et al, 2003; Larrieu et al; 2013).
230 Finally, these serological data should be considered as evidence of present and past WNV
231 activity in the different islands. Consequently, such results confirm that there is a potential
232 risk of exposure for the human population. In this context, and particularly because of the
233 favorable tropical conditions (temperature and hygrometry) for the development of insect
234 vectors and for the virus survival, a surveillance network including identification of mosquito
235 vectors and vertebrate hosts (mainly bats) involved in WNV transmission to birds, horses,
236 humans, and possibly other mammals living in these islands as well as environmental factors
237 must be set up to ensure potential preventive strategies. These findings raise the need of
238 collaboration between neighboring countries, especially when exposed to similar risks
239 of emergence to limit the risk of spread of infectious diseases.

240

241 **Acknowledgements**

242 This study was conducted in the frame of AnimalRisk/TROI, a research program on emerging
243 animal diseases in the Indian Ocean, funded by FEDER POCT (European Union, Regional
244 Council of Reunion and French government) under the DP One Health-OI. The authors thank
245 Floriane Boucher for her help on the map.

246

247 **Conflict of interests**

248 The authors have no conflict of interests to declare.

249

250 **References**

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357

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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| Characteristics | N=311 | |
|------------------------|-----------------------|------------|
| | 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) |

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

| Variables | Madagascar | | Reunion Island | | Mauritius Island | | Seychelles Island | | Total | |
|------------------------|-------------|-------------|----------------|-------------|------------------|-------------|-------------------|-------------|-------------|-------------|
| | N=121 | | N=104 | | N=77 | | N=9 | | N=311 | |
| | 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) |

380 Table 5 Multiple regression model for 1 risk factors associated with WN antibody
 381 prevalence in horses, Indian Ocean. CI stands for Confidence Interval

| Variables | Categories | Adjusted odds ratio (95% CI) | ³⁸² <i>p</i> -value ³⁸³ |
|---------------------|---------------------|---------------------------------|---|
| Age (yrs) | 1-5 | Reference | |
| | 6-10 | 1.44 (0.59-3.58) | ³⁸⁴ 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 |