Serological evidence for the circulation of flaviviruses in seabird populations of the western Indian Ocean

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SUMMARY

Birds play a central role in the epidemiology of several flaviviruses of concern for public and veterinary health. Seabirds represent the most abundant and widespread avifauna in the western Indian Ocean and may play an important role as host reservoirs and spreaders of arthropod-borne pathogens such as flaviviruses. We report the results of a serological investigation based on blood samples collected from nine seabird species from seven islands in the Indian Ocean. Using a commercial competitive enzyme-linked immunosorbent assay directed against the prototypic West Nile flavivirus, antibodies against flaviviruses were detected in the serum of 47 of the 855 seabirds tested. They were detected in bird samples from three islands and from four bird species. Seroneutralization tests on adults and chicks suggested that great frigatebirds (*Fregata minor*) from Europa were infected by West Nile virus during their non-breeding period, and that Usutu virus probably circulated within bird colonies on Tromelin and on Juan de Nova. Real-time polymerase chain reactions performed on bird blood samples did not yield positive results precluding the genetic characterization of flavivirus using RNA sequencing. Our findings stress the need to further investigate flavivirus infections in arthropod vectors present in seabird colonies.

Key words: Great frigatebird, masked booby, Meaban virus, red-footed booby, sooty tern, Usutu virus, West Nile virus.

INTRODUCTION

First described in Uganda in 1937 [1], West Nile virus (WNV) is a mosquito-borne flavivirus that can induce fatal encephalitis in humans and horses, as well as in domestic and wild birds [2]. WNV is considered to be an emerging pathogen, having the most widespread geographical distribution in flaviviruses, including Africa, India, Europe, Asia and America [3–5]. Usutu virus (USUV) is another mosquito-borne flavivirus originating from Africa [6]. Closely related to WNV, it has recently been of epidemiological concern

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due to its rapid spread in Europe [7]. Because such viruses represent a threat to public health [7, 8] and wildlife conservation [9–11], the development of surveillance and research programmes on these infectious agents is critical, particularly at lower latitudes where reporting efforts are the lowest and emerging infectious diseases are more likely to occur [12].

Birds play a central role in the epidemiology of WNV and USUV as they represent the main amplifying hosts in the wild [2, 7] and act as potential long-range vectors through their migratory behaviour [5]. Despite cases of human WNV infections reported in Madagascar [1–3], few studies have investigated the role of wild birds as reservoirs of this virus in the western Indian Ocean (WIO) [4, 5]. Moreover, to our knowledge, no attempt has been made to detect USUV in bird populations of the WIO islands although it originates from adjacent African countries [6–9]. Such information is important to assess the risk of emergence of flaviviruses in this region and to define appropriate preventive procedures for public and veterinary health.

With 30 breeding species totalling about 19 million individuals, seabirds represent the most abundant and widespread avifauna in the WIO [10, 11]. In this region, seabirds and their ectoparasites are involved in the transmission and dispersal of several infectious agents including viruses, bacteria and blood parasites [12–18]. In this study we therefore hypothesized that seabirds could also play an important role in the epidemiology of flaviviruses because most of them breed in colonies with high bird density and are infested by potential arthropod vectors [12, 19, 20]. Mosquitoes and ticks, specifically associated with seabirds [21–24], can be found in breeding colonies and be responsible for the circulation of arboviruses, including flaviviruses [25–28]. For instance, Meaban virus (MEAV) has been detected in soft ticks of the genus *Carios* (*Ornithodoros*), in gull colonies in Europe [4, 12, 27]. In the WIO, *Carios capensis* is commonly found in seabird colonies [9, 17, 22] but to date its role as a vector of flaviviruses remains to be assessed.

Our objective was therefore to determine if the seabirds of the WIO are exposed to flaviviruses, particularly to WNV, USUV, and MEAV. Blood samples were collected in nine seabird species from seven islands of the region. Different techniques were conducted to determine if such flaviviruses circulated in these bird populations. A commercial competitive enzyme-linked immunosorbent assay (ELISA) was used as a serological screening tool to detect

antibodies against flaviviruses cross-reacting with WNV [12, 29, 30]. Virus-specific neutralization tests were then used to identify the flaviviruses to which birds had been exposed in ELISA-positive serum samples [31]. Real-time polymerase chain reactions (real-time PCR) were also performed in an attempt to directly detect flaviviruses in blood samples from bird populations in which antibodies against flaviviruses were found.

MATERIALS AND METHODS

Blood samples (from 0·2 to 1 ml) were collected from the brachial or metatarsian vein of 855 seabirds between April 2011 and February 2012. We focused on nine seabird species (Table 1): sooty tern (*Onychoprion fuscatus*), white-tailed (*Phaethon lepturus*) and red-tailed (*Phaethon rubricauda*) tropicbirds, wedge-tailed shearwater (*Puffinus pacificus*), lesser (*Anous tenuirostris*) and brown (*Anous stolidus*) noddies, great frigatebird (*Fregata minor*), masked (*Sula dactylatra*) and red-footed (*Sula sula*) boobies, in seven islands of the WIO (Fig. 1): Aride (<1 km^2, 4° 12' S, 55° 39' E), Bird (<1 km^2, 3° 43' S, 55° 12' E), Cousin (<1 km^2, 4° 19' S, 55° 39' E), Europa (28 km^2, 22° 21' S, 40° 21' E), Juan de Nova (5 km^2, 17° 03' S, 42° 45' E), Réunion (2512 km^2, 21° 22' S, 55° 34' E), and Tromelin (1 km^2, 15° 53' S, 54° 31' E). These islands are uninhabited or host small human communities (these islands are mainly nature reserves, military camps, or privately owned), except for Réunion island (800 000 inhabitants). The same sampling strategy was performed as in a previous investigation on influenza A virus infection in seabirds performed on the same bird communities and islands [32]. The most abundant and diverse bird species were sampled on each island by taking into account geographical, safety, ethical and sample conservation constraints. Details that relate to the number of species and colony size are available in Lebarbenchon et al. [32]. When sampling was performed during chick rearing, samples from both adults and chicks were collected.

Two drops of blood were stored in 800 μl Longmire lysis buffer [33], the remaining blood was centrifuged and serum samples were conserved at −20 °C in the field. All samples were transported under refrigeration (<4 °C) to the laboratory, and maintained at −80 °C until tested. Screening of sera for flavivirus antibodies was first conducted using a commercial competitive ELISA kit designed to detect antibodies against the

Table 1. *Results of competitive ELISAs for the detection of flavivirus antibodies in seabird sera from the western Indian Ocean*

Island	Species		Status	Sample size	No. of positive samples	Percentage of positive samples (95% CI)
Aride	*Onychoprion fuscatus*	Sooty tern	Adult	50	0	0
	Phaethon lepturus	White-tailed tropicbird	Adult	19	0	0
	Puffinus pacificus	Wedge-tailed shearwater	Adult	13	0	0
Bird	*Anous stolidus*	Brown noddy	Adult	19	0	0
	Anous tenuirostris	Lesser noddy	Adult	20	0	0
	Onychoprion fuscatus	Sooty tern	Adult	50	0	0
	Phaethon lepturus	White-tailed tropicbird	Adult	30	0	0
Cousin	*Phaethon lepturus*	White-tailed tropicbird	Adult	43	0	0
	Puffinus pacificus	Wedge-tailed shearwater	Adult	50	0	0
Réunion	*Puffinus pacificus*	Wedge-tailed shearwater	Adult	53	0	0
Europa	*Onychoprion fuscatus*	Sooty tern	Adult	50	0	0
	Phaethon lepturus	White-tailed tropicbird	Adult	43	0	0
	Phaethon rubricauda	Red-tailed tropicbird	Adult	45	0	0
	Sula sula	Red-footed booby	Chick	10	0	0
			Adult	51	0	0
	Fregata minor	Great frigatebird	Chick	28	0	0
			Adult	20	2	10 (0–23·1)
Juan de Nova	*Onychoprion fuscatus*	Sooty tern	Chick	31	2	6·4 (0–15·1)
			Adult	115	10	8·7 (3·5–13·8)
Tromelin	*Sula dactylatra*	Masked booby	Chick	24	0	0
			Adult	28	22	78·6 (63·4–93·8)
	Sula sula	Red-footed booby	Chick	37	1	2·7 (0–7·9)
			Adult	26	10	38·5 (19·8–57·2)
Total				**855**	**47**	**5·5 (4·0–7·0)**

CI, Confidence interval.

structural pre-membrane (prM) and envelope (E) proteins of WNV (ID Screen® West Nile Competition, IDvet, France). Because WNV E protein shares conserved epitopes with other flaviviruses, this ELISA tool can be conveniently used as a surrogate test to detect antibodies to many flaviviruses, particularly those belonging to the Japanese encephalitis serocomplex [12, 29] but also to the seabird tick-borne group [30]. The analyses were implemented and interpreted as recommended in the manufacturer's protocol.

In order to determine the infecting flavivirus species, serum samples that tested positive in ELISA were then analysed in comparative micro virus neutralization tests (VNTs) [31] using a panel of three flaviviruses (WNV, USUV, MEAV) suspected to circulate in the WIO region. The limited volume of

serum that was available precluded testing other flaviviruses and did not allow for an extensive analysis of each serum sample with the three flaviviruses (Table 2). Neutralizing antibody titres to WNV (Israel 1998 strain, provided by Dr P. Desprès, Institut Pasteur, Paris) and USUV (SAAR 1776 strain) were determined by performing a 96-well plate VNT on Vero cells (ATCC® CCL-81) as previously described [34]. Samples that showed neutralization at dilutions ≥1:10 were considered positive (dilution range 1/10 to 1/640). The neutralizing antibody titre was considered to be the reciprocal value of the highest serum dilution that conferred complete protection to the cell layer. A plaque reduction neutralization test ($PRNT_{90}$) on SW13 cells (ATCC® CCL105) was also performed on these serum samples
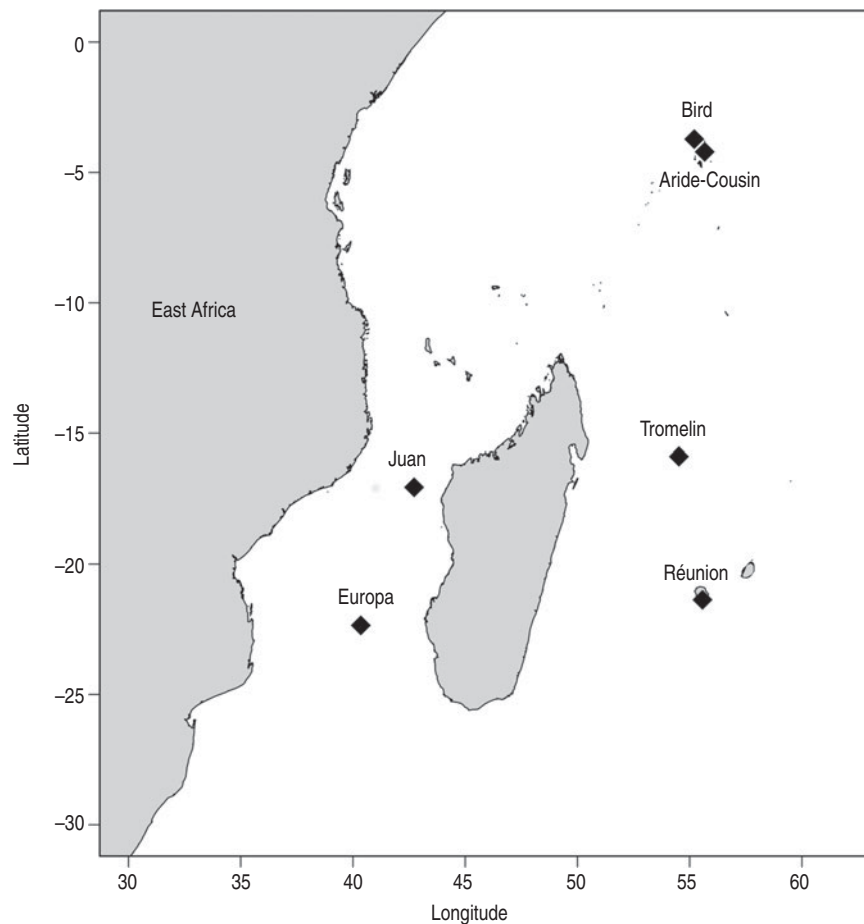
Fig. 1. Sampling locations (♦).

Table 2. *Results of virus neutralization tests (VNTs) for the detection of specific antibodies to West Nile virus (WNV), Meaban virus (MEAV) and Usutu virus (USUV) in samples tested positive with ELISA*

		ELISA pos.	WNV	MEAV	USUV
Europa	Great frigatebird	2	2/2	0/2	0/0*
Juan de Nova	Sooty tern	12	0/12	1/12	4/9*
Tromelin	Masked booby	22	0/21*	0/21*	15/15*
	Red-footed booby	11	0/10*	0/10*	8/8*
Total		47	2/45*	1/45*	27/32*

* Not enough serum volumes to perform VNTs for the three flaviviruses.
Results are presented as number of VNT-positive samples/number of tested samples.

to detect antibodies against MEAV (strain Brest ART707, provided by X. de Lamballerie), as described in [30]. A sample was considered positive if the number of viral plaques in wells treated with the serum was <10% of the number counted in the control well (without serum).

Real-time PCR was performed to detect flavivirus RNA in blood samples of populations in which

antibodies against flaviviruses were detected with the ELISA (sooty tern from Juan de Nova, great frigate-birds from Europa, masked booby and red-footed booby from Tromelin, $n = 309$). RNA was extracted from $250\,\mu$l avian blood in Longmire lysis buffer using the QIAamp viral RNA Mini kit (Qiagen, USA); samples were eluted in a final volume of 40 μl. Reverse transcription was performed on $20\,\mu$l

RNA product, using 0·1 µg of random hexamers (Promega, USA) and GoScript™ Reverse Transcriptase (Promega, USA), under the following thermal conditions: 80 °C for 5 min, 25 °C for 5 min, 42 °C for 60 min and 70 °C for 5 min [15]. The cDNAs were diluted 1:2 and stored at −20 °C until tested. Real-time PCR was performed following a previously published protocol with primers PF1S and PF2R-bis targeting a region of the NS5 gene [35]. ABsolute Blue SYBR Green mix (Thermo Fisher Scientific, UK) was used in a final volume of 25 µl containing 5 µl cDNA. Reactions were carried out in a Stratagene Mx3005P (Agilent Technologies, USA) PCR detection system. All PCR reactions were run with a negative and a positive (WNV) control.

χ^2 tests were used to test effects of species, island and bird age (i.e. chick or adult) on ELISA results. Analyses were performed using R v. 3.0.1 (R Foundation, Austria).

RESULTS

Using the competitive ELISA, antibodies against flaviviruses were detected in 47 (5·5%) of the 855 serum samples collected in nine seabird species from seven islands of the WIO. ELISA results showed a strong heterogeneity among islands ($\chi^2 = 26·03$, $P < 0·001$). Positive samples were collected in only three of the seven islands: Juan de Nova, Tromelin and Europa (Table 1). On Juan de Nova where two seabird species breed, the sooty tern (2 million pairs) and the crested tern (*Thalasseus bergii*, 250 pairs), only the former species was sampled and antibodies against flaviviruses were detected in 8·2 ± 4·5% of the birds (12/146). On Tromelin, two seabird species breed and were sampled, the masked booby and the red-footed booby. Antibodies were found in both species at 42·3 ± 13·4% (22/52) and 17·5 ± 9·4% (11/63), respectively. Eight seabird species reproduce on Europa. Of the five species sampled, only the great frigatebird tested seropositive to flaviviruses with a prevalence of 4·2 ± 5·6% (2/48). There was also a significant difference in seroprevalence between bird species ($\chi^2 = 7·01$, $P = 0·008$) and bird age ($\chi^2 = 48·87$, $P < 0·001$; Fig. 2). In fact, the prevalence in chicks was lower than in adults for masked and red-footed boobies from Tromelin ($\chi^2 = 29·55$, $P < 0·001$ and $\chi^2 = 11·18$, $P < 0·001$, respectively), but not for sooty terns from Juan de Nova ($\chi^2 = 0·01$, $P = 0·972$) and great frigatebirds from Europa ($\chi^2 = 0·95$, $P = 0·329$).

Based on VNTs, WNV-, MEAV- and USUV-specific antibodies were detected in two, one and 27 samples, respectively (Table 2). The two ELISA-positive samples collected from frigatebirds on Europa were positive for WNV (titres of 10 and 20) and negative for MEAV VNTs (not enough serum available to test USUV). USUV-specific antibodies (titres between 10 and 80) were detected in samples collected from the two booby species on Tromelin. One masked booby was both positive in WNV and USUV VNTs, at the test threshold and a titre of 40, respectively. For this particular sample, a comparison of the endpoint titres between the two viruses was performed. The virus with the highest neutralization capacity and at least a fourfold difference in titres was considered as the infecting flavivirus [36]. We thus reasonably assumed that this bird had been infected with USUV and not with WNV. On Juan de Nova, results were more difficult to interpret. No WNV-specific antibody was found in the sooty tern samples. One sample tested positive with the MEAV VNT at the test threshold (20) and four out of nine tested samples were weakly positive for USUV VNT (titres of 10). Moreover, five ELISA-positive sera did not lead to the identification of virus-specific antibodies based on the VNTs.

No flavivirus nucleic acids were detected by real-time PCR in the blood samples ($n = 309$) from populations in which antibodies against flaviviruses were found.

DISCUSSION

Overall, we found antibodies against flaviviruses in 5·5% of 855 seabirds tested using a competitive ELISA. Despite some strong spatial heterogeneity, this low seroprevalence indicates that seabirds are unlikely to be the main amplifying hosts for the tested flaviviruses in the WIO region. Seroneutralization assays identified two flaviviruses, USUV and WNV, as infecting seabird populations, which may represent a threat to public and veterinary health.

One may question whether detection of antibodies to flaviviruses represents merely the serological print of a remote infection or whether it indicates a recent, locally transmitted infection occurring at the breeding area and favoured by promiscuity between seabirds and high concentrations of arthropod vectors. Before further interpreting the results of the serological analyses, some issues regarding anti-flavivirus antibody dynamics in seabirds should be specified. First, it
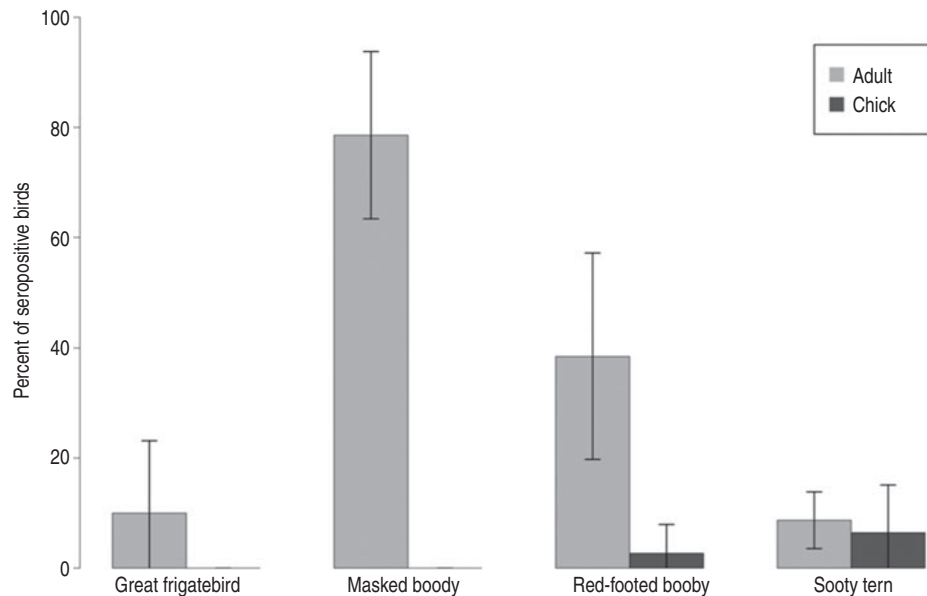
Fig. 2. Seropositivity rates (%) in birds with antibodies to flaviviruses detected by competitive enzyme-linked immunosorbent assays only for populations in which positive samples were found. Error bars represent ± confidence intervals.

has been suggested that following the exposure of an individual to a specific virus, virus-specific antibodies may persist over a long time period. Persistence of WNV antibodies in pigeons experimentally exposed to the virus has for instance been shown to last several months [37]. Hence, the seropositive status of an adult does not necessarily reflect a recent exposure to the virus. This may especially be the case for seabird species, which are long-lived animals, sometimes living for more than 20 years [38]. Second, seropositivity of a young chick to a flavivirus does not unequivocally mean that it was directly exposed to the virus, as this serological status may indicate the presence of maternal antibodies transferred through the egg yolk [37]. In most bird species, maternal antibody levels usually decline within the first 2 weeks of life, although recent work on shearwaters has shown that they can persist for more than 2 months post-hatching [39]. In the present study, antibody-neutralizing flaviviruses cross-reacting with WNV were not detected in the samples obtained from shearwaters and it is unlikely that maternal antibodies persist that long in non-Procellariiformes species, such as the masked and red-footed boobies [39]. Hence, in the present study, the low seroprevalence in chicks of boobies (fledglings) from Tromelin (Table 1) suggests a low local exposure and the high seroprevalence in adults fits with a likely long-term persistence of anti-flavivirus antibodies. Last, another methodological

issues should be considered. It is highly probable that not all flaviviruses to which seabirds are exposed are known, and in spite of the high specificity of VNTs, cross-neutralization by antibodies against closely related viruses and those within the same sero-complex may occur and interfere with test interpretations [29].

As mentioned above, the results show a strong spatial heterogeneity in seroprevalence patterns. Despite relatively balanced sample sizes among locations, antibodies against flaviviruses were found only on three of the seven islands and in four species. Antibodies neutralizing WNV were detected in sera from two adult great frigatebirds from Europa, which suggest that these birds have been exposed to this virus. As no seropositivity was detected in chicks, a local circulation of WNV on this island is unlikely. Frigatebirds are known to visit and roost in large number on other islands of the tropical Indian Ocean (Maldives, Comoros, Madagascar, Seychelles) outside their breeding season [40], and could have been infected during this period. The fact that no WNV-specific antibody was detected in the four other breeding species that we sampled on Europa also supports this assumption. By contrast, adults and chicks of seabird species breeding on Tromelin and Juan de Nova tested seropositive which suggest that flaviviruses circulate in these colonies and that birds were probably infected locally. Moreover, the

behaviour of these bird species during the non-breeding period is known to differ from that of frigate-birds. Although no studies have investigated the post-breeding dispersion of boobies, they are supposed to be sedentary and to remain on their breeding island all year round [41]. Antibodies neutralizing USUV detected in boobies on Tromelin thus suggested a local virus circulation on the island. High prevalences of infected birds (reaching more than 50% of the tested birds [42]) have sometimes been reported for USUV in Europe, with significant temporal variation likely resulting from herd immunity [42]. Together with the high seroprevalence we observed in boobies, the lack of PCR-positive samples could also suggest that herd immunity could limit virus circulation on Tromelin. It remains unclear why such high sero-prevalence has been detected on this island and the identification of epidemiological cycles as well as mechanisms of viral transmission will require add-itional investigation. Sooty terns migrate far from their breeding grounds, but they remain at sea with no land stopover (M. Le Corre, unpublished data). The likelihood that these birds became infected outside their breeding colony is lower than that of fri-gatebirds, which frequently roost in large numbers on remote islands. Positive ELISA samples for sooty terns from Juan de Nova suggest local circulation of flaviviruses, but results of VNTs were more difficult to interpret (positives at the test threshold, absence of virus neutralization for a subset of samples) and do not allow us to determine the infecting flavivirus species. Further investigations are required (see below).

It is noticeable that despite large sample sizes, several species and/or islands had no seropositive individuals. In particular, antibodies neutralizing flaviviruses were not detected in seabirds from the Seychelles islands (Bird, Cousin and Aride islands, $n = 294$), although most of these islands host large populations of mosquitoes (Cousin island) and ticks were observed in seabird colonies during bird sam-pling (Aride and Bird islands). Finally, the failure to detect flavivirus nucleic acids in any blood samples of individuals from colonies where seropositive birds were detected is probably due to the short viraemia observed for flaviviruses, usually <7 days [4]. Cloacal or oro-pharyngeal samples could be an inter-esting alternative to directly characterize these flavi-viruses from bird samples [4].

Overall, our findings suggest that the flaviviruses we have tested circulate at only a low level on islands of

the WIO and that the infections exhibited spatial het-erogeneity. These results could be explained by differ-ent assumptions, e.g. limited contacts between bird populations, or intrinsic resistance in certain bird spe-cies to infection or vector host preference. This study also highlights the need for further investigations on the occurrence of flaviviruses in potential arthropod vectors present on bird colonies. Within the seabird communities where flaviviruses are suspected to circu-late (Tromelin and Juan de Nova), detailed sampling of mosquitoes and ticks associated with the different hosts should notably be conducted to directly detect and characterize the circulating viruses. Given the highly seropositive boobies on Tromelin, efforts to characterize USUV on this island would be worth-while and further analyses of bird or arthropod sam-ples from Juan de Nova should explore whether VNT results are indicative of another flavivirus circu-lating on the island. Finally, phylogenetic studies of the identified flaviviruses should yield important insights on the origin and evolutionary history of viruses in the WIO islands [43].

DECLARATION OF INTEREST

None.

REFERENCES

1. **Smithburn KC, *et al.*** A neurotropic virus isolated from the blood of a native of Uganda. *American Journal of Tropical Medicine and Hygiene* 1940; **1**: 471–492.

2. **Komar N, *et al.*** Experimental infection of North American birds with the New York 1999 strain of West Nile virus. *Emerging Infectious Diseases* 2003; **9**: 311–322.

3. **Hubálek Z, Halouzka J.** West Nile fever-a reemerging mosquito-borne viral disease in Europe. *Emerging Infectious Diseases* 1999; **5**: 643–650.

4. **Rappole JH, Derrickson SR, Hubálek Z.** Migratory birds and spread of West Nile virus in the Western Hemisphere. *Emerging Infectious Diseases* 2000; **6**: 319–328.

5. **Reed KD, *et al.*** Birds, migration and emerging zoonoses: West Nile virus, lyme disease, influenza A and enteropathogens. *Clinical Medicine and Research* 2003; **1**: 5–12.

6. **Woodall JP.** The viruses isolated from arthropods at the East African Virus Research Institute in the 26 years ending December 1963. *Proceedings of the East African Academy* 1964; **2**: 141–146.

7. **Vazquez A, *et al.*** Usutu virus: potential risk of human disease in Europe. *European Communicable Disease Bulletin* 2011; **16**: 19935.

8. **Nash D, *et al.*** The outbreak of West Nile virus infection in the New York City area in 1999. *New England Journal of Medicine* 2001; **344**: 1807–1814.

9. **Daszak P, Cunningham AA, Hyatt AD.** Anthropogenic environmental change and the emergence of infectious diseases in wildlife. *Acta Tropica* 2001; **78**: 103–116.

10. **Weissenbock H, *et al.*** Emergence of Usutu virus, an African mosquito-borne flavivirus of the Japanese encephalitis virus group, central Europe. *Emerging Infectious Diseases* 2002; **8**: 652–656.

11. **Boyce WM, *et al.*** Surveillance for West Nile Virus and vaccination of free-ranging island scrub-jays (*Aphelocoma insularis*) on Santa Cruz Island, California. *Vector-Borne and Zoonotic Diseases* 2011; **11**: 1063–1068.

12. **Jones KE, *et al.*** Global trends in emerging infectious diseases. *Nature* 2008; **451**: 990–993.

13. **Lonchampt C, *et al.*** Persistence of an endemic circulation of West Nile virus in Madagascar. *Archives de l'Institut Pasteur de Madagascar* 2003; **69**: 33–36.

14. **Larrieu S, *et al.*** A fatal neuroinvasive West Nile virus infection in a traveler returning from Madagascar: Clinical, epidemiological and veterinary investigations. *American Journal of Tropical Medicine and Hygiene* 2013; **89**: 211–213.

15. **Fontenille D, *et al.*** The transmission cycle of West Nile virus Madagascar, Indian Ocean. *Annales de la Société Belge de Medecine Tropicale* 1989; **69**: 233–243.

16. **Nikolay B, *et al.*** Usutu Virus in Africa. *Vector-Borne and Zoonotic Diseases* 2011; **11**: 1417–1423.

17. **Sinclair I, Langrand O.** *Birds of the Indian Ocean Islands.* Cape Town: Struik, 2003.

18. **Converse JD, *et al.*** Soldado virus (Hughes group) from *Ornithodoros* (*Alectorobius*) *capensis* (Ixodoidea: Argasidae) infesting sooty tern colonies in the Seychelles, Indian Ocean. *American Journal of Tropical Medicine and Hygiene* 1975; **24**: 1010–1018.

19. **Converse JD, *et al.*** Aride virus, a new ungrouped arbovirus infecting *Amblyomma loculosum* ticks from roseate terns in the Seychelles. *Archives of Virology* 1976; **50**: 237–240.

20. **Lebarbenchon C, *et al.*** Absence of coronaviruses, paramyxoviruses, and influenza A viruses in seabirds in the southwestern Indian Ocean. *Journal of Wildlife Diseases* 2013; **49**: 1056–1059.

21. **Bastien M, *et al.*** *Haemoproteus iwa* in Great Frigatebirds (*Fregata minor*) in the Islands of the Western Indian Ocean. *PLoS ONE* 2014; **9**: e97185.

22. **Dietrich M, *et al.*** Rickettsia spp. in seabird ticks from western Indian Ocean islands, 2011–2012. *Emerging Infectious Diseases* 2014; **20**: 838–842.

23. **Wilkinson DA, *et al.*** Massive infection of seabird ticks with bacterial species related to *Coxiella burnetii*. *Applied and Environmental Microbiology* 2014; **80**: 3327–3333.

24. **Feare CJ.** Desertion and abnormal development in a colony of sooty terns *Sterna fuscata* infested by virus-infected ticks. *Ibis* 1976; **118**: 112–115.

25. **Dietrich M, Gomez-Díaz E, McCoy KD.** Worldwide distribution and diversity of seabird ticks: implications for the ecology and epidemiology of tick-borne pathogens. *Vector-Borne and Zoonotic Diseases* 2011; **11**: 453–470.

26. **Nuttall PA.** Tick-borne viruses in seabird colonies. *Seabird* 1984; **7**: 31–41.

27. **Chastel C, *et al.*** The isolation of Meaban virus, a new Flavivirus from the seabird tick *Ornithodoros* (*Alectorobius*) *maritimus* in France. *Archives of Virology* 1985; **83**: 129–140.

28. **Gritsun TS, Nuttall PA, Gould EA.** Tick-borne flaviviruses. *Advances in Virus Research* 2003; **61**: 317–371.

29. **Beck C, *et al.*** Flaviviruses in Europe: complex circulation patterns and their consequences for the diagnosis and control of West Nile disease. *International Journal of Environmental Research and Public Health* 2013; **10**: 6049–6083.

30. **Arnal A, *et al.*** Circulation of a meaban-like virus in yellow-legged gulls and seabird ticks in the western Mediterranean basin. *PLoS ONE* 2014; **9**: e89601.

31. **Weingartl HM, *et al.*** Comparison of assays for the detection of West Nile virus antibodies in chicken serum. *Canadian Journal of Veterinary Research* 2003; **67**: 128–132.

32. **Lebarbenchon C, *et al.*** Influenza A virus on oceanic islands: host and viral diversity in seabirds in the Western Indian Ocean. *PLoS Pathogens* 2015; **11**: e1004925.

33. **Longmire JL, *et al.*** Isolation and molecular characterization of a highly polymorphic centromeric tandem repeat in the family Falconidae. *Genomics* 1988; **2**: 14–24.

34. **Figuerola J, *et al.*** Prevalence of West Nile virus neutralizing antibodies in colonial aquatic birds in southern Spain. *Avian Pathology* 2007; **36**: 209–212.

35. **Moureau G, *et al.*** A real-time RT-PCR method for the universal detection and identification of flaviviruses. *Vector-Borne and Zoonotic Diseases* 2007; **7**: 467–478.

36. **Yeh J-Y, *et al.*** A diagnostic algorithm to serologically differentiate West Nile virus from Japanese encephalitis virus infections and its validation in field surveillance of poultry and horses. *Vector-Borne and Zoonotic Diseases* 2012; **12**: 372–379.

37. **Gibbs SEJ, *et al.*** Persistence of antibodies to West Nile virus in naturally infected rock pigeons (*Columba livia*). *Clinical and Vaccine Immunology* 2005; **12**: 665–667.

38. **Schreiber EA, Burger J.** *Biology of Marine Birds.* Hoboken: CRC Press, 2001.

39. **Garnier R, *et al.*** Maternal antibody persistence: a neglected life-history trait with implications from albatross conservation to comparative immunology. *Proceedings of the Royal Society of London, Series B: Biological Sciences.* 2012; **279**: 2033–2041.

40. **Weimerskirch H, *et al.*** Postbreeding movements of frigatebirds tracked with satellite telemetry. *The Condor* 2006; **108**: 220–225.

41. **Nelson B, Busby J.** *The Sulidae: Gannets and Boobies.* Oxford: Oxford University Press, 1978.

42. **Chvala S, *et al.*** Monitoring of Usutu virus activity and spread by using dead bird surveillance in Austria, 2003–2005. *Veterinary Microbiology* 2007; **122**: 237–245.

43. **Heinze DM, Gould EA, Forrester NL.** Revisiting the clonal concept of evolution and dispersal for the tick-borne flaviviruses by using phylogenetic and biogeographic analyses. *Journal of Virology* 2012; **86**: 8663–8671.