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Paper

Increasing Seroprevalence In Horses Evidences Widespread Circulation Of West Nile Virus In Albania

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Abstract

West Nile virus (WNV) is a zoonotic mosquito-borne flavivirus that causes West Nile disease, an emerging and re-emerging infection of major global importance. Horses are an important sentinel species for monitoring WNV circulation. Since viraemia in horses is brief and viral loads in the blood are low, serological assays are the primary tools used to monitor WNV exposure. This study aimed to estimate the seroprevalence of WNV antibodies in horses in Albania. Between 2017 and 2020, 509 serum samples were collected from apparently healthy, unvaccinated horses across different regions of the country. Samples were analysed using two commercial ELISA kits for the detection of total WNV-specific antibodies and WNV-specific IgM. Of the 509 samples tested, 143 (28.1%) were positive for total antibodies, while 12 (2.35%) were IgM-positive, indicating recent infection. As no horse tested positive in both assays, the combined seroprevalence was 30.5% (155/509; 95% CI: 26.6–34.6%), without double counting. A subset of 37 ELISA-positive sera was subsequently tested using the virus neutralisation test (VNT), the gold-standard confirmatory assay for WNV-specific antibodies. The VNT confirmed the presence of WNV-neutralising antibodies in 35/37 (94.6%) samples, with titres ranging from 1:10 to 1:640. These findings indicate widespread WNV circulation in Albania. Compared with previous reports based on samples collected in 2010–2011, seropositivity was higher and evidence of exposure was detected in previously unaffected areas, highlighting the need for continued integrated veterinary and public health surveillance.

Keywords

West Nile Virus, Horses, Seroprevalence, Albania

Introduction

West Nile virus (WNV) is a single-stranded RNA virus, a member of the *Orthoflavivirus* genus in the *Flaviviridae* family. It is a zoonotic arbovirus that can be transmitted to humans by the bite of an infected mosquito and can cause West Nile Neuroinvasive Disease (WNND), a severe clinical condition which is currently considered a serious public health problem worldwide, causing outbreaks and fatalities among humans and animals (Habarugira et al., 2020).

West Nile virus is maintained in a complex enzootic cycle between mosquitoes and birds, with several mosquito species and over 300 bird species implicated as potential vectors and hosts, respectively (Van der Meulen et al., 2005). *Culex* mosquitoes are the most common vectors. Besides humans, WNV can infect birds, horses, dogs and other vertebrates, with variable virulence. In temperate climate regions, outbreaks tend to be associated with late summer and autumn due to the mosquito vector's life cycle and the amplification from the bird-mosquito-bird cycle. In warmer climates, cases can occur throughout the year (Petersen et al., 2013). Horses, like humans, can develop disease, but neither of them develops sufficient to contribute to the transmission cycle, and are therefore considered as "dead-end" hosts.

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WNV was first isolated in 1937 from a febrile patient in the West Nile province of Uganda (Smithburn et al.,1940). Subsequent studies identified WNV in human sera in Egypt (Melnick et al., 1951), as well as in birds and mosquitoes (Work et al., 1953). WNV is now widespread, with intense circulation in Africa, the Middle East, Oceania, the Americas and Europe (Vilibic-Cavlek et al., 2019, Bakonyi et al.,2020, Ronca et al.,2021). West Nile virus antibodies were first detected in Europe during a serosurvey conducted in Albania in 1958 (Bárdos et al., 1959), which preceded the first outbreak in horses, reported in France in 1962 (Murgue et al., 2006). After a long period of epidemiological silence in Europe, the disease reappeared in Romania in 1996, with an unusually virulent outbreak. Three years later, an even more virulent outbreak occurred in Russia in 1999. Further outbreaks caused by lineage 1 of WNV occurred in the early 2000s in southern Europe (Bakonyi et al.,2006). In 2004, a new lineage (lineage 2) was detected for the first time in Europe. It was isolated from a northern goshawk (*Accipiter gentilis*) in Hungary (Bakonyi et al.,2006). This lineage spread rapidly to other southern European countries, causing a notable outbreak involving large numbers of human cases in Greece in 2010, and has expanded more recently also to Northern Europe, with outbreaks reported in 2019 in Germany and in the Netherlands in 2020 (Papa et al., 2011, Vlaskamp et al., 2020, Ziegler et al., 2020). In 2022, preceded by unusually high temperatures, Europe faced one of the worst waves of WNV outbreaks in history, with 1,340 human cases reported, second only to the 2018 season, with 1,888 cases (ECDC, 2022). While lineage 2 was predominant in most European territories affected, lineage 1 still continued to circulate endemically in southern Spain, France and Italy (Aguilera-Sepúlveda et al., 2024), co-circulating with lineage 2 in certain areas (northern Italy) (Barzon et al., 2022).

The first human clinical case in Albania was detected in 2010 (reported in 2011) in a 14-year-old child in Korça prefecture (south-east, bordering Greece) (Di Sabatino et al., 2014). In 2011, 15 human infections (WNV lineage 2) were confirmed (Di Sabatino et al.,2014). In 2011, a serological survey in horses found a 20.3% seroprevalence (Berxholi et al., 2013). Although no more recent surveillance studies have been performed in Albania, the virus is still circulating in the region as confirmed by a serosurvey conducted in horses and birds in neighbouring Kosovo in 2018-2019, where antibodies were found in 27 out of 260 equine sera (10.38%) (Rexhepi et al., 2021).

In our study, serum samples collected from equines were screened for WNV-specific antibodies in order to infer the current distribution and activity of WNV in Albania, as well as to assess the potential risk to humans and horses.

Materials and methods

Sample data

We analysed 509 banked equine blood samples for WNV antibodies. The samples, collected between July 2017 and October 2020, were originally submitted to the laboratory for screening for other equine viruses. Samples originated from horses of different ages (2-25 years), and mostly from local thoroughbreds. In this study, 9 regions were included covering a large area of the country: Durres (n = 14), Elbasan (n = 103), Fier (n = 80), Korça (n = 9), Kukes (n = 60), Lezhe (n = 57), Shkoder (n = 16), Tirana (n = 124) and Vlora (n = 46). Animals were raised on privately owned farms, where they perform heavy labour. The horses were not vaccinated against WNV and had never been outside Albania.

As this study was based on available banked equine sera, the sample size was constrained by the number and geographical distribution of eligible samples. The adequacy of the available sample size was evaluated using an expected prevalence of 30%, based on previous epidemiological studies conducted in Albania and neighbouring countries (Papa et al., 2011, Berxholi et al., 2013, Rexhepi et al., 2021, Lufo et al., 2023, Martin et al., 2023), and according to the formula described by Thrusfield (Thrusfield et al.,2023).

Blood collection and serum preparation

All animals were subjected to blood sampling according to best veterinary practices. Each blood sample was collected in a Vacutainer through venepuncture of the jugular vein. Samples were transported at 4 °C to the laboratory for serum separation within 24 h. Samples were centrifuged at 1,000 - 2,000 x g for 10 min, and the resulting sera were separated from the clot and stored at −20 °C to appropriately preserve antibody activity until serological analysis.

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Competitive Enzyme-Linked Immunosorbent Assay (ELISA tests)

Anti E (envelope protein) IgM antibodies were detected using the ID Screen® West Nile IgM Capture ELISA kit (ID-Vet, Montpellier, France).

Total antibodies were detected using the Ingezim WN Compac kit (Gold Standard Diagnostics, Madrid, Spain). It is a blocking ELISA capable of detecting all Ig isotypes that interfere with the binding of the monoclonal antibody provided in the kit to its specific epitope located in the domain III of the WNV E protein. This competitive ELISA was used to detect total antibodies rather than IgG alone, to increase sensitivity and capture all immunoglobulin isotypes, including early-stage responses that IgG-specific assays may miss. Hence, it is species-independent, i.e., it is able to detect antibodies in sera from different infected animal species (birds, horses, humans, etc.) even at very low titres, given its high analytical sensitivity. Although it is more specific than other ELISA assays commercially available, it is still susceptible to cross-reactions with other flaviviruses similar to WNV (WOAH, 2022), hence the need for confirmatory analysis using the more specific virus neutralisation test (VNT) method.

Virus Neutralisation Test (VNT)

A subset of 37 ELISA-positive samples was subjected to a confirmatory virus neutralisation test (VNT) in 96-well cell-culture microplates as described by Llorente et al. (2019). These sera were selected based on preliminary screening results, ensuring representation of positive, negative, and borderline samples, along with adequate sample quality and volume, to undergo virus neutralisation testing. This technique is based on the capability of the serum to neutralise the cytopathic effect of the virus. Due to the cross-reactivity observed with antigenically related flaviviruses, and to assess specificity against WNV, samples were tested in parallel against WNV (strain E101) and USUV (SAAR-1776) (GenBank accession numbers AF260968 and AY453412, respectively). Serum dilutions from 1:5 to 1:640 were used. Positive WNV and USUV control sera and negative control sera, were included in each assay. Readings were made between the fifth and seventh days by observing the presence of cytopathic effect (CPE) in each well. Samples with a neutralising titre equal to or higher than 1:10 were considered positive. Specificity for WNV antibodies was assigned when the observed VNT titre against WNV was at least four times higher than the observed titre against USUV. Samples not reaching this titre difference were considered positive for an undetermined flavivirus.

Statistical analysis

Data were aggregated by region to enhance representation and inference. Combined seropositivity was defined as positivity in either the whole-antibody blocking ELISA or the IgM capture ELISA. As no horse tested positive in both assays, the combined estimate represented unique seropositive animals and did not involve double counting. Confidence intervals for proportions were calculated using the Wilson Score method, a statistically robust method particularly useful for small sample sizes (Newcombe & Altman, 2000). This method is favoured over the traditional normal approximation interval because it better accounts for the discrete nature of binomial data and ensures that the calculated intervals fall within the 0-1 range. In this study, the method was used to estimate overall and regional WNV seroprevalence and corresponding 95% confidence intervals. Animals per thousand horses (APT) were calculated using the regional horse population as the denominator and used as population-standardised indicators of detected seropositivity across regions with differing horse population sizes and sampling fractions.

Results

As shown in Table I, 12 (2.35%) horses were IgM positive, distributed in five regions: Fier (1 -Asturkoj), Tirana (2-Vora), Vlora (4), Lezha (2 - Torovica), Elbasan (2- Gjinar, 1- Shushica). Regarding total antibodies detected by blocking ELISA, 143 out of 509 samples (28.09%) tested positive and 47 were doubtful (9.2%). The overall seropositivity, considering both tests, was 30.4%. Higher seropositivity rates were observed in Fier, Tirana, Vlora, Lezha and Elbasan.

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No	Region	Municipality	Samples	ELISA IgM (+)	ELISA WA (+)	ELISA WA (Doubtful)
1	Fier	Asturkoj	17	1	5	
		Baltëz	22		9	
		Ndërmenas	5		5	
		Mucoj	5		5	
		Dushk	31		5	4
2	Tirana	Vora	17	2	10	5
		Farka	33		13	5
		Berzhita	20			
		Selita	34		17	9
		Paskuqan	10			
		Peze	10			
3	Vlora	Vlora	46	4	3	
4	Durres	Lalez	7		2	
		Ishem	2		1	
		Shkallnur	3		1	
		Shijak	2			
5	Lezha	Torovica	39	2	21	8
		Balldre,	5		2	1
		Dragush	13		5	
6	Shkoder	Bushat	12			
		Koplik	4			
7	Kukes	Ujmisht	17		2	
		Golaj	13			
		Fajze	19		3	
		Gjinaj	11			
8	Elbasan	Labinot fusha	8			2
		Mengel	7		2	1
		Gjinar	27	2	6	3
		Komunak	23		5	3
		Linos	6		2	
		Selite	13		8	1
		Shushica	7	1	3	2
		Shergjan	3		2	1
		Burqes	3		2	
		Jagodine	3		1	
		Cerrik	3		1	1
9	Korce	Pirg	4		1	
		Vloçisht	5		1	1
TOTAL			**509**	**12**	**143**	**47**

Table I. Overall regional seropositivity rates in horses using IgM and whole antibodies (WA) ELISA tests.

Specificity of the antibodies detected by ELISA was further studied in a subset of 37 ELISA-positive serum samples using the gold-standard VNT test (WOAH, 2022). We also assessed also neutralisation activity against the closely related Usutu virus (USUV) in parallel, to discard possible cross-reactions (Llorente et al., 2019).

The presence of WNV specific antibodies was confirmed in 35 out of 37 samples. One sample was negative (VNT titre lower than 1:10) and another was classified as "undetermined flavivirus" as it did not meet the criterion for assignment of specificity to either flavivirus (Table II).

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Region	WA-ELISA (PI %)	WNV (VNT titre)	USUV (VNT titre)	Antibody specificity
Fier	98.13	≥1:640	1:10	WNV
	87.68	1:10	1:5	undetermined flavivirus
	98.31	≥1:640	1:20	WNV
	98.35	≥1:640	1:40	WNV
	97.56	1:40	1:5	WNV
	93.37	1:40	1:10	WNV
	91.59	1:40	1:5	WNV
	98.12	1:160	1:10	WNV
	97.99	≥1:640	1:20	WNV
	95.1	1:40	1:5	WNV
	97.97	1:320	1:40	WNV
	98.36	1:80	1:20	WNV
	94.57	1:80	1:10	WNV
	97.99	1:160	1:80	WNV
	98.08	1:80	1:20	WNV
	93.24	1:40	<1:5	WNV
	98.11	1:160	1:20	WNV
	98.11	1:320	1:80	WNV
	98.14	≥1:640	1:40	WNV
Tirana	98.41	≥1:640	1:20	WNV
	98.29	≥1:640	1:20	WNV
	98.3	1:160	1:20	WNV
	97.43	1:160	1:20	WNV
	98.46	1:320	1:20	WNV
	97.85	1:40	1:20	WNV
	98.4	≥1:640	1:10	WNV
Vlore	97.74	1:40	1:10	WNV
	97.98	1:80	1:10	WNV
	97.71	1:80	1:5	WNV
Lezhe	97.14	≥1:640	1:80	WNV
	98.16	≥1:640	1:40	WNV
	98.35	≥1:640	1:80	WNV
Elbasan	66.86	1:5	<1:5	negative
	98.23	≥1:640	1:20	WNV
	98.29	1:320	1:5	WNV
	98.2	≥1:640	1:40	WNV
	98.28	≥1:640	1:10	WNV

Table II. Data of the virus neutralisation test (VNT) from ELISA-positive horse samples.

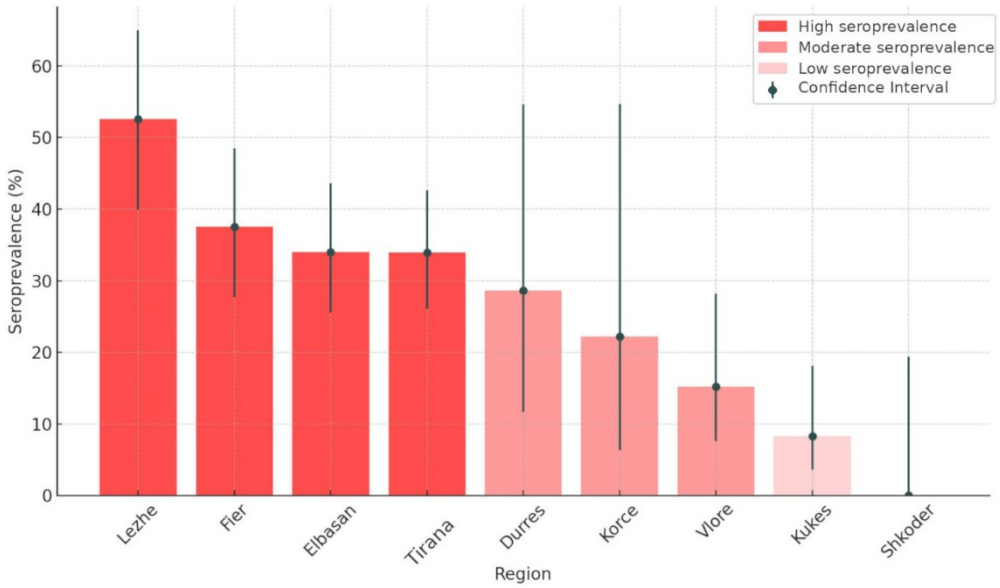

Figure 1. Percentage of seroprevalence in horses, showing variability among regions.

Overall combined seropositivity was 30.5% (95% CI: 26.6%–34.6%), including 143 (28.1%) horses positive by total-

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antibody ELISA and 12 horses (2.4%) positive by IgM ELISA, with no overlap between assays. Virus neutralisation testing confirmed WNV-specific antibodies in 35/37 (95%) ELISA-positive sera tested and excluded cross-reactivity with Usutu virus. This estimate is supported by VNT confirmation, with 95% of ELISA-positive samples verified as WNV-specific and non-reactive to Usutu virus. Regional seroprevalence varied considerably, reflecting differences in historical and recent exposure levels. High-seroprevalence regions (≥30%) included Lezhe, Fier, Elbasan, and Tirana, with Lezhe displaying the highest seroprevalence (52.6%) (Figure 1). The narrower confidence intervals in this group suggest stable estimates of extensive WNV exposure. Most seropositive samples were IgG-positive, while IgM antibodies detected in Elbasan (8.6%), Lezhe (6.7%), Tirana (4.8%), and Fier (3.3%) point to recent viral activity.

Moderate-seroprevalence regions (15–30%) included Durres (28.6%) and Korce (22.2%), both showing wide confidence intervals, indicating regional variability. In these regions, all seropositive cases were consistent with past exposure (IgM negative). In Vlore, the seroprevalence was 15.2%, with narrower CIs. Here, 8.7% of positive cases were IgM-positive, indicating recent viral activity alongside noteworthy historical exposure.

Regions with low seroprevalence, defined by rates below 15%, primarily reflected historical exposure, with no detected evidence of recent infection. In Kukes, seroprevalence was 8.3%, while Shkoder showed no positive cases, however, the upper confidence limit in Shkoder suggests that true seroprevalence could still be notable despite no cases being detected.

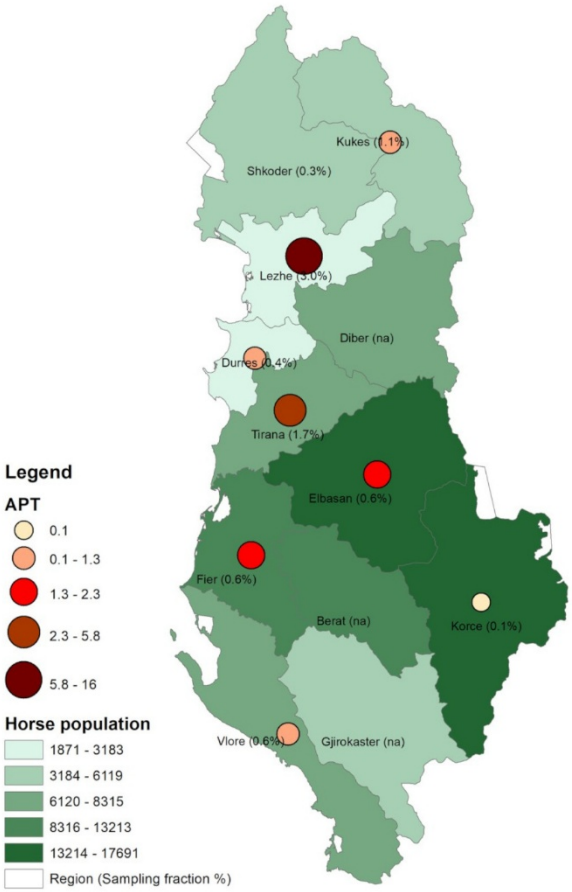

Figure 2. Geographical distribution of analysed horses. Colours of the regions corresponds to horse population and circles show the distribution of seropositivity, estimated as APT.

The overall APT was estimated at 1.5 (95% CI: 1.3 – 1.8), reflecting a substantive disease burden in horses. However, due to uneven sampling fractions across regions, the reliability of these results varies depending on the region. Regions with a higher sampling fraction (>1%) provide more reliable estimates of disease burden. Lezhe, with the highest APT of 16.0, indicates a concentrated infection rate within a small horse population, while Tirana's APT of 5.8, measured in a larger horse population, suggests substantial WNV exposure, though the infection rate is relatively lower. In contrast, Kukes, with an APT of 0.9, reflects limited activity. For regions with sampling fractions of ≤1%, estimates are less reliable. In Elbasan and Fier, moderate APTs of 2.1 and 2.3 suggest a measurable burden, though confidence remains limited due to low sampling. Similarly, Durres and Vlore show modest APTs of 1.3 and 1.0, likely

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reflecting partial under-detection. Korce's minimal APT of 0.1 is uncertain due to low sampling, making it difficult to accurately assess the true disease burden. Similarly, Shkoder's APT of 0.0 suggests no infection; however, further sampling would be necessary to confirm this finding.

Discussion

Data collected in this work indicate, as compared with previous studies (Berxholi et al.,2013), which found a 20.3% seroprevalence in horses in 2011, a marked geographical expansion of WNV circulation in the territory, covering 10 out of 12 districts (36 out of 38 municipalities) of Albania, mostly in the central and western part of the country. These results demonstrate that horses have been exposed to WNV, even though clinical signs were not observed.

Bearing in mind the presence of competent vectors (*Culex* spp. mosquitoes) in the territory, this WNV expansion could be due to the endemisation and expansion of WNV strains previously circulating in the territory, or to new introductions of the virus, through WNV infected carriers. Indeed, both mechanisms might have contributed to this spread, as occurs in Spain (Aguilera-Sepúlveda et al., 2024), another southern-European country where WNV is increasing its incidence and geographical range.

Given the high degree of antigenic similarity among members of the Japanese encephalitis serogroup, to which WNV belongs, it is necessary to check for cross-reactions against other serogroup members that could circulate in the study area, such as Usutu virus. This was effectively achieved by comparing neutralising antibody titres of tested sera against both WNV and USUV in parallel. The results of these analyses confirmed, for the vast majority of samples examined, that the antibodies detected by ELISA were induced by WNV infections and were not due to cross-reactions with related flaviviruses. Specifically, no USUV antibodies were detected in this study.

Our results showed considerable variability in WNV seroprevalence among regions, reflecting differences in historical and recent exposure levels. Seropositive animals were confirmed again in previously recognised endemic areas, as reported in previous studies (Berxholi et al., 2013, Di Sabatino et al., 2014, Morava et al., 2025), but additional affected areas were also identified. This is most likely the result of increased WNV activity across a wider area in Albania, probably due to ecological changes, among which warmer temperatures associated with climate change are likely to be one of the leading drivers involved, allowing mosquito populations to thrive in wider areas (Magallanes et al., 2024).

Detecting WNV-specific IgM in equine blood indicates recent infection. The brief duration of the anti-flavivirus IgM response, along with the high specificity of the assay, suggests that IgM ELISAs are useful for confirming recent WNV infections in horses. IgG antibodies are detected at a later stage and can persist in the bloodstream for longer periods. However, various studies have shown that antibodies to WNV in horses eventually diminish over the course of a few years (Jiménez-Clavero et al., 2010, Magallanes et al., 2023, Tolnai et al., 2025). These results imply that the horses analysed in this study may have been infected with the virus, likely within the last five years.

This recent WNV activity detected in horses in central Albania deserves particular attention, since it may reflect a new introduction of the virus into the area, or, as stated above, might be the result of WNV endemisation of previously circulating strains. Clarification of this point warrants further investigation. This should primarily address direct detection of the virus or its RNA in mosquitoes and/or vertebrate hosts, including birds, followed by molecular sequencing in order to ascertain the nature of the virus strain(s) circulating in the area and their origin.

The variability in prevalence rates and confidence intervals across districts underscores the importance of continuous and comprehensive surveillance to accurately assess and respond to the risk posed by WNV to horse and human populations.

Conclusion

In conclusion, the present study confirms increased WNV activity across extensive areas of Albania, where horses are highly exposed to infection. Epidemiological studies at the local level are paramount to forecast disease risks and implement preventive measures, such as vaccination of horses. WNV, already endemic in many regions of Albania, will likely continue to spread to naïve areas, as conditions for competent vectors become more favourable due to climate change. The results described here cannot completely rule out the circulation of USUV or other as-yet-unidentified flaviviruses in this area, in the absence of complementary information, such as isolation of flaviviruses from birds and/or mosquitoes. To clarify this point, further virological (isolation) and molecular (sequencing) investigations are needed in the country.

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Ethical Approval

No ethical authorisation was required. All samples included in this study were collected for routine testing.

Conflict of interest

The authors declare no conflicts of interest. Mention of trade names or commercial products in this article is solely for providing specific information.

Author Contributions

Conceptualisation: LL, KM; Methodology: LL, KM, FLL, EPR; Formal analysis: LL, KM, KB; Investigation: LL, KM, FLL; Writing original draft preparation: LL, KM; Writing, review and editing: LL, KM, FLL, EPR, MAJC; Visualisation: LL, KM; Supervision: KB, EPR, MAJC; Project administration: LL; EPR; MAJC; Funding acquisition: LL, EPR, MAJC.

All authors have read and agreed to the published version of the manuscript.

Data availability

The datasets generated in this study are available from the corresponding author upon reasonable request.

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