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# First serological evidence of West Nile virus among equines and birds in Kosovo, 2018-2019

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**Short report**

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# Abstract

**Background:** This study was conducted to survey the presence of the West Nile virus (WNV) in Kosovo by serological testing of the healthy autochthonous equine population and virus detection in birds and mosquitoes. Between January 2018 and June 2019, 260 equine serum samples, 626 mosquitoes (60 pools), 50 domestic birds and 51 wild birds were collected from different regions of Kosovo. Equine and domestic bird serum samples were tested by IgG ELISA while mosquitoes and bird viscera were tested for WNV RNA by RT-PCR. Positive ELISA samples were confirmed by Plaque Reduction Neutralization Test (PRNT) and eight by Virus Neutralization Test (VNT).

**Results:** This is the first report providing evidence of WNV antibodies among animals in Kosovo. WNV antibodies were present in 27 out of 260 equine sera (10.38%) and one out of 50 samples in domestic birds by ELISA and PRNT. Eight of 27 positive equine serum samples were confirmed by VNT. No WNV RNA was detected in birds or mosquitoes.

**Conclusions:** The occurrence of WNV antibodies in autochthonous equines from all regions of Kosovo indicates that the virus is circulating within the country. Public health authorities should therefore plan a risk assessment and disease control program.


# Background

West Nile virus (WNV) is an arthropod-borne virus of the Flaviviridae family with worldwide distribution [1]. The natural cycle of infection involves many species of birds and mosquitoes. In Europe, these are predominantly mosquitoes from the genus Culex [2, 3]. Birds are the natural reservoir of the virus, serving as amplifying hosts, while humans and horses are considered accidental or dead-end hosts [4]. WNV infection is often asymptomatic in humans and equines, with approximately 20% of human cases showing febrile syndrome and neuroinvasive disease in less than 1% of patients [5].

While 9 genetic lineages of WNV have been identified, only lineages 1 and 2 appear to have significant medical importance [6]. Both lineages are considered endemic in Europe, based on studies and reports of WNV infection in humans, horses, and birds [7]. Recent studies report that lineage 2 of WNV is dominant in Europe, including Balkan countries [8, 9]. Recent WN disease outbreaks have caused great concern for Europe's public health authorities [10, 11].

While WNV antibodies were first detected in Europe from Albania in 1958, WNV infection was only sporadically detected until 1996, when a large outbreak occurred in Romania [12, 13]. Since 1996, WN virus from humans, equines, birds, and mosquitoes has been detected or isolated in many European countries.

WN human cases in Kosovo were first reported in 2012, with four patients being confirmed clinically and in the laboratory. Between 2013 and 2015, 14 infected people were identified [14]. The latest outbreak of WNV infection in humans in Kosovo was in 2018, with 14 human cases, including three deaths [11].

Data about WNV infection in animals and mosquitoes is lacking in Kosovo. The first study in the country, in 2010, which serologically tested 29 equine serum samples, found no WNV antibodies. The same study found a seroprevalence rate of 22% in horses in neighboring Albania, although no antibodies were detected in birds [15]. Globally, WNV has been detected in more than 300 bird species, while over 75 mosquito species are competent

vectors for virus transmission [5, 16]. In Kosovo, the most predominant and abundant mosquito species is Culex pipiens, representing around 40% of the distribution of 13 identified species in the country [17]. No WNV infections have yet been documented in animals in Kosovo.

The aim of this study was to investigate the presence of WNV in horses, mosquitoes, and birds in Kosovo.

## Material And Methods

# Sampling

Equine sera: 260 equine blood samples (251 horses, 7 donkeys, and 2 mules; mean age = 10.9 years, range = 1–32 years) were collected from seven regions of Kosovo between January and September 2018: Prishtina n = 32, Mitrovica n = 28, Peja n = 25, Prizren n = 47, Gjakova n = 12, Ferizaj n = 50, and Gjilan, n = 66. The samples originated from 177 different animal owners. There are 2,353 equines in Kosovo [18]. Blood samples were collected randomly from healthy horses with no history of WN diseases or symptoms of neurological disorders. Sera were kept at -20 °C until use.

Bird sampling: 50 sera of domestic birds (backyard chickens) were collected. The birds, aged from 1 to 3 years old, were purchased from free-range farms. After slaughtering, 5 ml blood was collected from each animal while 5 g of viscera tissues from the brain, liver, heart, kidney, and lungs were pooled and stored in -80 °C freezer. In addition, 51 wild bird samples from the Corvidae family were collected randomly, as found dead by hunters and stored in -20 °C freezer. The birds' viscera (brain, liver, heart, kidney, and lungs) were pooled and stored at -80 °C.

Mosquito sampling: Most of the mosquito samples were collected from the same farms as the equine samples. Before RT-PCR screening, all mosquitoes were pooled from 5 to 30 individuals, depending on species and collection sites. Collections were made using an Insect Monitoring Trap (IMT) with dry ice. Collected specimens were stored in a -20° C freezer. The 626 mosquitoes (60 pools) were grouped in five genera, including Culex spp. (226 individuals; 26 pools), Aedes spp. (136 individuals; 16 pools), Anopheles spp. (184 individuals; 7 pools), Culiseta spp. (34 individuals; 4 pools), and Phlebotomus spp. (46 individuals; 7 pools). The sampling period coincided with the peak activity period for these mosquito species. Adult specimens were morphologically identified to species level using interactive identification keys for mosquitoes of the Euro-Mediterranean Region [19].

# Serological Testing

ELISA (Enzyme-linked immunosorbent assay): Sera were tested for WNV-specific antibodies using a commercially available ELISA-blocking enzyme immunoassay, which allowed recognition of WNV-IgG-antibodies (INGEZIM west Nile COMPAC, Madrid, Spain). ELISA was performed according to the manufacturer's instructions.

PRNT (Plaque Reduction Neutralization Test): All ELISA-positive serum samples were then subjected to PRNT to confirm the presence of virus specific antibodies. The test was performed on two-fold dilutions of the serum samples (starting from 1:20) against a 100 pfu/100 ∝L diluted NY99 (lineage 1) strain of the WNV. Serum samples blocking 90% of plaque occurrence were considered positive for WNV-specific antibodies.

VNT (Virus Neutralization Test): eight ELISA and PRNT positive horse sera were then confirmed by virus neutralization test (VNT), with neutralizing antibody titers from 1:320 to 1:1,280, using WNV strain NY99 and USUV strain 1477 on Vero E6 cells [23].

# RT-PCR Testing

RNA extraction from mosquito and bird organs: the mosquito pools (5–30 individuals) and 0.5 g of bird organ (0.1 g of brain, lung, heart, kidney, and lungs) were placed in a 2 ml Eppendorf tube containing three to five steel beads (7 mm, Qiagen, Hilden, Germany), frozen on liquid nitrogen and directly pulverized in a Tissue-Lyser LT (Qiagen, Hilden, Germany) with a − 20 °C pre-cooled rotor at 50 Hz for 2–5 min. Repeated freezing and lysis was necessary in some cases. The samples were then re-suspended in 300–500 µl PBS (depending on tissue size) containing 500 IU/ml penicillin, 10% fetal calf serum, and 500 µg/ml amphotericin, and centrifuged at 2,000 × g. Viral RNA extraction was done with QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

RT-PCR: 25 µl RNA of each sample extractions were tested using RealStar® WNV RT-PCR Kit 1.0, (Altona Diagnostics, Hamburg, Germany) for the detection of West Nile virus (WNV) RNA, according to the manufacturer's instructions.

## Results

Of the 260 equine serum samples, 10.38% (27 out of 260) tested positive for WNV-specific antibodies by ELISA. These samples were further tested for neutralizing antibodies using Plague Reduction Neutralization Test (PRNT) with titers ranging from 1:20 to 1:80. Samples testing positive from both the ELISA and PRNT were considered as positive for WNV-specific antibodies. Eight positive equine sera samples were then confirmed by VNT.

The results revealed that WNV-seropositive horses are widespread in Kosovo. WNV antibodies were detected in horses from seven regions with different seroprevalence rates. The highest rate was in Peja 20.00% (5/25), followed by Mitrovica 17.86% (5/28), Gjakove 16.67% (2/12), Ferizaj 10% (5/50), Gjilan 9.09% (6/66), Prizren 6.38% (3/47), and Prishtina 3.13% (1/32). No positive samples were detected from donkeys or mules.

One domestic bird (2%) resulted positive for WNV antibodies out of 50 chickens tested, near to Kosovo's capital city, Prishtina.

Table 1
Results for WNV in equines, birds, and mosquitoes in Kosovo.

| Region | Equines | | | Domestic birds | | | Mosquitoes | | Wild Birds | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Serological testing | | | | | | RT- PCR testing | | | |
| | No. Tested | ELISA Positive | PRNT Positive | No. Tested | ELISA Positive | PRNT Positive | No. pools Tested | RT-PCR | No. Tested | RT-PCR |
| Mitrovicë | 28 | 5 | 5 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Gjilan | 66 | 6 | 6 | 8 | 0 | 0 | 6 | 0 | 0 | 0 |
| Ferizaj | 50 | 5 | 5 | 3 | 0 | 0 | 13 | 0 | 0 | 0 |
| Gjakovë | 12 | 2 | 2 | 3 | 0 | 0 | 6 | 0 | 0 | 0 |
| Prizren | 47 | 3 | 3 | 8 | 0 | 0 | 7 | 0 | 0 | 0 |
| Pejë | 25 | 5 | 5 | 12 | 0 | 0 | 13 | 0 | 0 | 0 |
| Prishtinë | 32 | 1 | 1 | 16 | 1 | 1 | 14 | 0 | 51 | 0 |
| Total | 260 | 10.38% | 100% | 50 | 2% | 2% | 60 | 0% | 51 | 0% |

The most abundant mosquito species trapped close to WNV-positive locations for equines was Culex pipiens (in 21 out of 27 locations), representing 36% of all mosquito species. In contrast, no gRNA was detected by RT-PCR in 60 pools.

None of the 51 wild birds or 50 domestic birds was positive for viral RNA by RT-PCR.

# Discussion

This study provides the first report of WNV seropositivity in equines and birds in Kosovo. The wide distribution of WNV antibodies in autochthonous equines from all regions of Kosovo indicates that the virus is already circulating within the country. The clinical history of WN clinical disease in ELISA-positive horses has not been previously reported. A study in Kosovo from 2010 detected no antibody response to WNV infection in serum samples collected from 29 horses [15]. However, this is most likely due to the small sample size.

Surveys from neighboring countries have detected the following WNV infection rates in healthy equines: Croatia 0.41% (2007), Serbia 12% (2009/10), and Albania 22% (2010) [15, 20, 21].

WNV appears to have been introduced into Kosovo between 2008 and 2012, with the first reported human cases in 2012, when four patients were confirmed by laboratory tests [14]. WNV was introduced through the migratory birds before amplifying within local bird populations, which have become endemic sources of WNV in Kosovo [22].

Kosovo's most abundant mosquito species is Culex pipiens (40%) of the 13 identified species in the country [17]. In our study, too, the most frequent species was Culex pipiens (36%), which appeared in 21 out of 27 sampled

locations (78%) where equines tested positive for WNV. Our survey did not detect viral genomic RNA by RT-PCR, perhaps due to the small sample size for mosquitoes and birds.

Further surveillance studies in vectors and animals are thus necessary to clarify which WNV linages are circulated between birds and mosquitoes to assess the risk for humans and animals.

# Conclusions

This report provides the first evidence of WNV antibodies among autochthonous equines and birds in Kosovo. The dominant mosquito species was *Culex pipiens,* which has been identified as a main WNV vector in Europe. The occurrence of WNV in Kosovo should be a serious concern for public health authorities in planning their strategies for disease prevention and control.

# Abbreviations

ISA- Enzyme linked immunosorbent assay; IgG- Immunoglobuline G; RT-PCR- Reverse transcription polymerase chain reaction; RNA- Ribonucleic acid; WNV- West Nile virus.

# Declarations


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### Author Contributions

A.R., K.SH, B.X., K.B., and N.M-B. collected samples in the field.

K.SH., B.X., P.E., R.P., K.B., and A.O. performed laboratory analyses and interpreted the results.

A.R., K.SH., coordinated the study and drafted the manuscript.

All authors reviewed and approved the final version of the manuscript.

### Ethics declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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## Figures

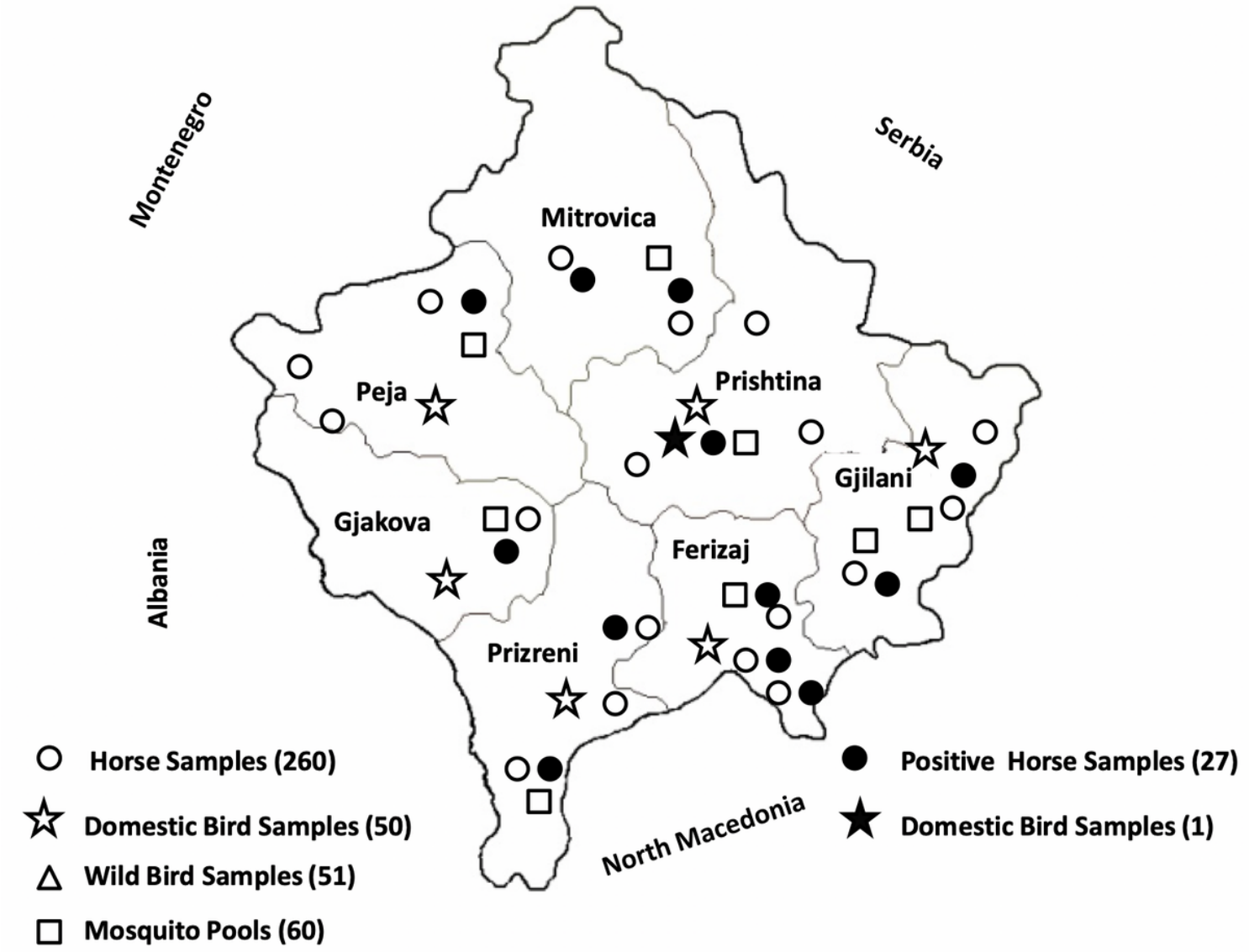



Figure 1

Geographical distribution of WNV-positive antibodies in equines and birds in Kosovo