

Serosurveillance of some emerging RNA viruses among horses in northwest Nigeria

By

Olaolu Tosin Olufemi

As part of PhD thesis at the School of Veterinary Medicine and
Science, University of Nottingham, United Kingdom

May 2023

Primary supervisor: Professor Janet Daly

Secondary Supervisors: Dr Stephen Dunham and Dr Alexander
Tarr

Abstract

Viruses of zoonotic and economic importance continue to expand with a growing threat to animal and human health globally. Monitoring and surveillance provide an early warning strategy for the prevention and control of these emerging viruses of public health importance. This cross-sectional study aimed to investigate the sero-epidemiology of equine influenza virus (EIV), West Nile virus (WNV) and *Equine Hepacivirus* (EqHV) in northwest Nigeria. A total of 830 horses were bled on a single occasion in Kaduna, Kano, Katsina and Sokoto States between October 2019 and January 2020. Antibodies to influenza A/equine/Richmond/2007 (H3N8) and A/equine/Worcestershire/2019 (H3N8) propagated in embryonated hen's eggs were measured using single radial haemolysis (SRH). A prevalence of 71.3% (95% 68–74%) for antibodies to equine influenza (EI) was detected with a mean SRH value of 78.2mm² (median = 88 mm², interquartile range = 0–121 mm²). Age of horses was significantly associated with seropositivity for EI. The burden of EI in northwest Nigeria is abundant with a 71.3% exposure among horses. Only 9% of the horses however had sufficient antibodies (SRH value > 150 mm²) protective against clinical disease or virus shedding.

Vaccination of horses with the circulating strain(s) should therefore be considered. A systematic review and meta-analysis of WNV seroprevalence studies among equids in Europe (2001 – 2018) was conducted followed by a scoping review on WNV antibodies among African equids. Antibodies to WNV and other flaviviruses were detected with the ID screen® West Nile competition multi-species ELISA (cELISA). Also, plant-based WNV NS1, WNV mutant envelope - 'Equad' and EqHV-core proteins ELISAs were used to screen a subset of 691 sera for antibodies against WNV and EqHV. Despite an observed seroprevalence of 9.8% (2765/28089) and 35% (1998/5746) in Europe and Africa, respectively, WNV among European equids (39 studies) was extensively more investigated than in African equids (16 studies). This is evidenced by the varying geographic widespread and number of studies in the two regions. Although both regions mainly used cELISA, only 37.5% of studies conducted on African samples probed further for specific flaviviruses (compared with 66.7% of studies conducted in Europe). With an overall WNV seroprevalence of 94.3% (783/830) obtained using cELISA, age and state were risk factors for flavivirus infection. WNV NS1 and Equad ELISAs revealed 33.9% (234/691) and 12.6% (89/691)

seroprevalence, respectively suggestive of more specificity than the cELISA. The seroprevalence ranging between 12.6%–94.3% revealed by the ELISAs establishes the presence of WNV and related flaviviruses in the study area and suggests a potential zoonotic threat. Plant-based ELISAs are cheap options to be considered for serosurveillance of WNV and related viruses among sentinel hosts. Antibodies to EqHV (*Hepacivirus equi*) were detected in 12% (83/691, 95% CI; 10–15%) of the horses screened. State and purpose of horses were associated with EqHV seropositivity. This project to the best of my knowledge is the first to describe the circulation of EqHV in Nigeria demonstrating the potential of plant-based ELISA for the serosurveillance of emerging viruses in developing countries. EI, WNV and EqHV are abundant among horses in northwest Nigeria. Continuous monitoring and surveillance for these emerging diseases with a one-health strategy is recommended to safeguard human and animal health.

Acknowledgement

Firstly, I would like to thank my supervisors. I deeply appreciate Professor Janet Mary Daly, a supervisor turned mentor. Focusing on a PhD at the start of a pandemic was made possible by your selfless commitment to impacting lives and transforming generations through dedication, motivation, patience, and excellent guidance. Thank you, Janet, for exemplifying competence, leadership and commitment. Your support inspiring me to work hard all the way. I am now more than a 'PCR specialist'.

Many thanks to my secondary supervisors: Dr Alexander Tarr and Dr Stephen Dunham for their immense support on this project. Alex, my time in your laboratory at the medical school would forever be cherished. The hospitality, reception and conducive environment provided for me by you and every member of the laboratory is a testament to the kindheartedness and professionalism of the team.

I would like to appreciate the University of Nottingham for the opportunity to complete my study here especially as a recipient of the Vice Chancellor's Research Excellence Scholar (International). I sincerely thank staff members of the School of Veterinary Medicine and Science, especially administrative

and technical staff who made my time memorable. Special greetings to Rachael Humphreys (and her wonderful team) who helped me with all necessary support from the start of this program, through the pandemic and afterwards.

I am grateful for the support I enjoyed from wonderful personalities in my group and in our laboratory. I appreciate Dr Ge Wu, Dr Anitha Varghese, Dr Mirelle Radley, Dr Meshach Maina and Dr Ola Elbony who helped me take off smoothly in the laboratory. I also thank Dr Toshana Foster, Dr Rob, Dr Karina Bingham, Dr Askar Alshammari, Ika Nurziah, Dr Ahmed Mahmoud and Hope Everest. I will forever cherish the moments we spent together. Though mostly in the workplace, I am hopeful that we get to spend more time at more social events in the near future. Sharing space with my seat mate Shannon Ward in the company of Dr Kieren Pitchers was awesome, thank you guys.

It was a great privilege to have Dr Marnie Brennan as an internal assessor, whose scholarly advice and scrutiny were always on point. Thank you, Marnie, for your commitment and dedication to this study. Thank you, Prof Thomas Chambers, for your helpful evaluation and constructive feedback. I also would like to thank Dr Richard J Newton from the University of Cambridge

for being part of this project, especially for providing robust guidance on the epidemiology and statistics of this study. I am grateful to my teachers, colleagues and friends in Ahmadu Bello University, Federal University Wukari and University of Jos.

I remain grateful for the gift of my father Engr Samuel Akingbade Olufemi, who sees things differently and beyond. Thank you to my mothers especially, Mrs Ayanfe Veronica Olufemi, Mummy Joko Jenyo, Mrs Wuraola Adeyanju and Mrs Christiana Olowodun. Thank you mummies for helping with Tehillah during your visits, this took a lot off our shoulder. God bless you richly. I am thankful to the Pastorate and members of RCCG, Rehoboth House Nottingham and especially New Dawn Choir, you gave me a place to always refresh my mind. My 'together forever family' - The Bellos, thank you for demonstrating familyhood. Thank you Dr Yemisi Adedeji for always checking up on me; you have consistently showed me what I did not pay attention to. Thank you. I am grateful to your husband Mr Tomi Adedeji for his support. My lovely wife Rhoda has been the most accommodating and loving: Sweet, you are indeed my helpmeet, right from inception and throughout the PhD, you remain loving, sacrificing for me and the kids. We love

you dearly, thank you. To my first day cheer leader, Yetty Mama, I know you are smiling from heaven thank you for guiding and leading me. May your gentle soul continue to rest in peace. This degree is our testimony and a testament of God's faithfulness.

To the God who created the heavens and the earth, I remain thankful. Thank you, Lord, for coming through for me. I trust you for the days ahead.

Declaration

I declare that this thesis titled *Serosurveillance of some emerging RNA viruses among horses in northwest Nigeria*; is my work and a result of my own effort. This thesis has not been submitted anywhere for any academic degree or professional qualification. Any ideas and text from others have been duly acknowledged. Data relating to this thesis has been archived in the Nottingham Research Repository: *Variables of horses and serostatus to some emerging equine RNA viruses in northwest Nigeria* <http://doi.org/10.17639/nott.7342>.

Olaolu Tosin Olufemi

May, 2023

Table of contents

Abstract	ii
Acknowledgement	v
Declaration.....	ix
Table of contents	x
List of figures	xiii
CHAPTER 1.....	1
Introduction	1
1.1 Background	1
1.2 Study area, study population and study design	2
Study Design.....	6
1.3 Emerging and re-emerging equine viruses – why serosurveillance?	7
1.4 Aims and Objectives	16
CHAPTER 2.....	18
Seroprevalence of equine influenza and its associated risk factors in north-western Nigeria.....	18
CHAPTER 3.....	19
Systematic review and meta-analysis of seroprevalence studies of West Nile virus in equids in Europe between 2001 and 2018.....	19
CHAPTER 4.....	20
A scoping review of West Nile virus seroprevalence studies among African equids	20
CHAPTER 5.....	21
Serosurveillance of West Nile virus and other flaviviruses among horses in northwest Nigeria.....	21

CHAPTER 6.....	22
Serosurveillance of West Nile virus and hepatitis A among horses in northwest Nigeria using ELISA based on plant-expressed proteins	22
CHAPTER 7.....	23
Summary	23
Appendix A.....	36
Sampling and data collection	36
Appendix A.1	38
Propagation of A/equine/Richmond/07 (H3N8) and A/equine/Worcestershire/2019 (H3N8)	38
Appendix A.2	41
Hemagglutination assay	41
Appendix B.....	43
Single radial haemolysis assay (SRH)	43
Sensitization of sheep RBC with A/equine/Richmond/07 (H3N8)	44
Preparation of Assay plates	45
Performing the SRH assay	45
Appendix C.....	48
Plant expression of WNV recombinant proteins (Mutant E (equad) and NS1).....	48
Infiltration of <i>Nicotiana benthamiana</i> with pEAQ-HT-Equad and pEAQ-HT-NS1	49
Extraction and Purification of <i>Nicotiana benthamiana</i> leaves infiltrated with pEAQ-HT-Equad and pEAQ-HT-NS1.....	51

Appendix D	54
Development and Optimization of WNV equad and NS1 Indirect ELISAs	54
Antigen Coating/Coating buffer	54
Blocking Buffers	54
Sera/Sera dilutions.....	55
Conjugates/Substrate	56
Controls	56
Protocol for developed plant based ELISA.....	60
Appendix E.....	62
Data Analyses (Regression analyses).....	62
Bibliography	64

List of figures

Figure 1. 1 Map showing African countries with Nigeria highlighted in the western region 3

Figure 1. 2 Map of Nigeria showing the study area with different colour shading used for Kano, Sokoto, Katsina, Jigawa, Kebbi and Zamfara States 4

Glossary

Purposive sampling: a non-probability sampling involving a prior judgement about the target population. Only apparently healthy horses were sampled in this study.

Gate-keeper sampling: a purposive sampling method involving prior permission from one who often represents the interest of others and allows access to them.

Snowball sampling: involves obtaining samples from an 'unclear' or discrete population where one sample unit leads (snowballs) to another.

Convenience sampling: is a non-probability sampling technique involving sample units that are accessible.

Epidemiology: is the study of disease patterns and associated risk factors that determine disease spread in a given population.

Seroepidemiology: is the study of epidemiology based on serological investigation of changing levels of antibodies to infectious agent(s).

Serosurveillance: is the systematic collection, collation, analyses, interpretation and dissemination of data obtained from seroepidemiological investigations for the benefit of public health.

Seroprevalence: is the proportion of host+ (horses) infected in a defined population at a given period, based on serological investigation.

CHAPTER 1

Introduction

1.1 Background

The purpose of this project was to investigate antibodies to some emerging and re-emerging equine viruses: equine influenza (EI), West Nile virus (WNV) and *equine Hepacivirus* (EqHV), in northwest Nigeria. While equine influenza and West Nile virus have a global spread, less is known about the epidemiology of *equine Hepacivirus*.

Northwest Nigeria is arguably home to the majority of Nigerian equids, but there is little data on the viruses affecting horses in the region. This study intended to provide up-to-date information that will enlighten policy makers on a national strategic plan for routine monitoring of EI, WNV and related viruses.

1.2 Study area, study population and study design

Nigeria is located in sub-Saharan Africa (Figure 1.1) with an estimated population of 200,964,000 people (UN 2019) and covers a landmass of 924,000 km². With over 250 ethnic groups; the Hausa and Fulani ethnic group majorly occupy Northern Nigeria and represent about 29% of the nation's population (World Bank Document 2016).

Sampling was conducted in northwest Nigeria, which comprises of seven states namely: Kaduna, Kano, Katsina, Kebbi, Jigawa, Zamfara, and Sokoto (Figure 1.2). Due to security reasons, only five states (Kaduna, Kano, Katsina, Jigawa and Sokoto) were accessible at the time of sampling; four states (Kaduna, Kano, Katsina and Sokoto) gave consent to sample. These four states are home to some of the historical and ancient emirates in Northern Nigeria; Zazzau emirate; Kano emirate, Daura emirate and Sokoto Caliphate. Northwest Nigeria borders the Republic of Benin to the west and Niger to the north. Equids are abundant in the north-west where they are used as draft animals, for sports and traditional and ceremonial purposes, especially in the emirates.



Figure 1. 1: Map showing African countries with Nigeria highlighted in the in the western region. Created using <https://mapchart.net//africa.html>.

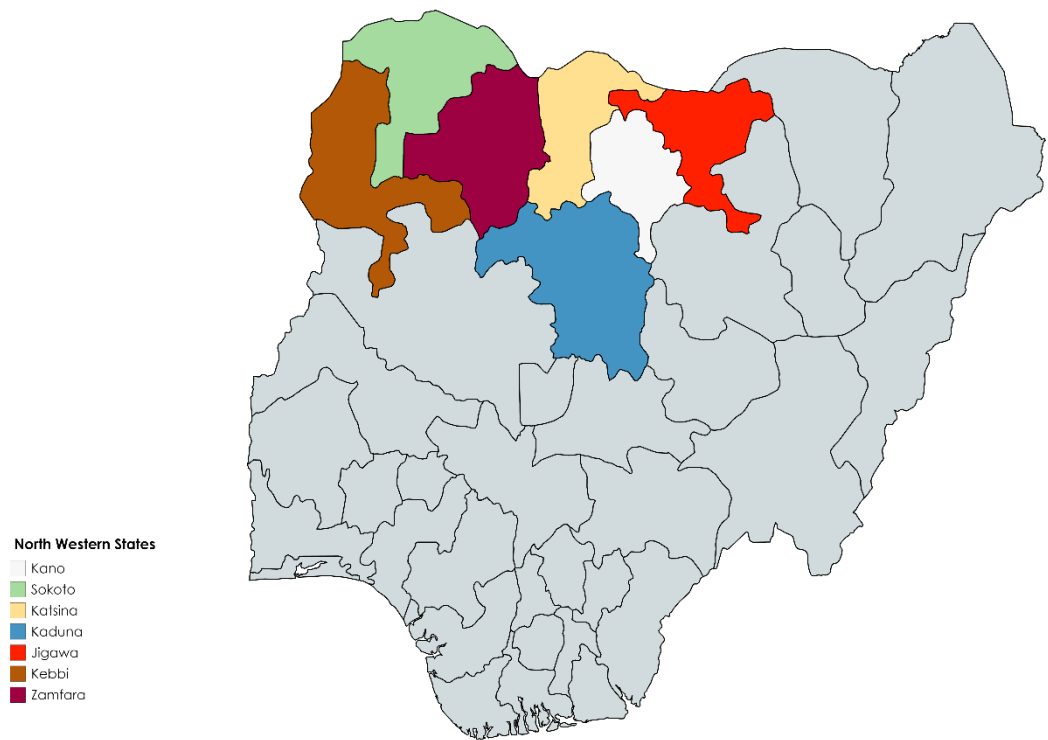


Figure 1. 2: Map of Nigeria showing the study area. Created using <https://mapchart.net//africa-detailed.html> with different colour shading used for Kano, Sokoto, Katsina, Jigawa, Kebbi and Zamfara States

Nigerian native horses belong to the Arab breed (Bukar et al., 2007) and are mostly used for leisure, research, security, sports, transportation and cultural events (Ehizibolo et al., 2011). Horses in northern Nigeria are associated with affluence and royalty with often engagement in traditional festivals – durbar (Bukar et al., 2007). Horses are often tethered in shades that serve as stables. Some horses have roofed housing with grooms living within the premises along with other domestic animals; pigeons nesting in horse sheds; turkeys, chicken and chicks brooding in pen, ruminants grazing freely in open field where horses are kept; free roaming guard dogs and bats roosting on trees providing shade for horses. These contacts prompted by equine management systems in tropical environments provide favorable conditions for the transmission of emerging equine viruses considering their inherent propensity to cross species. There could be a connection between the sero-epidemiology of these emerging equine viruses and the presence of other animals in North-western Nigeria.

Although there are successful efforts towards the development of WNV vaccines in horses (Saiz, 2020), equids in Nigeria, are not vaccinated against WNV. Also, EI is not routinely vaccinated

against. However, expensive horses imported from Argentina, Spain, South Africa, Chad, Sudan, and Niger for polo and racing often come with an up-to-date EI vaccination status. Wealthy and informed horse owners often secure and administer influenza vaccines following international travel (Adeyefa and McCauley, 1994). With the 2018/2019 outbreak being the first report of the OIE listed disease in Nigeria (World Organisation for Animal Health, 2019), a serosurveillance of some emerging RNA viruses among horses in northwest Nigeria will contribute towards an early warning and early detection thus minimizing economic losses due to diseases and safeguarding equine and human health.

Study Design

A cross-sectional approach was used to obtain serum samples from equids in north-western Nigeria and assess the risk factors for exposure to these emerging viruses.

This study received an approval from the School of Veterinary Medicine and Science Ethics Clinical Review Panel (approval

number: 2635181112), University of Nottingham, United Kingdom.

Consent was also sought, and approval secured, from the various Emirate councils in the study area and local authorities. Private horse stables, especially for polo, were accessed through resident veterinarians and veterinarians from the State Ministries of Agriculture. Jigawa State was not responsive. Four states (Kano, Kaduna, Katsina and Sokoto) in the northwest were visited for sampling.

1.3 Emerging and re-emerging equine viruses – why serosurveillance?

In the past decades, emergence and re-emergence of infectious diseases have become a public health threat requiring a global collaborative response (Pollard, 1995, Longbottom, 1997). Emerging infectious diseases are diseases caused by infectious agents that have freshly appeared in a population or have existed but rapidly increased their occurrence and geographical reach (Morse and Schluederberg, 1990, Morse, 1993).

Equids are single toed hindgut fermenters of plant cellulose which belong to the family Equidae and genus *Equus*. They include

domesticated horses and donkeys as well as wild asses and zebra; likely to have originated nearly 4.5 million years ago (Orlando, 2015). Horses (*Equus caballus*) are susceptible to a range of emerging viruses, most of which have zoonotic potential (Bender and Tsukayama, 2004). Equids often share these emerging viruses with humans. Although this makes them potentially at risk of transmitting these emerging infections to humans (and *vice-versa*), it is thought that because of the long history of interaction between domesticated equids and people, most interspecies transmission of viruses already known to affect both species occurred in the past. Equids can therefore serve as sentinel species for some infectious diseases of humans. Surveillance among dogs have proven the species as beneficial sentinels for monitoring WNV and related flaviviruses (Resnick et al., 2008, Durand et al., 2016) Serosurveillance provides a time point glance at the burden of infectious disease thus providing an early warning and early detection of the potential threat and possible public health implications. This allows stakeholders to prioritize and channel scarce resources towards monitoring, prevention and control of

emerging and re-emerging diseases of economic and public health importance.

Equine influenza (EI) remains a re-emerging threat that is being overlooked even with its economic and public health importance as well as zoonotic potential (Sack et al., 2019). In 2018/2019, EI, re-emerged in northwest Nigeria after the last reported outbreak in 1991, in Ibadan, southwest Nigeria, which was the first documented outbreak of EI in Nigeria with three viruses belonging to the Eurasian lineage of the H3N8 subtype (A/equine/Ibadan/4/91, A/equine/Ibadan/6/91 and A/equine/Ibadan/9/91) isolated (Adeyefa and McCauley, 1994). Phylogenetics revealed that A/equine/Ibadan/4/91 and A/equine/Ibadan/6/91 are similar to the virus isolated in Europe between 1989 and 1991 and related to viruses predominant in Europe since 1985 while A/equine/Ibadan/9/91 is closely related to viruses isolated in Europe after 1991 (Adeyefa et al., 1996b). Isolates from the recent outbreak belong to Florida Clade 1 (Shittu et al., 2020).

A national survey covering 1990–1993 discovered antibodies to H3 circulating among horses and donkeys in Nigeria (Adeyefa et al.,

1996a). There are reports of antibodies to H7 detected among polo horses in Nigeria (Olusa et al., 2010). A recent serological study also revealed antibodies to H3 and H7 among horses in Kaduna, North western Nigeria (Meseko et al., 2016). Although there have been serological reports of EI among equids across Nigeria, the recent outbreak reported by the World Organisation for Animal Health (WOAH) prompted the serosurveillance study, conducted as part of this project, to determine the protection level of horses in northwest Nigeria (Chapter 2).

Similarly, flaviviruses continually pose a threat to human and animal populations with a potential for disease emergence and re-emergence; West Nile virus (WNV) has an established public health importance globally (Shomaker et al., 2013). Epidemiological understanding is expedient to prevent infection and forestall emergence (Pierson and Diamond, 2020). WNV amongst other related flaviviruses cause equine encephalitis. These public health challenges pose a 'one health' risk to humanity in the 21st century (Centers for Disease Control and Prevention, 1998), endangering animal and human health. Currently, there are no vaccines against WNV in humans (Scherwitzl et al., 2017); raising concerns about

the potential for WNV emergence and reemergence makes monitoring antibodies in sentinel animals a mitigating strategy that will provide stakeholders with up to date status of the disease and the risk it poses to humans and other animals. The burden of flaviviruses is widespread especially in tropical nations where arthropod vectors are abundant. Knowledge on the spill over of WNV and related virus in sentinel host like horses could serve as a preparedness plan for the prevention and control of the disease among human host. Horses in northwest Nigeria were screened for antibodies to WNV and EqHV. This study attempted to ameliorate the limitation of cross-reactivity between antibodies hampering ELISAs in serodiagnosis of flaviviruses, while maximising the strengths (simplicity, sensitivity and rapidity) of the assay (Beck et al., 2013). Clones of WNV (non-structural protein 1, NS1 and modified envelope, Equad) and EqHV (core, C) proteins were transiently expressed in plants as a cheap option and used to develop in-house ELISA to detect antibodies to WNV and EqHV among horses in northwest Nigeria.

In this project, initially a systematic review of WNV in Europe was conducted (Chapter 3) from which it was apparent that most

studies (24/38) employed the commercially available ID-Vet ELISA to screen sera. Some studies used viral neutralization test (VNT)/ plaque reduction neutralization test (PRNT) and screened for other viruses like tick-borne encephalitis virus (TBEV) and Usutu virus (USUV) alongside WNV. In contrast, we could only scope for evidence on the seroprevalence of WNV among African equids (Chapter 4) as there were limited articles. The scoping review reaffirmed the use of ID-Vet ELISA in most studies (14/16). Other screening and/or confirmatory tests used included PRNT/VNT, western blotting (WB), microneutralization test (MNT), microsphere immunoassay (MIA) and complement fixation test (CFT). Some studies additionally screened for other flaviviruses like TBEV, USUV, Japanese encephalitis virus (JEV), Potiskum virus (POTV), Wesselsbron virus (WESSV) and Yellow Fever virus (YFV) in horses in Africa. The scoping review also unveiled a huge burden of flaviviruses among African equids. Screening for these viruses would be prohibitively expensive. Although VNT/PRNT is more specific than ELISA, the need for a specific antigen (for each virus), higher level of containment and cell culture is expensive and not practicable for routine serosurveillance. Though readily available

commercially, the cost of using WNV ID-Vet ELISA for serosurveillance is also substantially high, especially for surveillance in a developing nation like Nigeria. In December 2021, the cost of a kit containing four pre-coated 96-well plates was £1,118. Being zoonotic and a hazard group 3 pathogen complicates WNV manipulation in facilities without containment. Therefore, an ELISA using plant-expressed viral proteins was developed as a cheap alternative to the use of a category 3 facility for screening for WNV and EqHV specific antibodies among horses in northwest Nigeria (Chapter 6).

While EI and WNV have a global spread, relatively little is known about the epidemiology of equine Hepacivirus (EqHV), which was only discovered in 2011. Closely related to the Hepatitis C virus (HCV), EqHV was earlier called canine hepacivirus (CHV) as its was first isolated in dogs with respiratory disease outbreaks in the USA (Burbelo et al., 2012) before it was discovered to naturally infect horses, after which it was renamed non-human primate hepacivirus (NPHV). When it was realised that it predominantly infects equids, it was renamed EqHV. Equine hepacivirus belongs to the genus *Hepacivirus* and is a member of the family Flaviviridae

along with three other genera: *Flavivirus* (to which WNV belongs), *Pegivirus* and *Pestivirus*. Bats and rodents have been identified as probable major natural reservoir host for hepaciviruses owing to their vast global distribution and genetic diversity. The transmission cycle of EqHV remains unclear and is yet to be determined (Pacchiarotti et al., 2022).

There are currently no commercial kits to screen for EqHV in horses and routine laboratory diagnosis methods have not been validated based on international best practices outlined by WOA (WOA, 2022a, WOA, 2022b). To date, it has also not been possible to cultivate EqHV in the laboratory to perform cell-based neutralisation tests. However, published serological assays include Gaussian luciferase immunoprecipitation system (GLIPS), Luciferase immunoprecipitation systems (LIPS) and western blot (Pacchiarotti et al., 2022). Viral RNA detection targeting the conserved region of the genome for 5' UTR, NS3 and NS5B using RT-PCR (quantitative and nested) is often used for molecular diagnosis (Pacchiarotti et al., 2022).

From a meta-analysis, prevalence of EqHV has been reported to have a global seroprevalence of 47.1% (Pacchiarotti et al., 2022).

To date, two studies have reported EqHV seroprevalence in Africa (Bezerra et al., 2022); 65.7% in Morocco using Gaussia luciferase immunoprecipitation system (GLIPS) and 83.7% in South Africa using luciferase immunoprecipitation system (LIPS) (Abbadi et al., 2021, Badenhorst et al., 2018). This study, to the best of our knowledge, is the first to investigate EqHV in Nigeria (Chapter 6). Particularly in developing countries such as Nigeria, cheap and affordable screening assays such as those developed for this project could provide reliable data on the burden of flaviviruses and the potential threats to public health.

1.4 Aims and Objectives

The overall aim of this project was to investigate the sero-epidemiology of equine influenza virus (EIV), West Nile virus (WNV) and *Equine Hepacivirus* (EqHV) in northwest Nigeria. The objectives were as follows:

- a) Perform single radial haemolysis of equine sera using EIV isolates propagated in embryonated hens' eggs as antigen to detect serum antibodies.
- b) Perform a systematic review of seroprevalence studies of WNV among European equids
- c) Perform a scoping review of the seroprevalence of WNV among equids in Africa.
- d) Conduct a sero-epidemiological survey of WNV among horses in northwest Nigeria using a commercially available enzyme-linked immunosorbent assay (ELISA).
- e) Develop ELISAs with greater specificity for WNV using plant-expressed modified envelope protein (Equad) and NS1 as antigens to detect antibodies in serum.
- f) Conduct a sero-epidemiological survey of EqHV among horses in north-west Nigeria using plant expressed EqHV-

core protein as antigen in ELISA to detect antibodies in serum.

CHAPTER 2

Seroprevalence of equine influenza and its associated risk factors in north-western Nigeria

Note: This chapter is a published article:

Olufemi OT, Edeh ER, Isyaku MS, Haliru M, Samaila S, Philip Mshelia P, Owolodun OA, J. Richard Newton, Daly JM. Seroprevalence of Equine Influenza and Its Associated Risk Factors in Northwest Nigeria. *Pathogens*. 2022 Nov 17;11(11):1372.

MDPI journals permit the reuse of their articles provided the original article is appropriately cited.

Author contributions: Olufemi OT (42%)*, Edeh ER (2.38%), Isyaku MS (2.38%), Haliru M (2.38%), Samaila S (2.38%), Philip Mshelia P (2.38%), Owolodun OA (2.38%), J. Richard Newton (13%), Daly JM (31%).

*OTO: Field sampling, sample processing, data curation, formal analysis, methodology, Writing- original draft and Writing - reviewing and editing.

Article

Seroprevalence of Equine Influenza and Its Associated Risk Factors in Northwest Nigeria

Olaolu T. Olufemi ^{1,2} , Emmanuel R. Edeh ³, Mustapha S. Isyaku ⁴, Mustapha Haliru ⁵, Shafiu Samaila ⁵, Philip W. Mshelia ⁶, Olajide A. Owolodun ⁷, J. Richard Newton ⁸  and Janet M. Daly ^{1,*} 

- ¹ One Virology, Wolfson Centre for Global Virus Research, School of Veterinary Medicine and Science, University of Nottingham, Loughborough LE12 5RD, UK
- ² Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, University of Jos, Jos 930003, Nigeria
- ³ Department of Veterinary Medicine, Surgery and Radiology, Faculty of Veterinary Medicine, University of Jos, Jos 930003, Nigeria
- ⁴ Faculty of Veterinary Medicine, Bayero University Kano, Kano 700006, Nigeria
- ⁵ Ministry of Animal Health and Fisheries Development, Usman Faruk Secretariat, Sokoto 840103, Nigeria
- ⁶ Department of Veterinary Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria 810107, Nigeria
- ⁷ Biotechnology Centre, National Veterinary Research Institute, Vom 930103, Nigeria
- ⁸ Department of Veterinary Medicine, University of Cambridge, Cambridge CB3 0ES, UK
- * Correspondence: janet.daly@nottingham.ac.uk



Citation: Olufemi, O.T.; Edeh, E.R.; Isyaku, M.S.; Haliru, M.; Samaila, S.; Mshelia, P.W.; Owolodun, O.A.; Newton, J.R.; Daly, J.M.

Seroprevalence of Equine Influenza and Its Associated Risk Factors in Northwest Nigeria. *Pathogens* **2022**, *11*, 1372. <https://doi.org/10.3390/pathogens11111372>

Academic Editor: Nicola Pusterla

Received: 23 October 2022

Accepted: 14 November 2022

Published: 17 November 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Equine influenza (EI) is a fast-spreading respiratory disease of equids caused by equine influenza A virus (EIV), often resulting in high morbidity and a huge economic impact on the equine industry globally. In this cross-sectional study to determine the seroprevalence of EI and its associated risk factors, sera from 830 horses bled on a single occasion in Northwest Nigeria between October 2019 and January 2020 were screened for antibodies to A/equine/Richmond/1/2007 (H3N8) using the single radial haemolysis (SRH) assay. Antibodies were detected in 71.3% (592/830, 95% CI: 68–74%) of horses (SRH area ≥ 0.5 mm²). Although there were statistically significant univariable associations between seropositivity and age, sex, breed, purpose and coat colour, only age remained significant when included with each of the other variables in bivariable analyses. There was a clear trend for increasing odds of seropositivity with increasing age: OR 1.6, 95% CI: 1.05–2.40 ($p = 0.03$) for 5–14-year-olds and OR 8.13, 95% CI: 2.75–24.1 ($p < 0.001$) for ≥ 15 -year-olds compared to horses < 5 years old. The mean SRH value was 78.2 mm² (median = 88 mm², interquartile range = 0–121 mm²) with only 9% of the horses having an SRH value > 150 mm², considered sufficient to protect against clinical disease and virus shedding. Comparative screening of a subset of the horses ($n = 118$) with a 2019 H3N8 virus (A/equine/Worcestershire/2019) revealed a significantly greater seropositivity ($p = 0.0001$) than A/equine/Richmond/1/2007 consistent with exposure of the population during a widespread outbreak of EI in the region in 2019. In conclusion, there was an insufficient level of protection against EI in the region and introduction of a vaccination programme with vaccines containing recently circulating virus is recommended to mitigate against further outbreaks of EI in Nigeria.

Keywords: equine influenza; seroprevalence; Nigeria

1. Introduction

Equine influenza (EI) is a rapidly spreading, emerging and re-emerging respiratory disease of equids [1] caused by equine influenza virus (EIV), a member of the genus *Alphainfluenzavirus* (formerly Influenza A) within the family Orthomyxoviridae [2]. It is globally recognised as causing high morbidity in susceptible equids during outbreaks, resulting in a huge economic impact on the equine industry. Two subtypes of influenza A

virus have circulated in horses, H7N7 and H3N8, which were first isolated in 1956 and 1963 from horses in Czechoslovakia (A/equine/Prague/56) and the USA (A/equine/Miami/63), respectively [3,4]. Given that the last confirmed outbreak caused by the H7N7 subtype occurred in 1979, it is now believed to be extinct in horses or circulating at low levels in limited geographical regions [5–9]. In contrast, since 1980, to date, there have been recurring EI outbreaks caused by the H3N8 subtype, which has become an almost global threat to horses, especially in regions without routine immunization [10,11].

The equine H3N8 subtype started as a discrete genetic and antigenic lineage until divergent evolution (in around 1989) produced two distinct lineages: American and Eurasian (Europe and Asia) [12]. A further divergence of the American lineage produced the Florida and Kentucky sub-lineages [13]. The Florida sub-lineage, which diverged into two clades: Florida clade 1 (FC1) and Florida clade 2 (FC2), represented by A/equine/South Africa/4/2003-like or A/equine/Ohio/2003-like and A/equine/Richmond/1/07-like viruses, respectively, has been responsible for EI outbreaks across the globe [14]. Until recently, FC1 viruses predominated in the Americas and FC2 viruses in Europe. In 2018/2019, widespread outbreaks were caused by FC1 viruses first identified in South America [15] and subsequently in European countries [16].

In Nigeria, the first outbreak of EI was described over three decades ago among 253 horses that converged (from different parts of the country) for a polo tournament in western Nigeria [17]. The isolates from this outbreak corresponded to the strains causing disease in Europe at the time [18]. The widespread EI outbreaks caused by FC1 viruses in 2018/2019 also affected Nigeria [19] and some other countries in West and Central Africa [20]. There have been few serological reports of EI among equids in different parts of Nigeria and those that exist are focussed on stabled horses [21–24]. Most of these reports employed competition ELISA and haemagglutination inhibition (HI) tests. Antibodies against EIV can also be detected using the single radial haemolysis (SRH) assay, which offers a reproducible, reliable and continuous measure of antibody that correlates with protection level to EI. In experimental infection of vaccinated ponies, SRH values above 154 mm² conferred complete protection (no clinical signs or virus shedding) against related EI strains while animals with SRH values greater than 85 mm² did not exhibit clinical disease but still shed virus [25]. Findings from field studies of outbreaks confirmed 150 mm² as the limit above which protection against a related strain of EIV is provided by a vaccine [26,27].

There are about 1.1 million equids in Nigeria (around 200,000 horses and 900,000 donkeys) [28] but there is a paucity of information on the seroprevalence and geographic spread of EI. The 2018/2019 outbreak, which majorly involved equids in North-west Nigeria geopolitical zone, highlighted the need to investigate antibody levels of horses in the region to inform public health plans for the prevention and control of future outbreaks was recognised. Thus, this study investigated the seroprevalence of equine influenza in 830 horses in four of the Northwest Nigerian states and the risk factors associated with SRH seropositivity.

2. Materials and Methods

2.1. Ethical Statement

This study received approval (reference 2635181112) from the School of Veterinary Medicine and Science Ethics Clinical Review Panel, University of Nottingham, United Kingdom.

2.2. Sampling

In this cross-sectional study, blood samples were obtained on a single occasion from apparently healthy horses in four states (Kaduna, Kano, Katsina and Sokoto) in Northwest Nigeria (Figure 1). Horses were sampled based on availability and willingness of horse owners to participate in the study, and all horses included in this study were sampled with consent from relevant authorities and community inhabitants. The horses belonged to farms, individual horse owners, monarchs and their traditional rulers. The samples were

collected from October 2019 to January 2020 during different visits to the four states. In total, 830 horses were aseptically bled by jugular venipuncture, and sera were harvested then heat inactivated before shipping to the United Kingdom. Basic information (age, breed, coat colour, location, use, sex and vaccination status) was recorded for each animal at the time of sampling. Information on influenza vaccination status was inconsistent and unreliable, especially among non-stabled horses; therefore, this was not employed in further analyses.

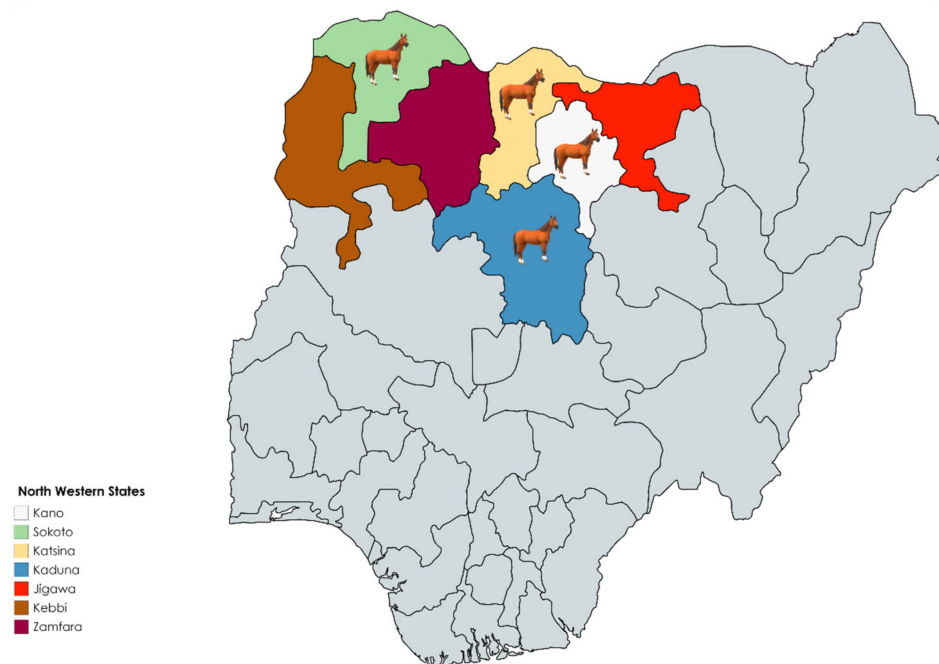


Figure 1. Map showing the seven states that comprise the geopolitical zone of Northwest Nigeria. Samples for this study were obtained from horses in the states of Kano, Sokoto, Katsina and Kaduna. Created using <https://mapchart.net/africa-detailed.html> (accessed on 25 May 2022).

2.3. Single Radial Haemolysis (SRH)

Single radial haemolysis (SRH) assay is an immunodiffusion assay based on virus-coupled erythrocytes. Influenza A/equine/Richmond/1/2007 (H3N8), representative of FC2, was kindly provided by Dr Debra Elton (Animal Health Trust) and A/equine/Worcestershire/2019 (H3N8), representative of FC1, by Dr Neil Bryant (Cambridge University). The viruses were propagated in embryonated hens' eggs and antibodies to both viruses were assessed by performing the SRH assay as prescribed in the World Organisation for Animal Health (WOAH) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [29]. Digital callipers were used to measure the diameter of the zones of lysis before calculating the area (mm²). Serum from a hyperimmune pony was used as a positive control on every plate.

2.4. Statistical Analysis

Overall seroprevalence was estimated by dividing the number of seropositive horses by the total number of horses sampled. Pearson's chi-square test was used to determine the association between explanatory variables and seropositivity to EI. The variables were categorized as follows:

States: Kaduna, Kano, Katsina and Sokoto
 Age: "young" (<5 years); "adult" (5–14 years); "old" (≥15 years)
 Breed: "exotic" (Argentine/Sudan/South African); "mixed" (Talon); "local" (West African Dongola/Arewa)
 Coat colour: "basic" (bay/chestnut/black/brown); "bright" (white/golden/pinto/roan); grey

Use: polo, durbar festival and mixed (more than one use including racing)
 Sex: male; female.

Ordinary logistic regression was used to determine the risk factors predictive of EI seropositivity (the dependent variable). Initially, Pearson's chi-square test was used on cross-tabulated data to examine the association between explanatory variables and seropositivity to EI. Ordinary logistic regression was used to determine the risk factors predictive of EI seropositivity (the dependent variable) with odds ratios (OR) and corresponding 95% confidence intervals (CI) calculated for each variable in univariable analyses. All non-collinear variables with $p \leq 0.2$ were selected for multivariable analyses by ordinary logistic regression conducted using a forward-stepwise approach, with improvement in model fit with the addition of further variables assessed using likelihood ratio tests, with significance for variable retention set at $p \leq 0.05$. Paired *t*-test was used to compare mean differences between paired SRH values obtained using the viruses isolated in 2007 or 2019. All analyses were performed using StataSE (17.1, StataCorp LLC, College Station, TX, USA).

3. Results

3.1. Demographic Characteristics of Horses Sampled in Northwest Nigeria

In total, 830 horses were sampled from Kaduna ($n = 256$), Kano ($n = 314$), Katsina ($n = 122$) and Sokoto ($n = 138$). Age of horses ranged from <3 months to 30 years, with a mean age $\pm$ SD of 8.05 ± 3.75 years. The ages of the 10 horses older than 18 years were estimated rounded to the nearest 5 years, i.e., 20 ($n = 5$), 25 ($n = 3$) and 30 ($n = 2$). In total, 535 (64.5%) of the horses were male and 295 (35.5%) were female. Most male horses were aged between 5 and 11 years and all horses aged <2 years and >15 years were female (Figure 2). Exotic (32.3%), local (38.7%) and mixed (29%) breeds of horses having basic (77.1%), bright (9.6%) and grey (13.3%) coat colours were sampled (Table 1).

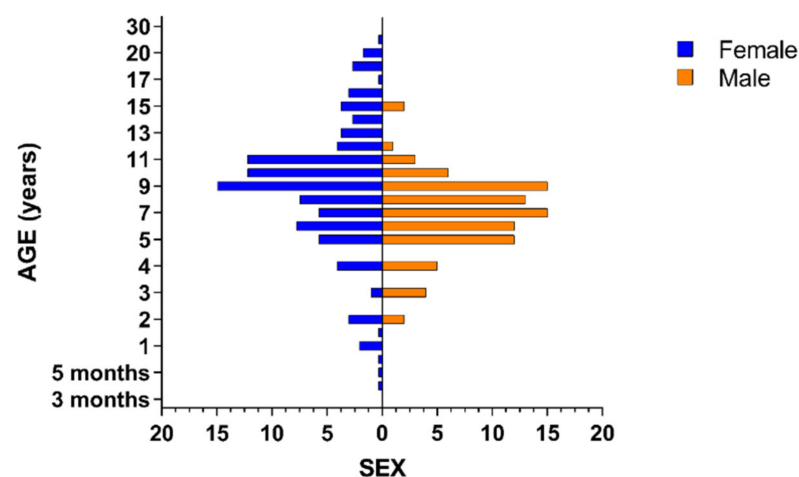


Figure 2. Population pyramid of raw age data versus sex.

Table 1. Factors associated with equine influenza seropositivity measured by single radial haemolysis assay using A/equine/Richmond/1/2007 (H3N8) virus among horses in Northwest Nigeria.

Factor	N	Positive	% Prevalence (95% CI)	<i>p</i> -Value ¹
AGE				
Young (<5 years)	113	69	61.1 (52–70)	0.0001
Adult (5–14 years)	662	472	71.3 (68–75)	
Old (≥15 years)	55	51	92.7 (82–97)	
SEX				
Male	535	369	68.9 (65–73)	0.043
Female	295	223	75.6 (70–80)	

Table 1. Cont.

Factor	N	Positive	% Prevalence (95% CI)	p-Value ¹
BREED				
Local	321	221	68.8 (64–74)	0.033
Exotic	268	207	77.2 (72–82)	
Mixed	241	164	68 (62–74)	
LOCATION				
Kaduna	256	192	75 (69–80)	0.443
Kano	314	221	70.4 (65–75)	
Katsina	122	85	69.7 (61–77)	
Sokoto	138	94	68.1 (60–75)	
PURPOSE				
Mixed	149	99	66.4 (58–74)	0.074
Polo	331	250	75.5 (71–80)	
Traditional Durbar	350	243	69.4 (64–74)	
COAT COLOUR				
Basic	640	469	73.3 (70–77)	0.042
Bright	80	55	68.8 (58–78)	
Grey	110	68	61.8 (52–70)	
TOTAL	830	592	71.3 (68–74)	

¹ p-values < 0.05 indicated in bold typeface, CI = 95% confidence interval, N = total number tested.

3.2. Prevalence of Antibodies against Influenza A/equine/Richmond/1/2007 (H3N8) among Horses Sampled in Northwest Nigeria

Antibodies to influenza A/equine/Richmond/1/2007 (H3N8) were detected in 592 of the 830 horses screened, giving an overall prevalence of 71.3% (95% CI: 68–74%) (Table 1). A mean SRH value of 78.2 mm² (median = 88 mm², interquartile range = 0–121 mm²) was obtained with a range of 0–208 mm² (the lowest area measured was 12 mm²). Only 9% of horses had an SRH antibody value above 150 mm²; 44.5% horses had values between 85 and 150 mm² and 46.5% had values less than 85 mm² (Figure 3).

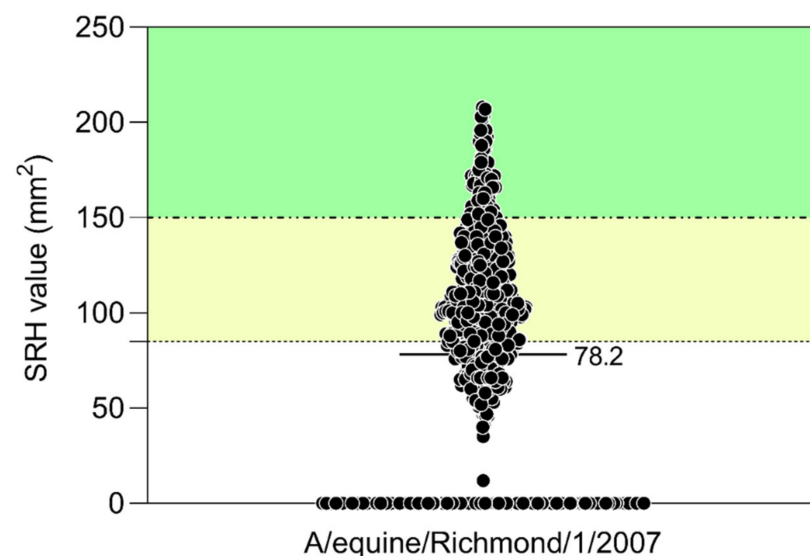


Figure 3. Single radial haemolysis (SRH) antibody values measured against A/equine/Richmond/1/2007 among 830 horses in Northwest Nigeria. Dashed lines and shading indicate cut-off above which clinical and virological protection (---, green shading) or clinical protection only (---, yellow shading) is expected. The horizontal bar shows the mean SRH value.

There were significant differences in seropositivity in horses of different age, sex, breed and coat colour (Table 1). Although not statistically significant, the highest mean SRH values were seen in horses in Kaduna state (75.0%) and in horses used for polo (75.5%).

Univariable logistic regression showed that, compared to the young horses, adult and old horses had an OR of 1.6 (95% CI, 1.05–2.40) and 8.1 (95% CI, 2.75–24.1), respectively (Table 2). Male horses had an OR of 0.7 (95% CI, 0.52–0.99) compared to female horses. “Exotic” breeds of horses had an OR of 1.54, 95% CI: 1.06–2.22 compared to 1.00 and 0.96 for local and mixed breeds, respectively. Grey horses had an OR of 0.59 (0.39–0.90), which was significantly different ($p = 0.015$) to “basic”-coloured horses (the referent category) whereas the OR for “bright” coloured horses (0.86) was not significantly different.

Table 2. Univariate logistic regression analyses of equine influenza virus seropositivity determined by single radial haemolysis assay with A/equine/Richmond/1/2007 (H3N8) among 830 horses in Northwest Nigeria.

FACTOR	N (n)	ODDS RATIO (95% CI)	<i>p</i> -Value ¹
AGE			
Young (<5 years)	113 (69)	1.00	-
Adult (5–14 years)	662 (472)	1.58 (1.05–2.40)	0.03
Old (≥15 years)	55 (51)	8.13 (2.75–24.1)	<0.0001
SEX			
Female	295 (223)	1.00	-
Male	535 (369)	0.72 (0.52–0.99)	0.04
BREED			
Local	321 (221)	1.00	-
Exotic	268 (207)	1.54 (1.06–2.22)	0.02
Mixed	241 (164)	0.96 (0.67–1.38)	0.84
LOCATION			
Kaduna	256 (192)	1.00	-
Kano	314 (221)	0.79 (0.55–1.15)	0.22
Katsina	122 (85)	0.77 (0.47–1.24)	0.27
Sokoto	138 (94)	0.71 (0.45–1.12)	0.15
PURPOSE			
Mixed	149 (99)	1.00	-
Polo	331 (250)	1.56 (1.02–2.37)	0.039
Traditional Durbar	350 (243)	1.15 (0.76–1.73)	0.51
COAT COLOUR			
Basic	640 (469)	1.00	-
Bright	80 (55)	0.80 (0.48–1.33)	0.39
Grey	110 (68)	0.59 (0.39–0.90)	0.015
TOTAL	830 (592)		

¹ *p*-values < 0.05 indicated in bold typeface; CI = 95% confidence interval, N = total number tested; n = number positive

In the multivariate logistic regression analysis to identify confounding factors, only age remained a significant predictor of EIV seropositivity with an OR of 1.6 in adult horses and OR of 8.1 in old horses.

3.3. Seroprevalence of A/equine/Worcestershire/2019 (H3N8) among a Sample of Horses Positive for A/equine/Richmond/1/2007 (H3N8) in Northwest Nigeria

The mean SRH value obtained with the 2019 virus (150.4 mm²) for 118 of the samples was significantly higher ($p = 0.00001$) than the mean value of 131.7 mm² obtained with the 2007 virus (Figure 4). Of the 118 samples tested against both viruses, only 26.3% had SRH values at or above the level expected to provide clinical and virological protection when measured using the 2007 virus. Furthermore, 6.8% had SRH values below the cut-off expected to provide clinical protection. In contrast, around double the number of samples

(52.5%) had SRH values above 150 mm² when measured using the 2019 virus and only 3.4% had values below the lower threshold of 85 mm².

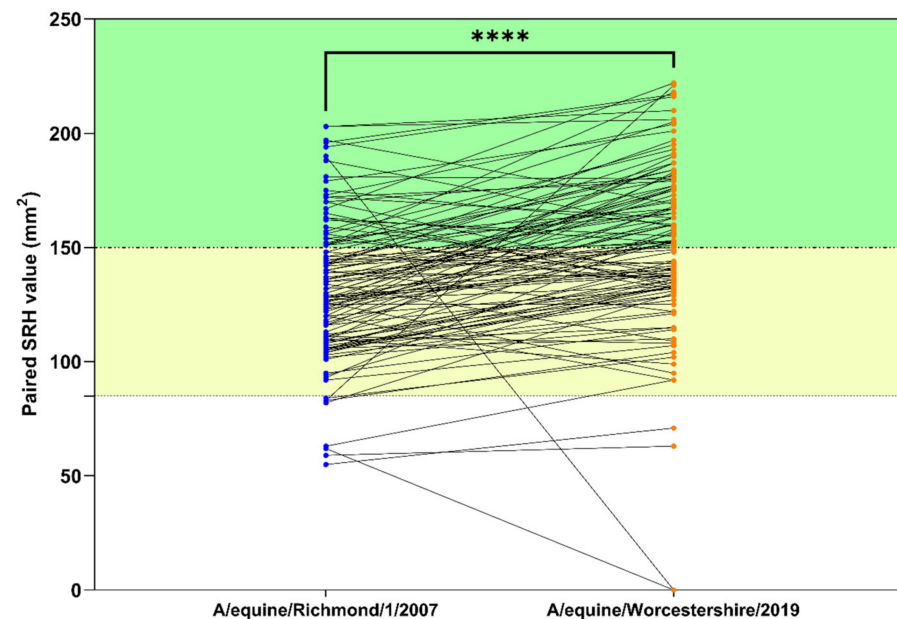


Figure 4. Comparison of single radial haemolysis antibody values measured against A/equine/Richmond/1/2007 or A/equine/Worcestershire/2019 among a subset of 118 horses in Northwest Nigeria. Dashed lines and shading indicate cut-off above which clinical and virological protection (---, green shading) or clinical protection only (- · -, yellow shading) is expected. **** indicates $p = 0.0001$ (Paired t -test).

4. Discussion

To the best of our knowledge, this is the first study to investigate the seroprevalence of EI among horses and the associated risk factors across states in Northwest Nigeria. Although EI virus was first isolated in tropical Africa in 1991 [17], earlier EI seroprevalence studies among horses in Nigeria focused only on polo horses during tournaments [22] and some selected stables in Kaduna State [24]. The results of this study therefore provide contemporary information that can be employed in steering the introduction of an effective action plan on the control of EI in Northwest Nigeria and the country at large. Nonetheless, donkeys might also contribute to the epidemiology of equine influenza as they represent around 80% of the equine population in Nigeria. Donkeys are predominantly found in northern Nigeria where they are used as work animals but as they are not bred locally (the majority are imported from the Republic of Niger [30]).

There have been growing concerns about the transboundary introduction and re-introduction of EI to Nigerian horses [19,20]. Sokoto State remains home to the old Sokoto caliphate with its boundaries including the present-day Burkina Faso, Cameroon, Niger and Nigeria. The border towns in Sokoto (Illela) and Katsina (Jibia and Daura) have large populations of horses. These border towns are arguably a gateway for movement of equids for trade and sports from Nigeria, through Niger to other West African countries. Kaduna and Kano are two states renowned for hosting colourful traditional durbars as well as local and international equine events in northern Nigeria. The overall seroprevalence of 71.3% (95% CI = 68.1–74.3) in this study corroborates the earlier finding of [18], which established that equine influenza exists among equids in all regions of Nigeria. However, the prevalence observed in this study is greater than previous reports from northern Nigeria [24], Brazil [31] and Spain [32]. The high prevalence observed might be due to a recent widespread exposure to equine influenza virus. Argentina has become an established source of equine importation into Nigeria [33], along with other sources like Europe and South Africa, due to the increasing interest in equestrian activities in Nigeria. The Argentine

Ministry of Agriculture facilitated the export of 206 horses to Nigeria between 2018 and 2019. This probably explains the first report of an FC1 strain in some west African countries [19] as well as in West and Central African countries [20] coinciding with the 2019 outbreak of FC1 EI in South America (Argentina and Uruguay) [34]. Furthermore, with the rising cross-border equestrian activities, it is difficult to rule out neighbouring countries as sources of EI, as they are potential sources of equine importation to Nigeria.

In univariable analyses, no significant association was found between location of horses and seropositivity, but polo horses had higher seropositivity and a significantly higher OR than horses used for other purposes. Polo horses might have had the highest EI seropositivity (75.5% (95% CI: 71–80)) because of the likelihood of being the most mobile compared to horses used for traditional durbar and mixed purposes. Although EI vaccines are not sold in Nigeria, vaccines are often imported [17], mostly along with importation of horses. The use of vaccines in the “exotic” breeds of horses, which includes 224 of the 331 horses involved in polo, might explain the higher seropositivity in these categories. The media reported that the 2018/2019 outbreak was followed by an EI vaccination campaign organized by the state government in July 2019, 6 months later, for a limited number of horses.

Significantly more female horses were seropositive than males in univariate analysis. However, despite the higher seroprevalence observed in female horses, sex was not a predictor of EI seroprevalence in the multivariable analyses. This is consistent with a report in USA and Canada [35].

Grey coat colour was significantly associated with lower seropositivity and a lower OR in univariable analyses than other coat colours, but this significance was lost in the multivariable analyses. This may be due to the under-representation of grey-coat horses (13.2%) compared to the “basic”-coat-coloured horses among older horses resulting in lowering of study power to detect this effect as significant. The “bright” and grey coat colour categories were also over-represented among horses used for traditional durbars whereas polo ponies were more likely to be darker-coloured.

Exploring the host factors associated with EIV seropositivity in both univariable and multivariable models, age was a consistently significant predictor of EI seropositivity. The significant progressive increase of EI seropositivity across age groups may be suggestive of recurring exposure in older horses. This finding agrees with studies from Brazil and Spain [30,31].

The mean SRH value obtained for all 830 horses with A/equine/Richmond/07 was only 78.2 mm² (median = 88 mm²; interquartile range = 0–121 mm²) with 46.5% of horses having antibody levels below the value associated with clinical protection against a homologous virus challenge. A small proportion (9%) of the sampled equine population had antibodies likely to be fully protective against EI infection, similar to the finding in a report from Brazil [30]. The significantly higher mean SRH value obtained with a subset of 118 antibody-positive sera with A/equine/Worcestershire/2019 (H3N8) compared to A/equine/Richmond/1/07 (H3N8), which represents FC1 and FC2, respectively, is consistent with the recent circulation of an FC1 virus among horses in Nigeria. If the high seropositivity of EI among horses in Northwest Nigeria is indicative of higher rates of EI infection, then this could increase the risk of interspecies transmission of EI owing to the common practices of housing other domestic species in close proximity. These animals could play a role in the transmission dynamics and epidemiology of EI. In 2007, investigation of an EI outbreak in New South Wales inferred the possible role of birds as mechanical propagators of equine H3N8 virus [36]. The severe H3N8 EI outbreak that occurred in Jilin and Heilongjiang provinces of Northeast China in 1989 suggests the innate potential of influenza viruses to emerge in horses without reassortment [37]. The close interaction of various avian species and other mammals could result in novel influenza viruses like the avian-like A/equine/Jilin/1/89 (H3N8) in the future.

Keeping other animals like dogs, cats, ducks, ostriches, peacocks, sheep, goats, cattle, rabbits and pigeons in proximity with horses was commonly observed while sampling the

horses in Northwest Nigeria. Bats were observed roosting in trees near where horses were tethered. Although influenza viruses have not been reported among bats in Nigeria, recent studies have demonstrated the abundance of some emerging viruses among bats sampled in Nigeria [38,39]. Since the first isolation of H3N8 equine influenza in dogs in 2004 [40], several reports have emerged of infection of dogs with equine influenza: a retrospective investigation in the UK disclosed the cause of the 2002 respiratory outbreaks among English foxhound to be an H3N8 virus of equine origin [41]. Just recently, some influenza A viruses including equine H3N8 were serologically detected in dogs in Poland [42]. Even though the equine/canine influenza lineages evolved independently from the avian H3 lineage and are unrelated to the human H3 lineage [43], the presence of other animals could provide opportunities for new interspecies transmission events.

5. Conclusions

Our study showed serological evidence for EI exposure in 71.3% of the tested population, with the majority of this presumed to result from infection rather than vaccination as vaccines are not routinely administered in Nigeria. The higher mean antibody values obtained using a more recent isolate in the SRH assay provides further evidence that the antibodies detected were likely due to infection during a recent outbreak rather than vaccination. Importantly, we also demonstrated an insufficient level of protection against EI in the region. Introduction of a vaccination programme with vaccines containing recently circulating FC virus, which could be targeted at horses that travel and intermingle, should mitigate against further outbreaks of equine influenza in Nigeria.

Author Contributions: Conceptualization, J.M.D. and O.T.O.; methodology, J.M.D., O.T.O. and J.R.N.; formal analysis, J.M.D., O.T.O. and J.R.N.; investigation, J.M.D., O.T.O., J.R.N., E.R.E., M.S.I., M.H., S.S., P.W.M. and O.A.O.; resources, J.M.D., O.T.O., J.R.N., E.R.E., M.S.I., M.H., S.S., P.W.M. and O.A.O.; data curation, O.T.O.; writing—original draft preparation, O.T.O.; writing—review and editing, J.M.D., O.T.O. and J.R.N.; visualization, J.M.D., O.T.O. and J.R.N.; supervision, J.M.D.; project administration, J.M.D. and O.T.O.; funding acquisition, J.M.D. and O.T.O. All authors have read and agreed to the published version of the manuscript.

Funding: O.T.O. is funded by a University of Nottingham Vice-Chancellor’s Scholarship for Research Excellence (International).

Institutional Review Board Statement: This study received approval (reference 2635181112) from the School of Veterinary Medicine and Science Ethics Clinical Review Panel, University of Nottingham, United Kingdom.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank the Emirate council of Zazzau, Kano and Sokoto for their support. Additionally, the Hon. Commissioner Ministry of Animal Health and Fisheries Development Sokoto State, Professor Abdulkadir Usman Junaidu and the staff of the Ministry of Agriculture in Katsina State.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sack, A.; Cullinane, A.; Daramragchaa, U.; Chuluunbaatar, M.; Gonchigoo, B.; Gray, G.C. Equine Influenza Virus—A Neglected, Reemergent Disease Threat. *Emerg. Infect. Dis. J.* **2019**, *25*, 1185. [\[CrossRef\]](#)
2. King, A.M.Q.; Lefkowitz, E.J.; Mushegian, A.R.; Adams, M.J.; Dutilh, B.E.; Gorbalenya, A.E.; Harrach, B.; Harrison, R.L.; Junglen, S.; Knowles, N.J.; et al. Changes to taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses. *Arch. Virol.* **2018**, *163*, 2601–2631. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Sovinova, O.; Tumova, B.; Pouska, F.; Nemec, J. Isolation of a virus causing respiratory disease in horses. *Acta Virol.* **1958**, *2*, 52–61. [\[PubMed\]](#)
4. Waddell, G.H.; Teigland, M.B.; Sigel, M.M. A new influenza virus associated with equine respiratory disease. *J. Am. Vet. Med. Assoc.* **1963**, *143*, 587–590. [\[PubMed\]](#)
5. Ismail, T.; Sami, A.; Youssef, H.; Abou Zaid, A. An outbreak of equine influenza type 1 in Egypt in 1989. *Vet. Med. J. Giza* **1990**, *38*, 195–206.

6. Webster, R.G. Are equine 1 influenza viruses still present in horses? *Equine Vet. J.* **1993**, *25*, 537–538. [\[CrossRef\]](#)
7. Singh, G. Characterization of A/eq-1 virus isolated during the equine influenza epidemic in India. *Acta Virol.* **1994**, *38*, 25–26.
8. Madić, J.; Martinović, S.; Naglič, T.; Hajsig, D.; Cvetnić, S. Serological evidence for the presence of A/equine-1 influenza virus in unvaccinated horses in Croatia. *Vet. Rec.* **1996**, *138*, 68. [\[CrossRef\]](#)
9. van Maanen, C.; Cullinane, A. Equine influenza virus infections: An update. *Vet. Q.* **2002**, *24*, 79–94. [\[CrossRef\]](#)
10. Oladunni, F.S.; Oseni, S.O.; Martinez-Sobrido, L.; Chambers, T.M. Equine Influenza Virus and Vaccines. *Viruses* **2021**, *13*, 1657. [\[CrossRef\]](#)
11. Paillot, R.; Hannant, D.; Kydd, J.H.; Daly, J.M. Vaccination against equine influenza: Quid novi? *Vaccine* **2006**, *24*, 4047–4061. [\[CrossRef\]](#)
12. Daly, J.M.; Lai, A.C.; Binns, M.M.; Chambers, T.M.; Barrandeguy, M.; Mumford, J.A. Antigenic and genetic evolution of equine H3N8 influenza A viruses. *J. Gen. Virol.* **1996**, *77*, 661–671. [\[CrossRef\]](#)
13. Lai, A.C.; Chambers, T.M.; Holland, R.E., Jr.; Morley, P.S.; Haines, D.M.; Townsend, H.G.; Barrandeguy, M. Diverged evolution of recent equine-2 influenza (H3N8) viruses in the Western Hemisphere. *Arch. Virol.* **2001**, *146*, 1063–1074. [\[CrossRef\]](#)
14. Bryant, N.A.; Rash, A.S.; Russell, C.A.; Ross, J.; Cooke, A.; Bowman, S.; MacRae, S.; Lewis, N.S.; Paillot, R.; Zanon, R.; et al. Antigenic and genetic variations in European and North American equine influenza virus strains (H3N8) isolated from 2006 to 2007. *Vet. Microbiol.* **2009**, *138*, 41–52. [\[CrossRef\]](#)
15. Moreira, R.; García, A.; Ahumada, C.; Badía, C.; Suárez, P.; Yangari, B.; Aguayo, C.; Herrera, J.; Espejo, G.; Pinto, E. Report of 2018 equine influenza outbreak in Chile. *Austral. J. Vet. Sci.* **2019**, *51*, 27–31. [\[CrossRef\]](#)
16. OIE. *OIE Expert Surveillance Panel on Equine Influenza Vaccine Composition*; The Official World Organisation for Animal Health: Paris, France, 2020; pp. 1–7. Available online: <https://oiebulletin.fr/?officiel=08-4-2-2020-1-panel&edition=12827&pdf=officiel&article=15169> (accessed on 18 October 2021).
17. Adeyefa, C.A.; McCauley, J.W. Outbreak of equine influenza in polo horses in Ibadan, Nigeria: Virus isolation, clinical manifestation and diagnosis. *Vet. Rec.* **1994**, *134*, 683–684. [\[CrossRef\]](#)
18. Adeyefa, C.A.O.; James, M.L.; McCauley, J.W. Antigenic and genetic analysis of equine influenza viruses from tropical Africa in 1991. *Epidemiol. Infect.* **1996**, *117*, 367–374. [\[CrossRef\]](#)
19. Shittu, I.; Meseko, C.A.; Sulaiman, L.P.; Inuwa, B.; Mustapha, M.; Zakariya, P.S.; Muhammad, A.A.; Muhammad, U.; Atuman, Y.J.; Barde, I.J.; et al. Fatal multiple outbreaks of equine influenza H3N8 in Nigeria, 2019: The first introduction of Florida clade 1 to West Africa. *Vet. Microbiol.* **2020**, *248*, 108820. [\[CrossRef\]](#)
20. Diallo, A.A.; Souley, M.M.; Issa Ibrahim, A.; Alassane, A.; Issa, R.; Gagara, H.; Yaou, B.; Issiakou, A.; Diop, M.; Ba Diouf, R.O.; et al. Transboundary spread of equine influenza viruses (H3N8) in West and Central Africa: Molecular characterization of identified viruses during outbreaks in Niger and Senegal, in 2019. *Transbound. Emerg. Dis.* **2021**, *68*, 1253–1262. [\[CrossRef\]](#)
21. Adeyefa, C.A.; Hamblin, C.; Cullinane, A.A.; McCauley, J.W. Nationwide serological survey of equine influenza in Nigeria. *Rev. D'élevage Méd. Vét. Pays Trop.* **1996**, *49*, 24–27.
22. Olusa, T.A.O.; Adegunwa, A.K.; Aderonmu, A.A.; Adeyefa, C.A.O. Serologic evidence of Equine H7 Influenza Virus in polo horses in Nigeria. *Sci. World J.* **2010**, *5*, 17–19. [\[CrossRef\]](#)
23. Olusa, T.A.O.; Adeyefa, C.A.O. Serosurveillance of equine H3 influenza virus in horses in Ibadan, Nigeria. *Trop. Vet.* **2009**, *27*, 15–19.
24. Meseko, C.A.; Ehizibolo, D.O.; Nwokike, E.C.; Wungak, Y.S. Serological evidence of equine influenza virus in horse stables in Kaduna, Nigeria. *J. Equine Sci.* **2016**, *27*, 99–105. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Mumford, J.A.; Wood, J. Establishing an acceptability threshold for equine influenza vaccines. *Dev. Biol. Stand.* **1992**, *79*, 137–146. [\[PubMed\]](#)
26. Newton, J.R.; Townsend, H.G.; Wood, J.L.; Sinclair, R.; Hannant, D.; Mumford, J.A. Immunity to equine influenza: Relationship of vaccine-induced antibody in young Thoroughbred racehorses to protection against field infection with influenza A/equine-2 viruses (H3N8). *Equine Vet. J.* **2000**, *32*, 65–74. [\[CrossRef\]](#)
27. Daly, J.M.; Yates, P.J.; Newton, J.R.; Park, A.; Henley, W.; Wood, J.L.; Davis-Poynter, N.; Mumford, J.A. Evidence supporting the inclusion of strains from each of the two co-circulating lineages of H3N8 equine influenza virus in vaccines. *Vaccine* **2004**, *22*, 4101–4109. [\[CrossRef\]](#)
28. Bourn, D.; Wint, W.; Blench, R.; Woolley, E. Nigerian Livestock Resources Survey. Available online: <http://www.fao.org/livestock/agap/frg/FEEDback/War/t1300b/t1300b0g.htm> (accessed on 10 May 2022).
29. OIE. Equine Influenza. 2019. Available online: <https://www.oie.int/app/uploads/2021/03/3-05-07-eq-inf.pdf> (accessed on 18 December 2021).
30. Blench, R.; De Jode, A.; Gherzi, E. Donkeys in Nigeria: History, Distribution and Productivity. A Resource Book of the Animal Traction Network for Eastern and Southern Africa (ATNESA) ACP-EU Technical Centre for Agricultural and Rural Corporation (CTA), Wageningen, The Netherlands. 2015. Available online: <https://www.atnesa.org/donkeys/donkeys-blench-productivity-NG.pdf> (accessed on 12 November 2022).
31. Daly, J.M.; Megid, J.; Langoni, H.; de Nardi Júnior, G.; Ribeiro, M.G. Retrospective serological survey for influenza in horses from Brazil. *Braz. J. Microbiol.* **2021**, *52*, 461–466. [\[CrossRef\]](#)
32. Jurado-Tarifa, E.; Daly, J.M.; Perez-Ecija, A.; Barba-Recreo, M.; Mendoza, F.J.; Al-Shuwaikh, A.M.; Garcia-Bocanegra, I. Epidemiological survey of equine influenza in Andalusia, Spain. *Prev. Vet. Med.* **2018**, *151*, 52–56. [\[CrossRef\]](#)

33. Sule, W.F.; Oluwayelu, D.O.; Adedokun, R.A.; Rufai, N.; McCracken, F.; Mansfield, K.L.; Johnson, N. High seroprevalence of West Nile virus antibodies observed in horses from southwestern Nigeria. *Vector Borne Zoonotic Dis.* **2015**, *15*, 218–220. [CrossRef]
34. De Agricultura, M.A. Exportaciones Argentinas de Équidos. 2019. Available online: https://www.magyp.gob.ar/sitio/areas/equinos/informacion_estadistica/_archivos/_000001_Exportaciones%20Equinas/002019_Informe%20Exportaciones%20de%20Equinos%202010%202019.pdf (accessed on 23 October 2022).
35. Nyaga, P.N.; Wiggins, A.D.; Priester, W.A. Epidemiology of equine influenza, risk by age, breed and sex. *Comparative Immunology. Microbiol. Infect. Dis.* **1980**, *3*, 67–73. [CrossRef]
36. Spokes, P.J.; Marich, A.J.; Musto, J.A.; Ward, K.A.; Craig, A.T.; McAnulty, J.M. Investigation of equine influenza transmission in NSW: Walk, wind or wing? *New South Wales Public Health Bull.* **2009**, *20*, 152–156. [CrossRef]
37. Guo, Y.; Wang, M.; Kawaoaka, Y.; Gorman, O.; Ito, T.; Saito, T.; Webster, R.G. Characterization of a new avian-like influenza A virus from horses in China. *Virology* **1992**, *188*, 245–255. [CrossRef]
38. Ameh, V.O.; Wu, G.; Goharriz, H.; Shipley, R.; Fooks, A.R.; Sabeta, C.T.; McElhinney, L.M. Serum Neutralization Profiles of Straw-Colored Fruit Bats (*Eidolon helvum*) in Makurdi (Nigeria), against Four Lineages of Lagos Bat Lyssavirus. *Viruses* **2021**, *13*, 2378. [CrossRef]
39. Kia, G.S.N.; Tao, Y.; Umoh, J.U.; Kwaga, J.K.P.; Tong, S. Identification of Coronaviruses, Paramyxoviruses, Reoviruses, and Rotaviruses among Bats in Nigeria. *Am. J. Trop. Med. Hyg.* **2021**, *104*, 1106–1110. [CrossRef]
40. Crawford, P.C.; Dubovi, E.J.; Castleman, W.L.; Stephenson, I.; Gibbs, E.P.; Chen, L.; Smith, C.; Hill, R.C.; Ferro, P.; Pompey, J.; et al. Transmission of Equine Influenza Virus to Dogs. *Science* **2005**, *310*, 482–485. [CrossRef]
41. Daly, J.M.; Blunden, A.S.; Macrae, S.; Miller, J.; Bowman, S.J.; Kolodziejek, J.; Nowotny, N.; Smith, K.C. Transmission of Equine Influenza Virus to English Foxhounds. *Emerg. Infect. Dis.* **2008**, *14*, 461–464. [CrossRef]
42. Kwasnik, M.; Smreczak, M.; Rola, J.; Urbaniak, K.; Rozek, W. Serologic investigation of influenza A virus infection in dogs in Poland. *J. Vet. Diagn. Investig.* **2020**, *32*, 420–422. [CrossRef]
43. Zhang, W.; Jiang, Q.; Chen, Y. Evolution and Variation of the H3 Gene of Influenza A Virus and Interaction among Hosts. *Intervirology* **2007**, *50*, 287–295. [CrossRef]

CHAPTER 3

Systematic review and meta-analysis of seroprevalence studies of West Nile virus in equids in Europe between 2001 and 2018

Note: This chapter is a published article:

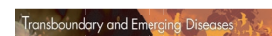
METZ MBC, **OLUFEMI OT**, DALY JM BARBA M. (2020). Systematic review and meta-analysis of seroprevalence studies of West Nile virus in equids in Europe between 2001 and 2018. *Transboundary and Emerging Diseases* 68, 1814-1823 doi: <https://doi.org/10.1111/tbed.13866>

Transboundary and Emerging Diseases journal permits the reuse of their articles provided the original article is appropriately cited.

Author contributions: METZ MBC (26.4%), OLUFEMI OT (17.2%)*, DALY JM (29.8%) BARBA M (26.4%).

*OTO: Extracting and charting data, data analyses, Writing - reviewing and editing. Specifically, OTO confirmed correct data extraction from the papers and generated the Forest Plot (Figure 3) and the funnel plot (Figure 4), assisted in drafting the manuscript (in particular in regard to the methods to generate figures 3 and 4) and proof-read the manuscript.

REVIEW ARTICLE



WILEY

Systematic review and meta-analysis of seroprevalence studies of West Nile virus in equids in Europe between 2001 and 2018

Marine B. C. Metz¹ | Olaolu T. Olufemi² | Janet M. Daly² | Marta Barba¹

¹Agentes Microbiológicos asociados a la Reproducción Animal (ProVaginBio), Veterinary Faculty, Universidad Cardenal Herrera-CEU, CEU Universities, Valencia, Spain

²School of Veterinary Medicine and Science, University of Nottingham, Nottingham, UK

Correspondence

Marta Barba, Agentes Microbiológicos asociados a la Reproducción Animal (ProVaginBio), Veterinary Faculty, Universidad Cardenal Herrera-CEU, CEU Universities, Valencia, Spain
Email: martabrvt@gmail.com

Abstract

There is some evidence that West Nile virus (WNV), which causes encephalomyelitis in equids, is an emerging disease in Europe. The aim of this study was to perform a systematic review and meta-analysis to analyse seroprevalence studies of WNV in equids in European countries between 2001 and 2018. Two electronic databases, PubMed and Scopus, were searched for relevant publications published from 2001 to 2018 using predetermined keywords. A total of 1,484 papers were initially found. After applying the eligibility criteria, 39 papers were finally included in the systematic review. Analysis of 28,089 equids from 16 European countries revealed a pooled seroprevalence of 8% (95% CI 5%–12%, $p < .001$, $I^2 = 99.3\%$) in Europe. The pooled seroprevalence was slightly higher in Mediterranean basin countries than other countries and when calculated for samples collected between 2001 and 2009 compared to 2010 to 2018. Differences in study design (e.g. sampling associated with recent outbreaks of WNV) contributed to a high degree of variability among studies. Further studies with harmonized study design and reporting of the results are recommended to better estimate and monitor European seroprevalence of WNV in equids.

KEYWORDS

equids, Europe, horses, Mediterranean basin, seroprevalence, WNV

1 | INTRODUCTION

West Nile virus (WNV) is a mosquito-borne zoonotic virus from the genus *Flavivirus* and family *Flaviviridae* (Anon, 2017). Its transmission cycle involves birds and mosquitoes, especially from the *Culex* species, which act as vectors of the virus. Several vertebrate species can be infected by the virus, but mammals, particularly humans and equids, are considered 'dead-end' hosts as they do not usually develop sufficiently high levels of viraemia for transmission to blood-feeding mosquitoes (Komar, 2000). The virus was first isolated from a human being in 1937 in the West Nile region of Uganda (Smithburn et al., 1940). Since the first reported case of WNV in horses in Egypt in 1963 (Schmidt & Mansoury, 1963), the disease has expanded in range causing significant human and animal health issues. An example of these are the outbreaks reported in EU member

countries since the start of the 2020 transmission season, and as of 10 September 2020, 173 human cases of WNV infection and 15 deaths (Greece, Spain, Italy and Romania) and 60 outbreaks among equids (Spain, Italy, France, Portugal and Germany) have been reported through the European Animal Disease Notification System (ADNS).

Several genetic lineages of the virus have been found, but isolates from lineages 1 and 2 have mainly been responsible for the disease in humans and equids in European countries, with lineage 1 predominant until the mid-2000s (Ciccozzi et al., 2013; Long, 2014). As a neurotropic virus causing encephalomyelitis, clinical signs in horses include ataxia, paralysis of the limbs, prolonged recumbency, muscle fasciculations and abnormal mentation (Long, 2014). The mortality rate in horses has been estimated at 35%–45% (Long, 2014). However, studies suggest that only

around 10% of infected horses present neurological signs (Gardner et al., 2007). For diagnosis, laboratory testing is necessary to confirm the infection as the neurological signs are not pathognomonic for the disease. Treatment is mostly supportive as there are no known effective antiviral medications (Long, 2014). An equine WNV vaccine was first licensed in the United States in 2003, and further types of WNV vaccine have since been approved for use in horses, but an equine WNV vaccine was not licensed for use in Europe until 2009. Due to the low viral titres in horses, *ante-mortem* PCR-based detection of viral RNA is unreliable (Kleiboeker et al., 2004). Therefore, suspected cases of WNV infection are usually confirmed by IgM capture ELISA and/or measuring seroconversion using a plaque reduction neutralization test (PRNT). Equine WNV-specific IgM antibodies are usually detectable from around 8 days post-infection (so most horses with encephalitis test positive at the time that clinical signs are first observed) and remain detectable for up to 3 months (Beck et al., 2017). Neutralizing (IgG) antibodies are detectable in equine serum by 2 weeks post-infection and can persist for more than 1 year. The OIE Terrestrial Manual (OIE, 2018) suggests that IgG indirect and competitive ELISAs, virus neutralization test (VNT) or PRNT are suitable methods for determining prevalence of infection. However, as ELISA methods are less specific, where related flaviviruses co-circulate with WNV, positive results obtained should be confirmed by PRNT or VNT and testing against other flaviviruses in parallel. Other flaviviruses detected in Europe include Bagaza virus (BAGV), louping ill virus (LIV), tick-borne encephalitis virus (TBEV) and Usutu virus (USUV) (Llorente et al., 2015; Long, 2014).

The aim of this study was to conduct a systematic review and meta-analysis to analyse seroprevalence studies of WNV in equids in Europe from the year 2001 to 2018 inclusive, to compare the prevalence in countries of the Mediterranean basin with other European countries and to evaluate the prevalence of two periods: from 2001 to 2009 and from 2010 to 2018.

2 | MATERIALS AND METHODS

2.1 | Search strategy

A systematic search strategy was performed in the databases PubMed and Scopus to identify all published studies reporting the prevalence of WNV in equids in Europe from 1 January 2001 to 20 March 2019 (the date the search was performed). The following keywords and Boolean operators ('AND' and 'OR') were used: (prevalence OR incidence OR frequency OR occurrence OR detection OR identification OR isolation OR characterization OR investigation) AND (WNV OR WNV OR Flavivirus) AND (horse OR equine OR equid OR donkey OR mule OR foal). In Scopus, the search terms were applied to the title, abstract and the keywords. In PubMed, the search terms were applied to all fields. No language restrictions were applied. Retrieved searches were entered into a Microsoft Excel (2018) file.

The reference lists of the selected publications were reviewed manually to identify all potential studies that could have been missed in the two databases.

2.2 | Eligibility criteria

Inclusion criteria were divided into two categories: inclusion criteria related to the literature search and inclusion criteria inherent to the studies. First, the studies had to be published between 1 January 2001 and 20 March 2019 and the full text had to be available in English, Spanish or French. In addition, studies had to be prospective or retrospective serosurveys with animal-level prevalence and animals of the genus *Equus* (excluding zebra) reported, carried out in a European country and have performed a VNT and/or PRNT to confirm the specificity of antibodies detected by ELISA.

Studies were excluded if the titles and abstracts were not relevant to the subject of interest, did not fulfil the above eligibility criteria, had data missing or duplicated data published in another included study.

2.3 | Study selection and data extraction

In the first screening of all searched studies, duplicates were eliminated. The titles and abstracts of all retrieved studies were then independently screened by two authors (MBCM and MB) to identify potentially relevant studies. When the study could not be assessed from the title and abstract, the full text was screened. The full text of the studies retained after the first screening were further scanned independently and in a standardized manner by two authors (MBCM and MB) applying the eligibility criteria.

After the eligibility assessment process, data were extracted independently by two authors (MBCM and MB) and classified in three categories: general data related to the study, data related to the diagnostic techniques and data related to the animals. Any disagreements that arose between the authors were resolved through discussion with a third author (JMD). All the extracted data were summarized in a Microsoft Excel (2018) file.

The general data related to the study were as follows: title, first author's name, name of the journal, year of publication, database where the study was identified (PubMed or Scopus), type of study (i.e. prospective or retrospective serosurvey), language, country and region, sampling protocol (e.g. convenience or random sample), year and season of testing. The data related to the diagnostic techniques were as follows: initial serological test to detect the presence of antibody (immunoglobulin G), type of confirmatory test, strain of the confirmatory test and additional serological tests performed. The data related to the animals included number of equids, mean age, sex, breed, vaccination and clinical signs.

The total number of equids tested and the number testing positive specifically for WNV antibodies (and without a reported history of vaccination) were also extracted independently by two authors

(JMD and OTO) and any disagreement confirmed by a third author (MB).

2.4 | Data analysis and presentation

Statistical meta-analysis of the proportion of WNV antibody-positive animals was conducted and a forest plot generated using *metaprop* in Stata 16 (Nyaga et al., 2014). Subgroup meta-analysis was done for the Mediterranean and non-Mediterranean countries of Europe to investigate whether greater prevalence was reported in Mediterranean countries where the climate is more favourable for mosquitoes. As well, analysis of two balanced 9-year-period subgroups (2001–2009 and 2010–2018) was performed. This allowed the impact of the introduction of a WNV vaccine on seroprevalence studies to be determined. Estimates from individual studies were transformed using the Freeman–Tukey double-arc-sine transformation to stabilize the variance. Heterogeneity was assessed using the I-squared statistic (I^2). A funnel plot to assess publication bias was generated and outliers identified using R (R Core Team, 2014).

2.5 | Maintenance of study standard

This study has been performed in accordance with guidelines for meta-analysis of observational studies (MOOSE statement) and preferred reporting items for systematic reviews and meta-analyses (PRISMA statement) (Moher et al., 2015; Stroup et al., 2000).

3 | RESULTS

3.1 | Search results and study selection

From the initial database search, 1,484 potentially relevant publications were identified of which 663 were found in Scopus and 821 in PubMed. After removing the duplicates, the title and abstract of 950 studies were screened. Of the 104 studies that remained, 65 studies were excluded for reasons listed in Figure 1. The lack of a confirmatory test to measure neutralizing antibodies was one of the main reasons for exclusion ($n = 13$). The other exclusion factors were as follows: review articles, type of study, language other than English, French or Spanish, insufficient data, full text not available, duplicated data, type of study and year of study.

Finally, a total of 39 publications satisfied the inclusion criteria and were included in the systematic review, of which 38 were in English and one in French. Of the 39 publications, 3 were found in Scopus, 8 in PubMed and 28 in both databases. No additional studies that satisfied the inclusion criteria were found in the reference lists of selected studies. Table 1 presents the studies included in the systematic review.

3.2 | Study characteristics

Of the 39 studies included in the systematic review, the majority ($n = 36$) were prospective serosurveys; 3 studies were retrospective. In 14 studies (35.9%), it was stated that the equine serum samples were taken randomly. It was assumed that in the other studies, convenience samples were obtained. In total, 28,089 equids were tested, of which 375 were donkeys or mules. The prevalence was described only in horses in 34 studies, only in donkeys and mules in one study (García-Bocanegra, Arenas-Montes, Jaén-Téllez, et al., 2012) and in both horses and donkeys in 3 studies (Bosiljka et al., 2013; Ozkul et al., 2013; Raleigh et al., 2012). The mean number of equids sampled in each study was 720 with a wide range (68 to 5,178).

Of the 16 European countries in which studies were conducted, 7 (Albania, Croatia, Spain, France, Italy, Portugal and Turkey) are part of the Mediterranean basin (Figure 2). The highest number of studies was found for Spain ($n = 9$), followed by France and Serbia ($n = 4$). Prevalence data were available for both date ranges in the following ten countries: Croatia, Czech Republic, France, Germany, Ireland, Poland, Portugal, Serbia, Spain and Turkey (34 studies). Twelve of the studies were carried out in the first date range (2001–2009) and 19 in the second period (2010–2018). In one study (Raleigh et al., 2012), prevalence data were separated in the two periods of time. There were five studies that started in the first period and finished in the second period and two for which the year(s) of sampling was not specified.

In the majority of studies ($n = 24$), samples were first screened by ELISA and some or all of the positive-testing samples were confirmed by testing for WNV-specific neutralizing antibodies. In 10 studies, a neutralization test was performed without prior screening by ELISA, and in one study, both ELISA and VNT were used to screen the samples. In three studies, the initial screening test was either agar gel immunodiffusion (AGID), immunofluorescence antibody test (IFAT) or multiplex immuno-assay (MIA). Additional tests performed included western blot and haemagglutination inhibition (HI) test.

The virus strain used for the VNT or PRNT was described in 24 studies. The strain 'Eg101' was used in 12 studies, 'New York (NY99)' in 7 studies and 'Israel 1998 (IS98-ST1)' in one study. These three strains belong to genetic lineage 1. Only five studies used genetic lineage 2 strains: 'Austrian' ($n = 3$), 'Hungary 578/2010' ($n = 1$) or unspecified ($n = 1$). Of the five studies that used genetic lineage 2 strains, one study also used a strain from genetic lineage 1. The remaining studies ($n = 15$) did not specify the strain used.

In 18 of the 39 studies, samples were additionally screened for neutralizing antibodies to other flaviviruses; TBEV only in 2, USUV only in 6, USUV and TBEV in 9 and Bagaza virus in 1. Positive titres were detected against TBEV or USUV in five and seven studies, respectively. In four studies, some samples had similar titres against both WNV and USUV.

Of the 39 selected studies, only 3 described minimal demographic data (age, sex and breed) and 8 reported whether or not any of the tested equids were vaccinated or showed any clinical signs.

More than half of the studies ($n = 23$) reported the season of year when the animals were sampled; the majority of the studies were carried out in autumn (September to November).

FLOW DIAGRAM OF SEARCH STRATEGY

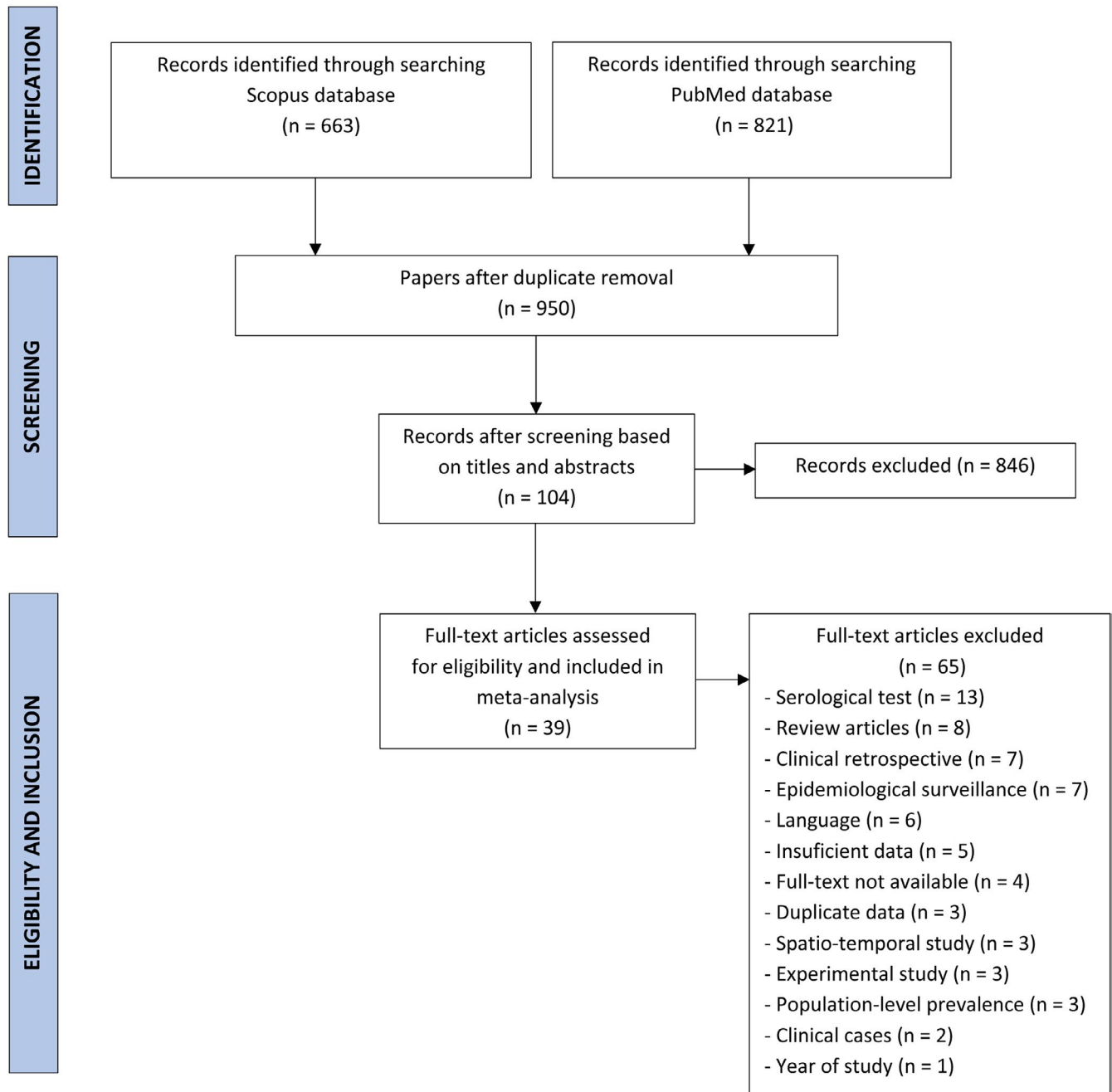


FIGURE 1 Flow diagram of article selection for West Nile prevalence in equids in Europe [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1111/tbed.13866)]

3.3 | Meta-analysis of WNV seroprevalence

The pooled seroprevalence was 8% (95% CI 5%–12%) with substantial heterogeneity ($I^2 = 99.3\%$) (Figure 3). Pooled seroprevalence was slightly higher in Mediterranean (9%, 95% CI 5%–14%) than non-Mediterranean countries (7%, 95% CI 3%–14%) and in the first sampling period (8%, 95% CI 2%–17%) than in the second sampling period (7%, 95% CI 4%–10%) (Table 2). A funnel plot (Figure 4) did not identify significant publication bias. However, two studies

(Calistri et al., 2010; Petrović et al., 2014) were identified as outliers in the studentized residual test.

4 | DISCUSSION

This systematic review sought to highlight important trends in WNV seroprevalence in equids in Europe; prevalence data of WNV reported in 39 studies of equids in Europe were analysed from the

TABLE 1 Characteristics of studies included in the systematic review

Publication	Country ^a	No. positive	No. tested	Seroprevalence (%)	Year	Screening test	Tests for other flaviviruses
1. Abad-Cobo et al. (2017)	Spain (ES)	5	369	1.36	2011–2013	ELISA	USUV
2. Alba et al. (2014)	Spain (ES)	0	178	0	2011	ELISA	
3. Bakonyi et al. (2013)	Hungary (HU)	79	276	28.62	2009	IFAT	
4. Barbić et al. (2012)	Croatia (HR)	72	2098	3.43	2010–2011	ELISA	TBEV + USUV
5. Barbić et al. (2013)	Croatia (HR)	48	1,380	3.48	2011	ELISA	TBEV + USUV
6. Barros et al. (2011)	Portugal (PT)	40	1,313	3.05	2004–2010	ELISA	
7. Barros et al. (2017)	Portugal (PT)	18	989	1.82	2011–2016	ELISA	
8. Bażanów et al. (2018)	Poland (PL)	62	411	15.09	2012–2013	VNT	USUV
9. Berxholi et al. (2013)	Albania (AL)	37	167	22.16	N.S.	ELISA & VNT	TBEV
10. Bosiljka et al. (2013)	Serbia (RS)	45	1199 ^b	3.75	2008–2012	AGID	
11. Busani et al. (2011)	Italy (IT)	348	2,528	13.77	2008 & 2009	ELISA	TBEV + USUV
12. Busquets et al. (2019)	Spain (ES)	9	138	6.52	2017 & 2018	ELISA	BAGV
13. Cabre et al. (2005)	France (FR)	0	94	0	2003	ELISA	
14. Calistri et al. (2010)	Italy (IT)	794	2030	39.11	2008	PRNT	
15. Csank et al. (2018)	Slovakia (SK)	10	145	6.90	2013	ELISA	TBEV + USUV
16. Durand et al. (2005)	France (FR)	304	906	33.55	2003	ELISA	
17. Ergunay et al. (2014)	Turkey (TR)	48	389	12.34	2011–2013	PRNT	
18. García-Bocanegra, et al. (2012)	Spain (ES)	36	510	7.06	2010	ELISA	
19. García-Bocanegra, et al. (2012)	Spain (ES)	12	109	11.01	2010–2011	ELISA	
20. García-Bocanegra, et al. (2012)	Spain (ES)	12	165 ^c	7.27	2011	ELISA	
21. Hubálek et al. (2008)	Poland (PL)	0	78	0	2006	PRNT	USUV
22. Hubálek et al. (2013)	Czechia (CZ) & Slovakia (SK)	19	395	4.81	2008–2011	PRNT	TBEV + USUV
23. Jiménez-Clavero et al. (2007)	Spain (ES)	13	157	8.28	2005	VNT	USUV
24. Jiménez-Clavero et al. (2010)	Spain (ES)	0	68 ^f	0	2008	VNT	USUV
25. Lupulovic et al. (2011)	Serbia (RS)	42	349	12.03	2009–2010	ELISA	USUV
26. Madić et al. (2007)	Croatia (HR)	4	980	0.41	2010–2011	ELISA	
27. Maquart et al. (2017)	France (FR)	9	96	9.38	2014	ELISA	USUV
28. Medić et al. (2014)	Serbia (RS)	72	252	28.57	2007–2011	ELISA	
29. Monaco et al. (2010)	Italy (IT)	271	770	35.19	2008	VNT	TBEV + USUV
30. Ozkul et al. (2006)	Turkey (TR)	36	299 ^d	12.04	N.S.	PRNT	
31. Ozkul et al. (2013)	Turkey (TR)	57	180	31.67	2011	PRNT	
32. Petrović et al. (2014)	Serbia (RS)	64	130	49.23	2012	ELISA	
33. Pradier et al. (2014)	France (FR)	143	1,159	12.34	2007–2008	ELISA	
34. Raleigh et al. (2012)	Ireland (IE)	0	490 ^e	0	2005–2006 (n = 90) & 2010 (n = 400)	ELISA	
35. Vanhomwegen et al. (2017)	Spain (ES)	11	172	6.40	2011–2012	MIA	TBEV + USUV
36. Weissenböck et al. (2003)	Austria (AT)	0	350	0	2001	PRNT	
37. Ziegler et al. (2012)	Germany (DE)	1	1,282	0.08	2007–2009	ELISA	TBEV + USUV

(Continues)

TABLE 1 (Continued)

Publication	Country ^a	No. positive	No. tested	Seroprevalence (%)	Year	Screening test	Tests for other flaviviruses
38. Ziegler, et al. (2013)	Germany (DE)	2	5,178	0.04	2010–2012	ELISA	TBEV + USUV
39. Ziegler, et al. (2013)	Ukraine (UA)	42	310	13.55	2010–2011	ELISA	TBEV

Abbreviations: AGID, agar gel immunodiffusion; BAGV, Bagaza virus; ELISA, enzyme-linked immunosorbent assay; IFAT, immunofluorescence antibody test; MIA, multiplex immuno-assay; N.S., not specified; PRNT, plaque reduction test; TBEV, tick-borne encephalitis virus; USUV, Usutu virus; VNT, virus neutralization test.

^aTwo-letter ISO country code.

^b1133 horses and 66 donkeys.

^c82 donkeys and 83 mules.

^d259 horses and 40 mules.

^e386 horses and 104 donkeys.

^fOnly results from samples collected in 2008 were included in the meta-analysis.

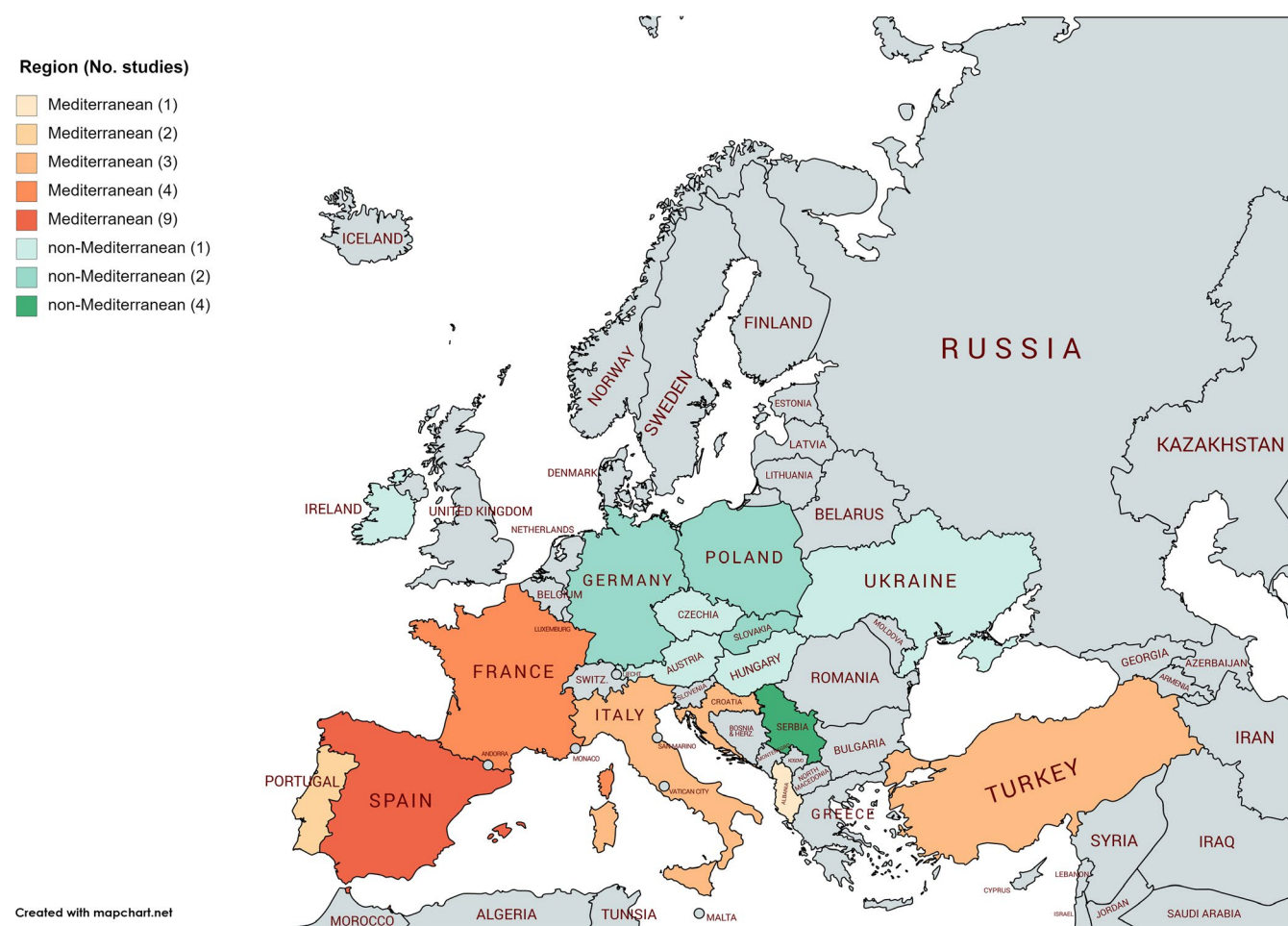


FIGURE 2 Map showing European countries for which data were included in the systematic review. Created using <https://mapchart.net/europe.html> with different colour shading used for Mediterranean and non-Mediterranean countries and depth of shading indicating number of studies performed in each country [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/terms-and-conditions)]

year 2001 to the year 2018. The pooled seroprevalence obtained was 8% (95% CI 5%–12%). However, few studies reported using random sampling methods; therefore, caution must be applied when generalizing the seroprevalence estimates to the target population. The substantial heterogeneity ($I^2 = 99.3\%$) meant that meaningful conclusions could not be drawn about differences in seroprevalence

between Mediterranean and non-Mediterranean countries or the two periods evaluated.

There was no evidence that small studies with small effect sizes were missing. However, the two studies that were identified as outliers (Calistri et al., 2010; Petrović et al., 2014) were also the two studies with the highest seroprevalence: 39% and 49%, respectively.

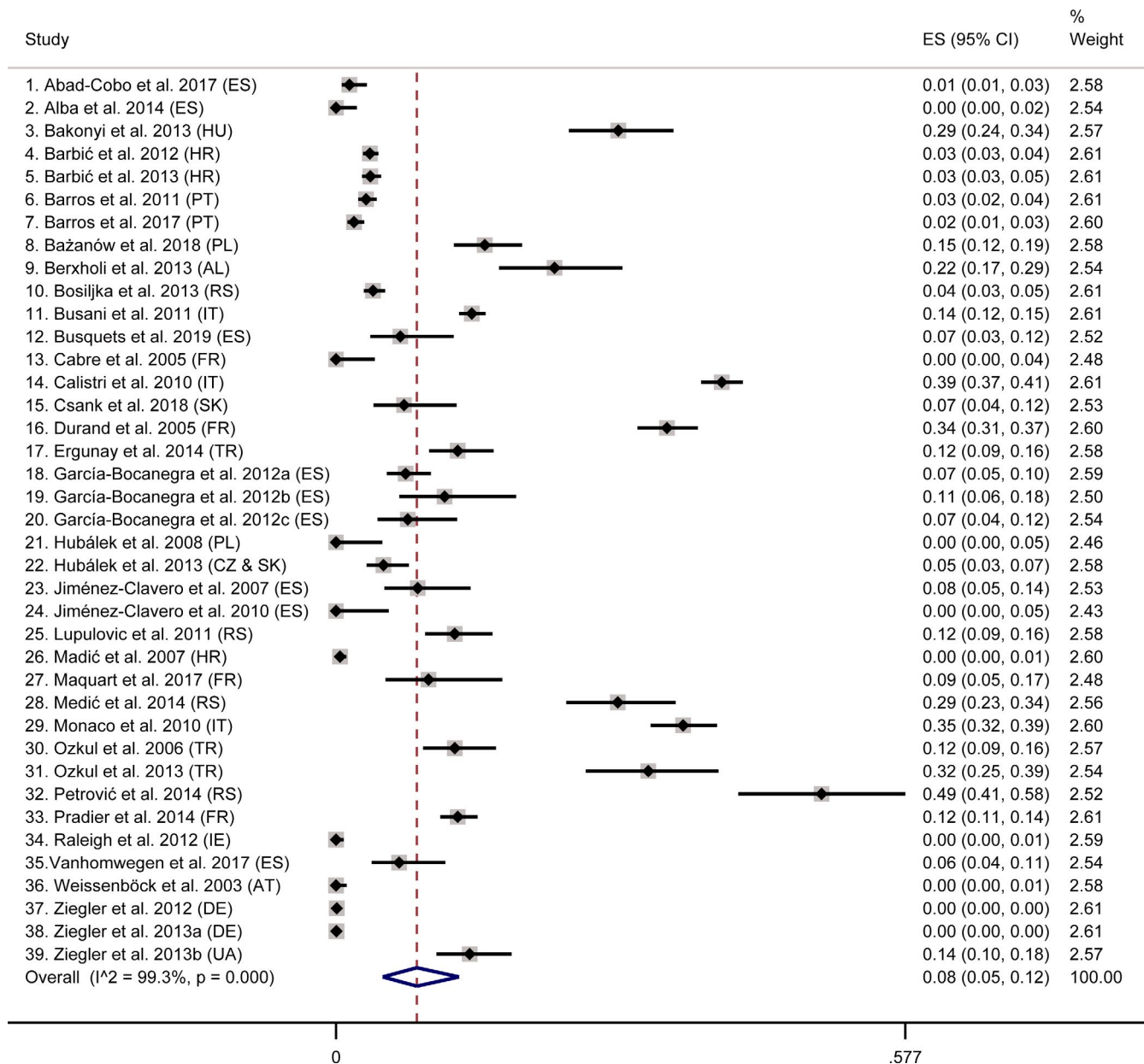


FIGURE 3 Forest plot showing the pooled estimated seroprevalence (ES) of West Nile virus among equids in Europe. Horizontal lines represent 95% confidence intervals (CIs). Each square box denotes the seroprevalence rate point estimate, and the area is proportional to the weight of the study [Colour figure can be viewed at wileyonlinelibrary.com]

		No. positive	No. tested	% (95% CI)	D.F.	I² (%)
Region	Mediterranean	2,327	17,244	9 (5–14)	24	99.1
	Non-Mediterranean	438	10,845	7 (3–14)	13	99.0
Sampling period	2000–2009	1953	9,788	8 (2–17)	12	99.4
	2010–2018	521	14,327	7 (4–10)	19	98.3
Overall		2,765	28,089	8 (5–12)	38	99.3

TABLE 2 Pooled seroprevalence of WNV in Europe

The study by Calistri et al. (2010) was associated with investigation of an outbreak of WNV. Similarly, in the study by Petrović et al. (2014), samples were collected from horses in November and

December 2012 after the first human outbreak of WNV reported in Serbia, which started in August 2012. Other studies with high seroprevalence were also associated with recent outbreaks.

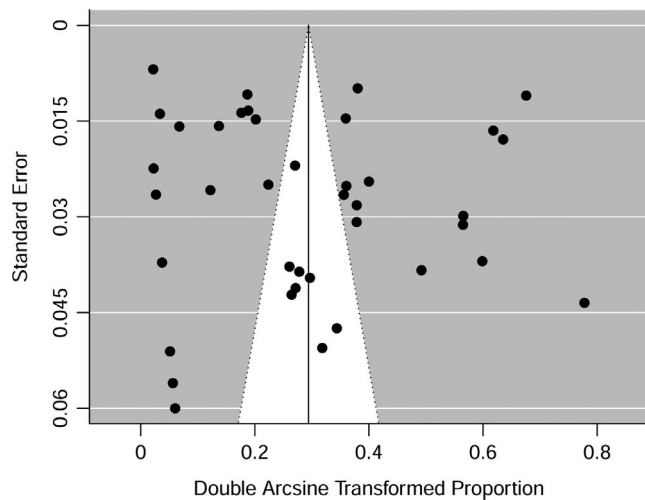


FIGURE 4 Funnel plot of standard error by Freeman-Tukey double-arc-sine-transformed proportion for all studies ($n = 39$)

The quality of data reporting varied between studies, for example, only three studies provided information on animal characteristics such as age, sex and breed, each of which could influence risk of exposure to and/or susceptibility to WNV infection, for example, older animals are more likely to have been exposed to virus. Reporting these demographic data is important for comparisons to be made between the outcomes of different studies. Recruitment criteria are important in understanding disease transmission in mobile animal populations such as horses where animals may have been exposed to the virus somewhere other than the study location. Furthermore, vaccination status became important after an equine WNV vaccine was first licensed in Europe in 2009. For example, Ziegler et al. (2012) found four samples positive for WNV antibodies in a study conducted in Germany, but three of these were from vaccinated horses (and were therefore removed from the seroprevalence estimation in this study) and one was from a horse from Hungary. However, vaccination status was only reported in 8 studies although 19 were conducted on samples collected after 2009.

ELISA is often the assay of choice for conducting seroepidemiological studies because it is simple, sensitive, rapid and often commercially available. However, due to extensive cross-reactivity between antibodies raised against different flaviviruses, the ELISA can yield false-positive results where different flaviviruses co-circulate (Beck et al., 2013). Therefore, this systematic review only included studies that used virus/plaque reduction neutralization tests to confirm positive samples; however, 21 out of 39 studies did not test other flaviviruses in parallel, which could have introduced errors in their prevalence estimation. The issue of cross-reactivity in ELISA was illustrated in some of the studies, for example Berxholi et al. (2013) found that two of seven samples that were positive in ELISA but negative in WNV VNT were positive for TBEV antibodies. Similarly, Ziegler, et al. (2013) found that four samples that were positive by ELISA but negative by WNV VNT were positive for TBEV (but not for USUV). One of the included

studies (Lupulovic et al., 2011) was the first to report neutralizing antibodies to USUV in horses; however, as the PRNT titres were 120 and 90 for WNV and USUV, respectively, they were not able to conclude whether this represented cross-reactive antibodies or prior exposure to both viruses. Calistri et al. (2010) mention that USUV was circulating in Italy in the year before samples were obtained in their study, but they did not test for USUV antibodies. In most cases, neutralization tests were positive for one virus only or titres were markedly higher (e.g. at least twofold) for one virus. However, neutralization tests were not always discriminatory, particularly where VNT titres were low (Jiménez-Clavero et al., 2007). Furthermore, Vanhomwegen et al. (2017) concluded that of 21 samples that were positive for flavivirus antibodies, 11 were specifically positive for WNV, 2 for USUV and 1 for TBEV, while 8 were positive for an unidentified flavivirus (1 of which they reported as positive for both WNV and an unidentified flavivirus). Ziegler, et al. (2013) reported four samples that were positive by WNV ELISA but VNT negative with WNV and USUV. Although this could be due to the lower sensitivity of VNT compared to ELISA (Beck et al., 2017). Furthermore, a high prevalence of tick-borne encephalitis virus (TBEV) has been reported in other studies in some European countries (Rushton et al., 2013). Therefore, there is evidence of non-WNV flaviviruses circulating in Europe, which should be taken into account when performing a serosurvey study.

Although most studies did not specify if seropositivity was caused by lineage 1 or 2 WNV strains, this is probably not critical, because it is not possible to tell by which virus lineage the immune response was elicited as the two strains differ only in three amino acids within domain III of the E protein, against which most neutralizing antibodies are directed (Berxholi et al., 2013).

Most studies were performed in autumn, probably after the beginning of the transmission season and occurrence of outbreaks in the summer. This should not affect the results of the meta-analysis other than the already mentioned bias caused by association of a serosurvey study after regional outbreaks, which could falsely increase the prevalence estimation.

Despite the fact that WNV transmission and outbreaks in equids occur in EU countries, the limitations detected in this study precluded the evaluation of an increase in seroprevalence over time. An analysis of the number of reported outbreaks could be useful to determine the re-emerging status of this virus; however, this was not the scope of this study.

Horses have been suggested as useful sentinels for WNV surveillance. However, the true seroprevalence of WNV in European equids remains uncertain due to variation in study design and reporting, and difficulty discriminating between cross-reactive antibodies. Standardized seroprevalence studies are critical to better understand the current epidemiological status of WNV in Europe and to monitor future changes.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No

ethical approval was required as this is a review article with no original research data.

CONFLICT OF INTEREST

No conflict of interest to declare by the authors.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

ORCID

Olaolu T. Olufemi  <https://orcid.org/0000-0001-5312-8591>

Janet M. Daly  <https://orcid.org/0000-0002-1912-4500>

Marta Barba  <https://orcid.org/0000-0002-3677-7239>

REFERENCES

- Abad-Cobo, A., Llorente, F., Barbero, M. D. C., Cruz-López, F., Forés, P., & Jiménez-Clavero, M. A. (2017). Serosurvey reveals exposure to West Nile virus in asymptomatic horse populations in central Spain prior to recent disease foci. *Transbound Emerg Dis*, 64(5), 1387–1392. <https://doi.org/10.1111/tbed.12510>
- Alba, A., Allepuz, A., Napp, S., Soler, M., Selga, I., Aranda, C., & Busquets, N. (2014). Ecological surveillance for West Nile in Catalonia (Spain), learning from a five-year period of follow-up. *Zoonoses Public Health*, 61(3), 181–191. <https://doi.org/10.1111/zph.12048>
- Anon (2017). Chapter 29 - Flaviviridae. In N. J. MacLachlan, & E. J. Dubovi (Eds.), *Fenner's Veterinary Virology*, 5th ed. (pp. 525–545). Academic Press.
- Bakonyi, T., Ferenczi, E., Erdélyi, K., Kutasi, O., Csörgő, T., Seidel, B., & Nowotny, N. (2013). Explosive spread of a neuroinvasive lineage 2 West Nile virus in Central Europe, 2008/2009. *Veterinary Microbiology*, 165(1–2), 61–70. <https://doi.org/10.1016/j.vetmic.2013.03.005>
- Barbić, L., Listeš, E., Katić, S., Stevanović, V., Madić, J., Starešina, V., & Savini, G. (2012). Spreading of West Nile virus infection in Croatia. *Veterinary Microbiology*, 159(3–4), 504–508. <https://doi.org/10.1016/j.vetmic.2012.04.038>
- Barbić, L., Vilibić-Čavlek, T., Listeš, E., Stevanović, V., Gjenaro-Margan, I., Ljubić-Sternak, S., & Savini, G. (2013). Demonstration of Usutu virus antibodies in horses. *Croatia. Vector Borne Zoonotic Dis*, 13(10), 772–774. <https://doi.org/10.1089/vbz.2012.1236>
- Barros, S. C., Ramos, F., Fagulha, T., Duarte, M., Henriques, A. M., Waap, H., & Fevereiro, M. (2017). West Nile virus in horses during the summer and autumn seasons of 2015 and 2016, Portugal. *Veterinary Microbiology*, 212, 75–79. <https://doi.org/10.1016/j.vetmic.2017.11.008>
- Barros, S. C., Ramos, F., Fagulha, T., Duarte, M., Henriques, M., Luís, T., & Fevereiro, M. (2011). Serological evidence of West Nile virus circulation in Portugal. *Veterinary Microbiology*, 152(3–4), 407–410. <https://doi.org/10.1016/j.vetmic.2011.05.013>
- Bažanów, B., Jansen van Vuren, P., Szymański, P., Stygar, D., Fracka, A., Twardón, J., & Paweńska, J. T. (2018). A survey on West Nile and Usutu viruses in horses and birds in Poland. *Viruses*, 10(2), <https://doi.org/10.3390/v10020087>
- Beck, C., Jiménez-Clavero, M. A., Leblond, A., Durand, B., Nowotny, N., Leparc-Goffart, I., & Lecollinet, S. (2013). Flaviviruses in Europe: Complex circulation patterns and their consequences for the diagnosis and control of West Nile disease. *Int J Environ Res Public Health*, 10(11), 6049–6083. <https://doi.org/10.3390/ijerph10116049>
- Beck, C., Lowenski, S., Durand, B., Bahuon, C., Zientara, S., & Lecollinet, S. (2017). Improved reliability of serological tools for the diagnosis of West Nile fever in horses within Europe. *PLoS Neglected Tropical Diseases*, 11(9), e0005936. <https://doi.org/10.1371/journal.pntd.0005936>
- Berxholi, K., Ziegler, U., Rexhepi, A., Schmidt, K., Mertens, M., Korro, K., & Groschup, M. H. (2013). Indigenous West Nile virus infections in horses in Albania. *Transbound Emerg Dis*, 60(Suppl 2), 45–50. <https://doi.org/10.1111/tbed.12141>
- Bosiljka, D., Vasić, A., Rogožarski, D., Vojinović, D., Elezović-Radovanović, M., Manić, M., & Gligić, A. (2013). Seroepizootiological-epidemiological investigation and mapping of West Nile infection in the republic of Serbia. *Acta Veterinaria*, 63(5–6), 569–579. <https://doi.org/10.2298/AVB1306569D>
- Busani, L., Capelli, G., Cecchinato, M., Lorenzetto, M., Savini, G., Terregino, C., & Marangon, S. (2011). West Nile virus circulation in Veneto region in 2008–2009. *Epidemiology and Infection*, 139(6), 818–825. <https://doi.org/10.1017/S0950268810001871>
- Busquets, N., Laranjo-González, M., Soler, M., Nicolás, O., Rivas, R., Talavera, S., & Napp, S. (2019). Detection of West Nile virus lineage 2 in North-Eastern Spain (Catalonia). *Transboundary and Emerging Diseases*, 66(2), 617–621. <https://doi.org/10.1111/tbed.13086>
- Cabre, O., Durand, J. P., Prangé, A., Gomez, J., Maurizi, L., Tolou, H., & Davoust, B. (2005). West Nile virus infection: Serological investigation among horses in France and in Africa. *Médecine Tropicale : Revue Du Corps De Santé Colonial*, 65(5), 439–443.
- Calistri, P., Giovannini, A., Savini, G., Monaco, F., Bonfanti, L., Ceolin, C., & Lelli, R. (2010). West Nile virus transmission in 2008 in north-eastern Italy. *Zoonoses and Public Health*, 57(3), 211–219. <https://doi.org/10.1111/j.1863-2378.2009.01303.x>
- Ciccozzi, M., Peletto, S., Cella, E., Giovanetti, M., Lai, A., Gabanelli, E., & Zehender, G. (2013). Epidemiological history and phylogeography of West Nile virus lineage 2. *Infect Genet Evol*, 17, 46–50. <https://doi.org/10.1016/j.meegid.2013.03.034>
- Csank, T., Drzewnioková, P., Korytár, L., Major, P., Gyuranecz, M., Pistl, J., & Bakonyi, T. (2018). A Serosurvey of Flavivirus Infection in Horses and Birds in Slovakia. *Vector Borne Zoonotic Dis*, 18(4), 206–213. <https://doi.org/10.1089/vbz.2017.2216>
- Durand, B., Dauphin, G., Zeller, H., Labie, J., Schuffenecker, I., Murri, S., & Zientara, S. (2005). Serosurvey for West Nile virus in horses in southern France. *The Veterinary Record*, 157(22), 711–713. <https://doi.org/10.1136/vr.157.22.711>
- Ergunay, K., Gunay, F., Erisoz Kasap, O., Oter, K., Gargari, S., Karaoglu, T., & Ozkul, A. (2014). Serological, molecular and entomological surveillance demonstrates widespread circulation of West Nile virus in Turkey. *PLoS Neglected Tropical Diseases*, 8(7), e3028. <https://doi.org/10.1371/journal.pntd.0003028>
- García-Bocanegra, I., Arenas-Montes, A., Jaén-Téllez, J. A., Napp, S., Fernández-Morente, M., & Arenas, A. (2012). Use of sentinel serosurveillance of mules and donkeys in the monitoring of West Nile virus infection. *The Veterinary Journal*, 194(2), 262–264. <https://doi.org/10.1016/j.tvjl.2012.04.017>
- García-Bocanegra, I., Arenas-Montes, A., Napp, S., Jaén-Téllez, J. A., Fernández-Morente, M., Fernández-Molera, V., & Arenas, A. (2012). Seroprevalence and risk factors associated to West Nile virus in horses from Andalusia. *Southern Spain. Vet Microbiol*, 160(3–4), 341–346. <https://doi.org/10.1016/j.vetmic.2012.06.027>
- García-Bocanegra, I., Jaén-Téllez, J. A., Napp, S., Arenas-Montes, A., Fernández-Morente, M., Fernández-Molera, V., & Arenas, A. (2012). Monitoring of the West Nile virus epidemic in Spain between 2010 and 2011. *Transbound Emerg Dis*, 59(5), 448–455. <https://doi.org/10.1111/j.1865-1682.2011.01298.x>
- Gardner, I. A., Wong, S. J., Ferraro, G. L., Balasuriya, U. B., Hullinger, P. J., Wilson, W. D., & MacLachlan, N. J. (2007). Incidence and effects of West Nile virus infection in vaccinated and unvaccinated horses in California. *Veterinary Research*, 38(1), 109–116. <https://doi.org/10.1051/vetres:2006045>

- Hubálek, Z., Ludvíková, E., Jahn, P., Trembl, F., Rudolf, I., Svobodová, P., & Stašíková, Z. (2013). West Nile Virus equine serosurvey in the Czech and Slovak republics. *Vector Borne Zoonotic Dis*, 13(10), 733–738. <https://doi.org/10.1089/vbz.2012.1159>
- Hubálek, Z., Wegner, E., Halouzka, J., Tryjanowski, P., Jerzak, L., Šikutová, S., & Włodarczyk, R. (2008). Serologic survey of potential vertebrate hosts for West Nile virus in Poland. *Viral Immunology*, 21(2), 247–253. <https://doi.org/10.1089/vim.2007.0111>
- Jiménez-Clavero, M. A., Gómez-Tejedor, C., Rojo, G., Soriguer, R., & Figuerola, J. (2007). Serosurvey of West Nile virus in equids and bovids in Spain. *The Veterinary Record*, 161(6), 212. <https://doi.org/10.1136/vr.161.6.212>
- Jiménez-Clavero, M. A., Llorente, F., Sotelo, E., Soriguer, R., Gómez-Tejedor, C., & Figuerola, J. (2010). West Nile virus serosurveillance in horses in Donana, Spain, 2005 to 2008. *The Veterinary Record*, 167(10), 379–380. <https://doi.org/10.1136/vr.c3155>
- Kleiboeker, S. B., Loiacono, C. M., Rottinghaus, A., Pue, H. L., & Johnson, G. C. (2004). Diagnosis of West Nile virus infection in horses. *Journal of Veterinary Diagnostic Investigation*, 16(1), 2–10. <https://doi.org/10.1177/104063870401600102>
- Komar, N. (2000). West Nile viral encephalitis. *Rev. Sci. Tech. off. Int. Epiz.*, 19, 166–176. <https://doi.org/10.20506/rst.19.1.1201>
- Llorente, F., Pérez-Ramírez, E., Fernández-Pinero, J., Elizalde, M., Figuerola, J., Soriguer, R. C., & Jiménez-Clavero, M. Á. (2015). Bagaza virus is pathogenic and transmitted by direct contact in experimentally infected partridges, but is not infectious in house sparrows and adult mice. *Veterinary Research*, 46(1), 93. <https://doi.org/10.1186/s13567-015-0233-9>
- Long, M. T. (2014). West Nile virus and equine encephalitis viruses: New perspectives. *The Veterinary Clinics of North America. Equine Practice*, 30(3), 523–542. <https://doi.org/10.1016/j.cveq.2014.08.009>
- Lupulovic, D., Martín-Acebes, M. A., Lazic, S., Alonso-Padilla, J., Blázquez, A. B., Escribano-Romero, E., & Saiz, J. C. (2011). First serological evidence of West Nile virus activity in horses in Serbia. *Vector Borne Zoonotic Dis*, 11(9), 1303–1305. <https://doi.org/10.1089/vbz.2010.0249>
- Madić, J., Savini, G., Di Gennaro, A., Monaco, F., Jukić, B., Kovač, S., & Listeš, E. (2007). Serological evidence for West Nile virus infection in horses in Croatia. *The Veterinary Record*, 160(22), 772–773. <https://doi.org/10.1136/vr.160.22.772>
- Maquart, M., Dahmani, M., Marié, J. L., Gravier, P., Leparç-Goffart, I., & Davoust, B. (2017). First serological evidence of West Nile virus in horses and dogs from Corsica Island. *France. Vector Borne Zoonotic Dis*, 17(4), 275–277. <https://doi.org/10.1089/vbz.2016.2024>
- Medić, S., van den Hoven, R., Petrović, T., Lupulović, D., & Nowotny, N. (2014). Serological evidence of West Nile virus infection in the horse population of northern Serbia. *J Infect Dev Ctries*, 8(7), 914–918. <https://doi.org/10.3855/jidc.3885>
- Moher, D., Shamseer, L., Clarke, M., Ghersi, D., Liberati, A., Petticrew, M., Shekelle, P., & Stewart, L. A. (2015). Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Systematic Reviews*, 4(1), 1. <https://doi.org/10.1186/2046-4053-4-1>
- Monaco, F., Lelli, R., Teodori, L., Pinoni, C., Di Gennaro, A., Polci, A., & Savini, G. (2010). Re-emergence of West Nile virus in Italy. *Zoonoses Public Health*, 57(7–8), 476–486. <https://doi.org/10.1111/j.1863-2378.2009.01245.x>
- Nyaga, V. N., Arbyn, M., & Aerts, M. (2014). Metaprop: A Stata command to perform meta-analysis of binomial data. *Archives of Public Health*, 72(1), 39. <https://doi.org/10.1186/2049-3258-72-39>
- OIE (2018). *West Nile fever*. In OIE Terrestrial Manual, 8th ed. (pp. 697–710). OIE.
- Ozkul, A., Ergunay, K., Koysuren, A., Alkan, F., Arsava, E. M., Tezcan, S., & Us, D. (2013). Concurrent occurrence of human and equine West Nile virus infections in Central Anatolia, Turkey: The first evidence for circulation of lineage 1 viruses. *International Journal of Infectious Diseases*, 17(7), e546–551. <https://doi.org/10.1016/j.ijid.2013.02.005>
- Ozkul, A., Yildirim, Y., Pinar, D., Akcali, A., Yilmaz, V., & Colak, D. (2006). Serological evidence of West Nile Virus (WNV) in mammalian species in Turkey. *Epidemiology and Infection*, 134(4), 826–829. <https://doi.org/10.1017/S0950268805005492>
- Petrović, T., Lazić, S., Lupulović, D., Lazić, G., Bugarski, D., Vidanović, D., & Petrić, D. (2014). Serological study on WNV presence in horses in Vojvodina after the human outbreak in Serbia in 2012. *Archives of Biological Sciences*, 66, 473–481. <https://doi.org/10.2298/ABS1402473P>
- Pradier, S., Sandoz, A., Paul, M. C., Lefebvre, G., Tran, A., Maingault, J., & Leblond, A. (2014). Importance of wetlands management for West Nile Virus circulation risk, Camargue, Southern France. *Int J Environ Res Public Health*, 11(8), 7740–7754. <https://doi.org/10.3390/ijerph110807740>
- R Core Team (2014). *R: A language and environment for statistical computing*. Retrieved from <http://www.R-project.org/>.
- Raleigh, P. J., Sammin, D. J., Connell, J., Markey, B. K., & O'Connor, M. (2012). Surveillance for antibodies to West Nile virus in Ireland. *The Veterinary Record*, 170(7), 180. <https://doi.org/10.1136/vr.100333>
- Rushton, J. O., Lecollinet, S., Hubálek, Z., Svobodová, P., Lussy, H., & Nowotny, N. (2013). Tick-borne Encephalitis virus in horses, Austria, 2011. *Emerg Infect Dis*, 19(4), 635–637. <https://doi.org/10.3201/eid1904.121450>
- Schmidt, J. R., & Mansoury, H. K. E. (1963). Natural and experimental infection of Egyptian Equines with West Nile Virus. *Annals of Tropical Medicine & Parasitology*, 57(4), 415–427. <https://doi.org/10.1080/00034983.1963.11686194>
- Smithburn, K. C., Hughes, T. P., Burke, A. V., & Paul, J. H. (1940). A neurotropic virus isolated from the blood of a native of Uganda. *American Journal of Tropical Medicine and Hygiene*, 20, 471–492.
- Stroup, D. F., Berlin, J. A., Morton, S. C., Olkin, I., Williamson, G. D., Rennie, D., & Thacker, S. B. (2000). Meta-analysis of observational studies in epidemiology: A proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *JAMA*, 283(15), 2008–2012. <https://doi.org/10.1001/jama.283.15.2008>
- Vanhomwegen, J., Beck, C., Després, P., Figuerola, A., García, R., Lecollinet, S., & Serra-Cobo, J. (2017). Circulation of zoonotic arboviruses in equine populations of Mallorca Island (Spain). *Vector Borne Zoonotic Dis*, 17(5), 340–346. <https://doi.org/10.1089/vbz.2016.2042>
- Weissenböck, H., Hubálek, Z., Halouzka, J., Pichlmair, A., Maderner, A., Fragner, K., & Nowotny, N. (2003). Screening for West Nile virus infections of susceptible animal species in Austria. *Epidemiology and Infection*, 131(2), 1023–1027. <https://doi.org/10.1017/s0950268803001031>
- Ziegler, U., Angenvoort, J., Klaus, C., Nagel-Kohl, U., Sauerwald, C., Thalheim, S., & Groschup, M. H. (2013). Use of competition ELISA for monitoring of West Nile virus infections in horses in Germany. *Int J Environ Res Public Health*, 10(8), 3112–3120. <https://doi.org/10.3390/ijerph10083112>
- Ziegler, U., Seidowski, D., Angenvoort, J., Eiden, M., Müller, K., Nowotny, N., & Groschup, M. H. (2012). Monitoring of West Nile virus infections in Germany. *Zoonoses Public Health*, 59(Suppl 2), 95–101. <https://doi.org/10.1111/zph.12015>
- Ziegler, U., Skrypnik, A., Keller, M., Staubach, C., Bezymenyyi, M., Damiani, A. M., & Groschup, M. H. (2013). West Nile virus antibody prevalence in horses of Ukraine. *Viruses*, 5(10), 2469–2482. <https://doi.org/10.3390/v5102469>

How to cite this article: Metz MBC, Olufemi OT, Daly JM, Barba M. Systematic review and meta-analysis of seroprevalence studies of West Nile virus in equids in Europe between 2001 and 2018. *Transbound Emerg Dis*. 2021;68:1814–1823. <https://doi.org/10.1111/tbed.13866>

CHAPTER 4

A scoping review of West Nile virus seroprevalence studies among African equids

Note: This chapter is a published article:

Olufemi OT, Barba M, Daly JM. A Scoping Review of West Nile Virus Seroprevalence Studies among African Equids. *Pathogens*. 2021;10(7):899. Published 2021 Jul 15. doi:10.3390/pathogens10070899

MDPI journals permit the reuse of their articles provided the original article is appropriately cited.

Author contributions: Olufemi OT (50%)*, Barba M (15%), Daly JM (35%).

*OTO: Methodology, Extracting and charting data, Writing- original draft and Writing - reviewing and editing.

Review

A Scoping Review of West Nile Virus Seroprevalence Studies among African Equids

Olaolu T. Olufemi ^{1,2} , Marta Barba ^{3,4}  and Janet M. Daly ^{1,*} 

¹ One Virology—Wolfson Centre for Global Virus Research, School of Veterinary Medicine and Science, University of Nottingham, Loughborough LE12 5RD, UK; olaolu.olufemi@nottingham.ac.uk

² Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, University of Jos, Jos 930003, Nigeria

³ Microbiological Agents Associated with Animal Reproduction (ProVaginBio), Veterinary Faculty, University Cardenal Herrera-CEU, CEU Universities, 46115 Alfara del Patriarca, Spain; martabrvet@gmail.com

⁴ Innovation Centre in Rural Bioeconomy, Agrifood Research and Technology Centre of Aragon (CITA), Av. Montañana, 930, 50059 Zaragoza, Spain

* Correspondence: janet.daly@nottingham.ac.uk

Abstract: West Nile virus (WNV) is an emerging and re-emerging zoonotic flavivirus first identified in and endemic to Africa. The virus is transmitted between birds by biting mosquitoes, with equids and humans being incidental hosts. The majority of infected incidental hosts display no or only mild clinical signs, but a fraction develop encephalitis. The aim of this scoping review was to identify and evaluate primary research on the presence of antibodies to WNV among African equids. Three bibliographic databases and the grey literature were searched. Of 283 articles identified, only 16 satisfied all the inclusion criteria. Data were collated on study design and outcomes. The overall seroprevalence reported ranged from 17.4 to 90.3%, with 1998 (35%) of the 5746 horses, donkeys and mules having screened positive for WNV antibodies. Several articles determined that seroprevalence increased significantly with age. Due to co-circulation of other flaviviruses in Africa, in the majority of studies that screened samples by ELISA, positive results were confirmed using a more specific neutralization test. However, only eight studies tested against other flaviviruses, including Potiskum, Uganda S, Wesselsbron and yellow fever virus in one, Japanese encephalitis and Usutu virus (USUV) in one, tick-borne encephalitis and USUV in one and USUV only in three. Equids are regarded as useful sentinel animals for WNV, but variation in study design poses challenges when trying to determine risk factors for, and trends in, WNV seroprevalence.

Keywords: West Nile virus; flavivirus; antibodies; Africa; horses; donkeys; mules; seroprevalence



Citation: Olufemi, O.T.; Barba, M.; Daly, J.M. A Scoping Review of West Nile Virus Seroprevalence Studies among African Equids. *Pathogens* **2021**, *10*, 899. <https://doi.org/10.3390/pathogens10070899>

Academic Editors:
Tatjana Vilibic-Cavlek and
Vladimir Savic

Received: 29 June 2021

Accepted: 12 July 2021

Published: 15 July 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

West Nile virus (WNV) is a mosquito-borne virus that affects humans and equids. It belongs to the Japanese encephalitis virus (JEV) sero-complex group within the family *Flaviviridae* and genus *Flavivirus* [1]. The virus was first isolated from humans in 1937, specifically from the blood of a woman who suffered mild febrile illness in the West Nile province of Uganda [2], and from horses in 1963 [3]. West Nile virus is believed to be the most pervasive arbovirus, with impacts on animal and human health [4]. The disease now has an almost worldwide distribution across Africa, Asia, Australia, the Middle East, North America and Europe [5–7]. Wild birds serve as primary reservoir hosts and the enzootic transmission cycle is maintained by infected *Culex* spp. mosquitoes [5]. Horses are excellent sentinels for WNV and can be used to forecast WNV risk patterns in humans [8]. Equids are more predisposed to WNV than humans, with possible manifestation of nervous signs in around 10% of infected horses [9].

West Nile virus is endemic to Africa, which is probably the source of all its lineages and genotypes [10]. The continual emergence and re-emergence of WNV in parts of Africa pose

veterinary and public health threats due to the resultant increased morbidity and mortality among humans, horses and birds [11,12]. The abundance of wild birds and ornithophilic mosquitoes on the African continent provides a suitable scenario for WNV transmission to horses and other susceptible hosts. Migratory birds are abundant in Africa [13,14] and their migratory routes play a significant role in the transmission of WNV [15]. Climate change has greatly influenced the expansion of vectors that may transmit WNV and other vector-borne diseases from endemic to non-endemic environments [16]. Human and animal health are largely impacted negatively by climate change, especially in low-income countries [17].

Transient and low-level viremia produced by WNV limits the use of RT-PCR for molecular diagnosis [18–21]. While antibody testing is extensively used for WNV diagnosis, cross reactivity with other flaviviruses makes diagnosis based on immunoglobulin M (IgM) and IgG detected by enzyme-linked immunosorbent assay (ELISA) alone unreliable [22]. Neutralization tests, which are more specific, are considered the gold standard for WNV diagnosis and are used to validate ELISA results [23–25].

Although the disease has been reported in horses in a few countries in Africa, there is no published study on the prevalence of antibodies to WNV in equids across Africa or the drivers of its emergence. The aim of this scoping review was to integrate the existing data on the prevalence of antibodies to WNV among African equids. The primary objective was to evaluate the reliability of what is known about the seroprevalence of WNV among equids in different regions of Africa. A further objective was to determine what is known about risk factors associated with the emergence of the disease in these regions. This scoping review provides veterinarians and agencies with an overview of the existing literature on the prevalence of WNV antibodies in horses, including providing evidence on the burden of WNV in African equids and highlighting knowledge gaps, thereby giving direction to future surveillance efforts.

2. Results

2.1. Literature Search

A total of 283 unique articles from an initial database search of 382 were screened. Title and abstract screening resulted in the exclusion of 242 articles. Of the 41 full-text articles assessed for eligibility, 25 were excluded with reasons listed in Figure 1. Finally, 16 articles satisfied the inclusion criteria.

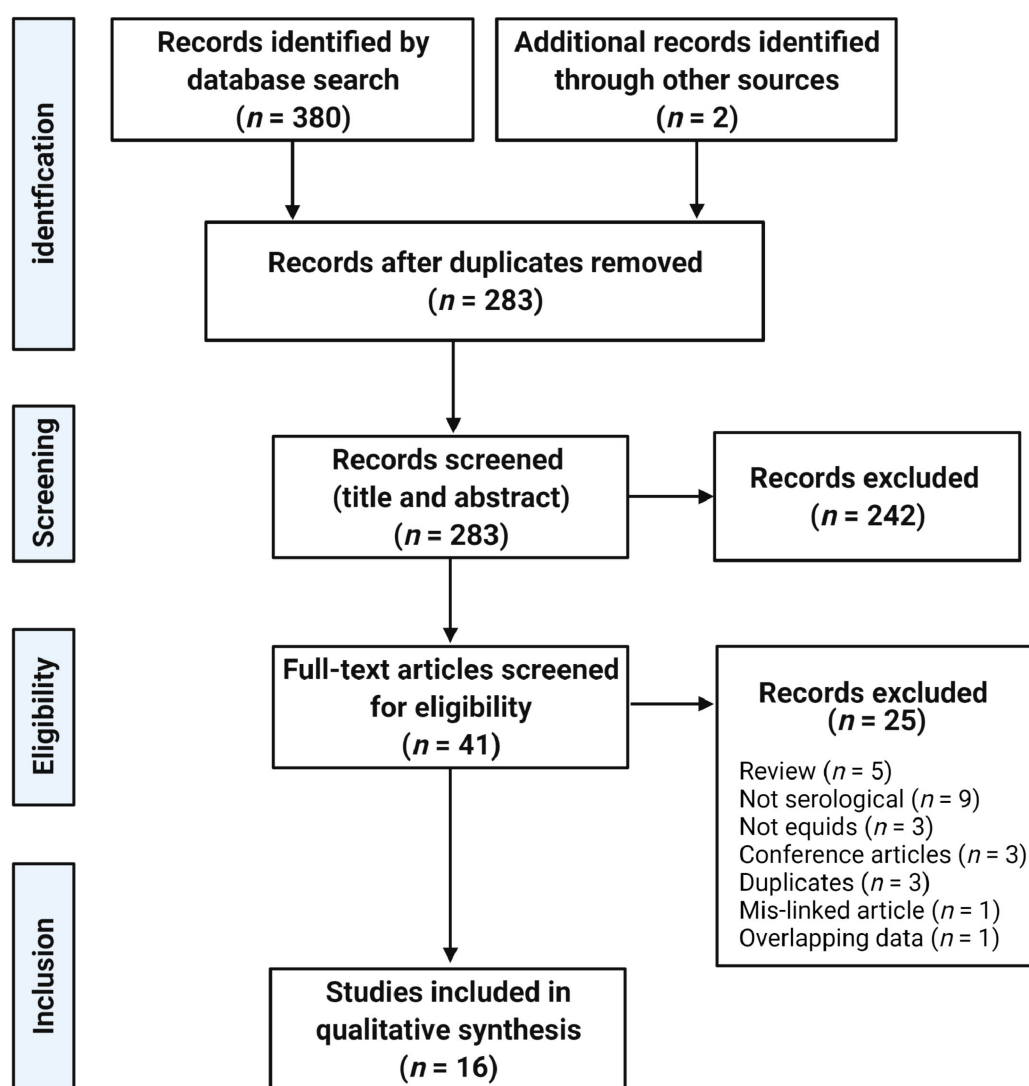


Figure 1. PRISMA flow diagram of literature search and screening articles. (Created with BioRender.com, accessed on 10 July 2021).

2.2. Study Characteristics

Most studies involved samples from a single country (Table 1) and were unevenly distributed across the continent. Eight studies (50%) were from North Africa only while five studies (31.3%) were from West Africa. One study (6.3%) from East Africa involved four islands: Madagascar, Mauritius, Réunion and Seychelles. There was no independent study in Central Africa although one study involved equids in West, East and Central African countries. Only one study (6.3%) was conducted in the South African region. Of the 16 articles included, 15 (93.8%) were published since 2000 (Table 1).

Table 1. Summary of study characteristics of the 16 included studies.

Study	Study Year	Country/ies ¹	Region	Study Design	Sampling Technique
Assaid 2020 [26]	NS	MA	North	Descriptive	Convenience
Bargaoui 2015 [27]	2009	TN	North	Cross-sectional	Multistage (Random)
Ben Hassine 2011 [28]	2008	TN	North	Cross-sectional	Cluster
Ben Hassine 2014 [29]	2012	TN	North	Cross-sectional	Random
Benjelloun 2017 [30]	2011	MA	North	Cross-sectional	Convenience

Table 1. Cont.

Study	Study Year	Country/ies ¹	Region	Study Design	Sampling Technique
Cabré 2006 [31]	2002–2005	DJ CG, GA, TD BJ, CI, SN	East Central West	Cross sectional	Convenience
Cardinale 2017 [32]	2010	MG, MU, RE, SC	East	Cross-sectional	Convenience
Chevalier 2006 [33]	2003	SN	West	Cross-sectional	Convenience
Chevalier 2010 [34]	2005	SN	West	Cross-sectional	Convenience
Davoust 2016 [35]	2014	SN	West	Cross-sectional	All available
Durand 2016 [36]	2012	MA	North	Cross-sectional	Judgmental/ Purposive
Guthrie 2003 [37]	2000–2001	ZA	South	Cohort	Judgmental/ Purposive
Lafri 2017 [38]	2014	DZ	North	Cross-sectional	Convenience
Olaleye 1989 [39]	1987	NG	West	Cross-sectional	Convenience
Selim 2020 [40]	2019	EG	North	Cross-sectional	Judgmental/ Purposive
Sule 2015 [41]	2011–2012	NG	West	Cohort	All available

NS, not specified. ¹ Two-letter ISO code: BJ, Benin; CG, Republic of the Congo; CI, Cote d'Ivoire; DJ, Djibouti; DZ, Algeria; EG, Egypt; GA, Gabon; MA, Morocco; MG, Madagascar; MU, Mauritius; NG, Nigeria; RE, Reunion; SC, Seychelles; SN, Senegal; TD, Chad; TN, Tunisia; ZA, South Africa (ZA).

Study designs included two cohort studies, while others were cross-sectional and one was descriptive (Table 1). Most studies used non-probability sampling techniques, including convenience and purposive/judgmental. In a few studies, probability sampling techniques were used, including random, cluster and multistage sampling. All equids of volunteer owners were sampled in Northwest Senegal and every horse available without WNV vaccination was sampled in Southwestern Nigeria. Two recent studies conducted a survey of vectors alongside the seroprevalence of WNV in equids [26,40].

Sample size varied from 62 to 1189 equids (Table 2). Only three studies (18.8%) presented a sample size calculation for the equids to be sampled. A total of 5746 equids comprising of 4323 (75.2%) horses, 1277 (22.2%) donkeys, 144 (2.5%) mules and 2 (0.03%) unspecified equids were screened across all the studies selected for this scoping review (Table 2). Eleven (68.8%) studies investigated antibodies to WNV in horses only while others also screened donkeys, mules, dogs, cattle, sheep and goats as well as unstated equids. Most studies screened equids older than 2 years with an overall range of 1–32 years. Four studies did not give an age description of the equine population, while two gave a general description of 'adults'. Whether male and/or female equids were included was only stated in half of the studies and the proportions of each was not revealed or was unclear in some. Thoroughbred, Argentine criollo, Dongola, Arab-Barb, European and Moroccan studs, local and mixed breeds were among the breeds screened for antibodies to WNV, although breed was not mentioned in most of the studies (Table 2).

Table 2. Summary of population demographics from 16 studies.

Study	N	Species (n)	Breed(s)	Age	Sex
Assaid 2020 [26]	92	Horse	Mixed	>2 years	M, F
Bargaoui 2015 [27]	1189 ¹	Horse (272) Donkey (807) Mule (107) NS (2)	NS	Adults	NS
Ben Hassine 2011 [28]	133 ¹	Horse (23) Donkey (93) Mule (17)	NS	NS ²	NS ²

Table 2. Cont.

Study	N	Species (n)	Breed(s)	Age	Sex
Ben Hassine 2014 [29]	284	Horse (28) Donkey (219) Mule (37)	NS	2–18 years	M, F
Benjelloun 2017 [30]	840	Horse	Mixed	>3 years	M, F
Cabré 2006 [31]	245	Horse	NS	NS	NS
Cardinale 2017 [32]	303	Horse	NS	Mostly adults	M, F
Chevalier 2006 [33]	120	Horse	Local	1–21 years (7.5) ³	NS
Chevalier 2010 [34]	367	Horse	NS	2–24 years	NS
Davoust 2016 [35]	283	Horse (64) Donkey (29) Sheep (136) Goat (29) Cattle (14) Dog (11)	NS	Adult	NS
Durand 2016 [36]	580	Horse (349) Dog (231)	NS	1.5–32 years (14.5)	M, F
Guthrie 2003 [37]	731	Horse	Thoroughbred	Foal: 2.3–10.2 months (6.6) ⁴ Yearling 15.2–20 months (18.1) ⁴ Dam: 6–22 years (11) ⁴	NS for foals/yearlings
Lafri 2017 [38]	293	Horse (71) Donkey (222)	NS	Adult	M, F
Olaleye 1989 [39]	62	Horse	Dongola and Arab-Barb	NS	M
Selim 2020 [40]	500 ¹	Horse	NS	NS	NS
Sule 2015 [41]	145	Horse	Argentine and local Dongola	≥3 years	M, F

¹ Sample size calculation performed; ² Information on age and sex was collected in a questionnaire but not reported; ³ Mean age; ⁴ Median age. Abbreviations: NS, not specified; M, male; F, female.

2.3. Seroprevalence

A wide range of assays was used to screen equids for antibodies to WNV across studies (Table 3). The notable reported assays were IgG enzyme-linked immunosorbent assay (ELISA), either in-house, commercial, antigen capture, competitive (cELISA) or IgM. Other tests used included micro-immunoassay, Western blot and neutralization tests, plaque reduction neutralization (PRNTs) ($n = 7$), virus neutralization ($n = 2$) and micro-neutralization ($n = 2$). Two studies did not confirm the results of the samples screened by cELISA. One study detected antibodies against non-structural 1 protein using an in-house ELISA to differentiate between infected and vaccinated horses. Most assays were combined with other methods except the complement fixation test (CFT) used in the earliest study.

The virus used for the neutralization test was clearly stated in 10 studies (Table 3); Eg101 was used in 4 studies, Israel 1998 (IS-98-ST1) in 3 studies, and AR 381/00, B956, Morocco 96.111, and NY99 were each used in one study. One study used both lineage 1 and 2 viruses (Eg101 and B956, respectively), while one study used a virus belonging to genetic lineage 2 (AR 381/00) only. Two studies did not specify the virus used in the neutralization test. One study used Ib-AN 7019 for the complement fixation test.

Table 3. Summary of serological tests and seroprevalence outcomes from 16 studies.

Study	Test	WNV Used in NT	Other Flaviviruses	Seroprevalence % (CI)
Assaid 2020 [26]	ELISA ¹ , MIA, MNT	IS-98-STI	TBEV, USUV	33.7
Bargaoui 2015 [27]	ELISA ¹ , MNT	IS-98-STI	-	28
Ben Hassine 2011 [28]	ELISA ¹	-	-	27.1
Ben Hassine 2014 [29]	ELISA ¹ , PRNT	Eg101	USUV	42.3
Benjelloun 2017 [30]	ELISA ¹ , VNT	Morocco 96.111	-	31.1
Cabré 2006 [31]	ELISA, WB, PRNT	Eg101	-	36
Cardinale 2017 [32]	ELISA ¹ , PRNT	NY99	JEV, USUV	27.39
Chevalier 2006 [33]	ELISA, PRNT	NS	-	78.3 (64.0–92.7)
Chevalier 2010 [34]	ELISA, PRNT	B956 and Eg101	USUV	85 (81–89)
Davoust 2016 [35]	ELISA, WB	-	-	74.2
Durand 2016 [36]	ELISA ¹ , VNT	IS-98-STI	USUV	60 (55–65)
Guthrie 2003 [37]	VNT	AR 381/00	-	35.3
Lafri 2017 [38]	ELISA, WB, PRNT	Eg101	-	17.4
Olaleye 1989 [39]	CFT	Ib-AN 7019	POTV, UGSV, WESSV, YFV	71
Selim 2020 [40]	ELISA ¹	-	-	19.4
Sule 2015 [41]	ELISA ¹ , PRNT	NS	-	90.3 (84.3–94.6)

¹ Commercially-available IDvet ELISA used. Abbreviations: CI, confidence intervals; CFT, complement fixation test; ELISA, enzyme-linked immunosorbent assay; JEV, Japanese encephalitis virus; MIA, multiplex immune-assay; MNT, micro-neutralization test; NS, not specified; POTV, Potiskum virus; PRNT, plaque reduction neutralization test; TBEV, tick-borne encephalitis virus; UGSV, Uganda S virus; USUV, Usutu virus; VNT, virus neutralization test; WB, Western blot; WESSV, Wesselsbron virus; YFV, yellow fever virus.

Although the majority of the studies ($n = 10$) did not screen further for neutralizing antibodies to other flaviviruses, Usutu virus (USUV) was the most investigated, with USUV only investigated in 3, USUV and JEV in 1, and USUV and tick-borne encephalitis (TBEV) in 1 (Table 3). The South African strain of USUV, SAAR-1776, was used to detect neutralizing antibodies in three studies while one study used the recombinant E domain III (rEDIII) of USUV (USUV.EDIII) and TBEV (TBEV.EDIII). One study did not mention the strain of virus used in PRNT to detect JEV-specific neutralizing antibodies. Other flaviviruses investigated included: yellow fever virus, Wesselsbron virus, Potiskum virus, and Uganda S virus. Positive titers were detected against USUV and undetermined flaviviruses in 2 and 1 studies, respectively. In one study, results of multiplex immune-assays and micro-neutralization tests differed for 5 samples; 2 samples were positive for WNV and USUV, 2 were positive for USUV and 1 undetermined by MIA while all 5 were negative for USUV by MNT and the undetermined flavivirus was WNV positive. In one study, complement-fixing antibodies were detected against yellow fever virus, Wesselsbron virus, Potiskum virus and Uganda S virus.

In total, 1998 equids (35%) were positive for WNV antibodies out of the 5746 animals screened in Africa. The seroprevalence reported by studies ranged from 17.4 to 90.3% (Table 3). However, although Cabré et al. [31] reported an overall seroprevalence of 36% for samples collected from six countries between 2002 and 2005, this ranged from 3% in Gabon in Central Africa to 92% in Senegal in Western Africa. The prevalence in Northern Africa ranged from 17.4% in Algeria to 42.3% in Tunisia, while in Western Africa, it ranged from 35.9% in Nigeria to 85% in Senegal. In Southern Africa and sub-Saharan Africa, a

prevalence of 35.3 and 35.9%, respectively, was reported (Figure 2). There was no apparent trend in seroprevalence over time. The earliest study [39] found a seroprevalence of 71% in Nigeria in 1987 whereas the most recent study [40] found a seroprevalence of 19.4% in Egypt in 2019. Similarly, two independent studies conducted in 2014 in Algeria and Senegal had a seroprevalence of 17.4% and 74.2%, respectively [35,38].

In 4 of the 16 studies, longitudinal samples were obtained from 615 equids and seroconversion was observed in 12% of them. In one study [37], seroconversion was observed in 50 equids (11.1%) that became seropositive as yearlings after testing negative as foals. A further 4 seroconverted a year after the first screening. More of the foals born earlier in the foaling season (August to December) seroconverted than those foaled later [37]. In Tunisia, of 84 horses that initially tested negative, 13 were lost to follow-up and only 2 (2.4%) seroconverted [28]. In one study, 36 horses (18 each in Chad and Côte d'Ivoire) were sampled again one year later [31]. In Chad, 2 of 3 horses that initially tested negative were positive and 5 of the 15 initially positive horses were no longer positive. In Côte d'Ivoire, none of the eight initially negative horses had seroconverted a year later and only one of ten horses remained positive. In one study [38], two horses became seropositive 8 months after arriving in El Kala, Algeria.

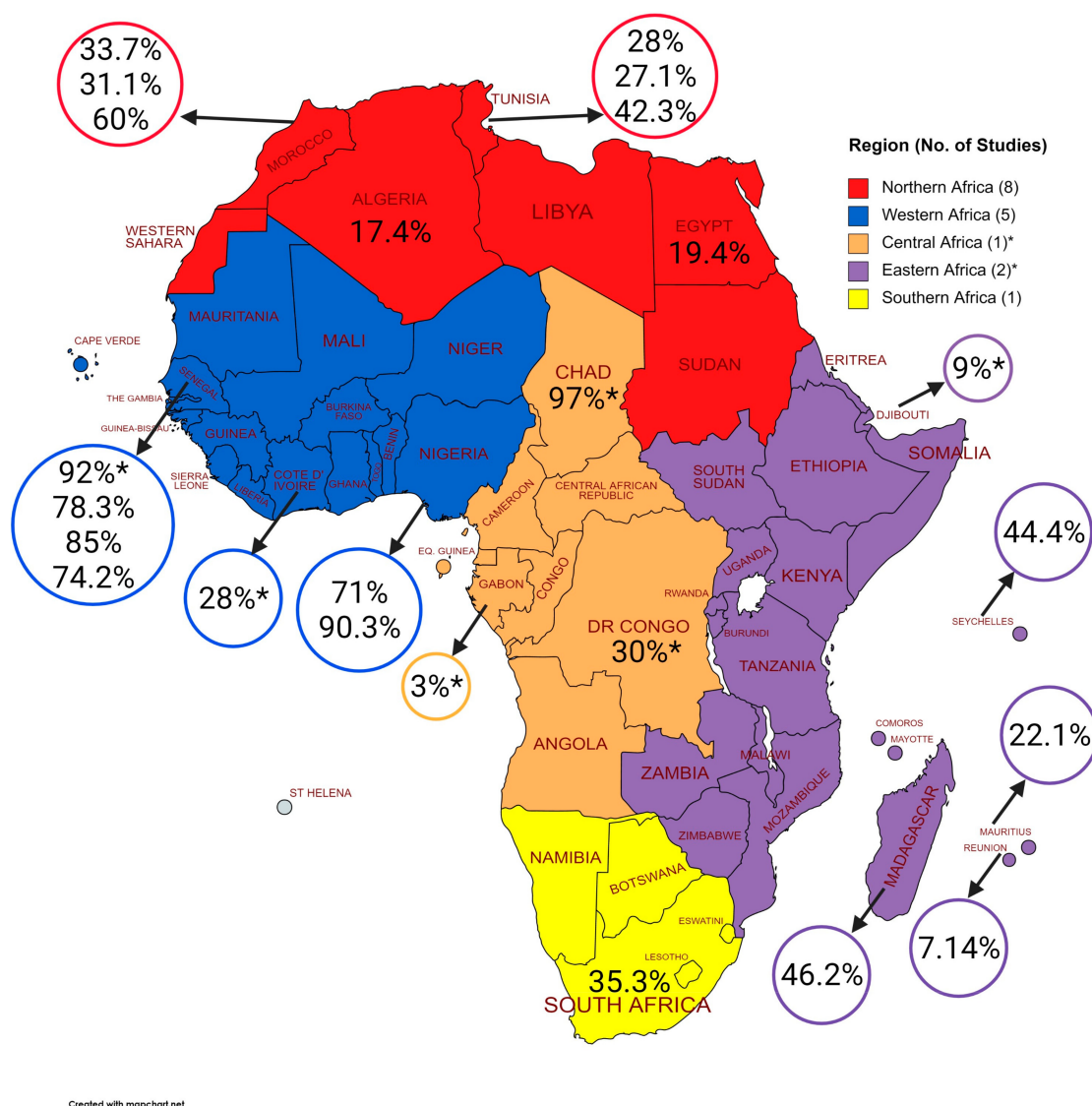


Figure 2. Map of Africa showing seroprevalence reported in different countries (* indicates study where samples were screened from multiple countries [31]). Created with MapChart.net (accessed on 26 May 2021).

2.4. Risk Factors

Risk factors for seroprevalence of WNV among the equids were assessed by 13 studies. Risk factors may be host dependent (e.g., species, breed, age, sex) or non-host dependent (e.g., environmental). Ten studies discussed both host and non-host dependent factors in relation to the seroprevalence and three [30,31,40] discussed only non-host dependent factors.

In one study, a significant difference between seroprevalence of horses and donkeys was identified [38]. In another study, breed-specific seroprevalence of WNV varied significantly with a higher likelihood of a local breed, Dongola, being seropositive than the imported Argentine [41]. WNV seroprevalence increased with age [37]. This increase was reported to be significant in several studies [32–34]. Cardinale et al. [32] reported odds of infection about three times higher in horses aged 11–15 years than those aged 1–5 years. Similarly, Guthrie et al. [37] found the odds of being seropositive were about 5 times higher for older mares (aged 10–22 years) compared to younger mares (aged 6–9 years). Being a stallion was a protective factor [36]. As mentioned above, in a study conducted in South Africa, foals born in August–October 1999 had a higher risk of seroconverting in their first year of life than those born November–December 1999 [37]. Furthermore, those born to seropositive mares had about half the risk of seroconverting of those born to seronegative mares [37].

In a study conducted in Senegal, Chevalier et al. [34] randomly sampled equids at five locations with different landscape types. In a statistical model that included equid age, they found that lower seroprevalence was associated with areas with saline water and arid salty mud flats, environments that are unfavorable for *Culex* mosquito larvae [34]. Similarly, WNV infection did not occur in equids from coastal lowlands in contrast to a widespread infection in Highveld/plateau and Western Cape Province regions of South Africa [37]. Other studies reported significant differences in seroprevalence in horses in different locations in Northeast Algeria and Morocco [36,38]. Only four studies described the mobility of the equids sampled [27,31,32,38].

3. Discussion

In this scoping review, 16 studies were identified in which authors described the seroprevalence of WNV among African equids. Two recently published studies [42,43], were outside our search period. An article referenced in one of these articles was not found in our search [44].

Overall, this review revealed an uneven spread of research on WNV seroprevalence across the five regions of Africa. More studies were reported in North and West Africa with only one report in Southern and Eastern Africa, while another study had equid representation from central and Eastern Africa. East Africa, the origin of the virus [2], is under-represented despite the burden of the disease in the region [45–47]. The two studies that involved the region are not a true representation of WNV seroprevalence among equids in the region as only animals from Djibouti [31] and four Indian Ocean islands [32] were sampled. Africa has a wide range of climatic conditions. The overall seroprevalence reported by Cabré et al. [31], who screened samples obtained from East, Central and West Africa, was 36% but there was a wide range of seroprevalence for individual countries. The highest seroprevalence (92–97%) was observed in the western and central parts of the Sahelian zone, which is typified by semi-arid climate and steppe and bush grass vegetation. In contrast, the sub-Saharan zone, with an arid climate and a semidesert vegetation had lower seroprevalence (3–30%). A regional difference in seroprevalence was also observed in equids sampled from five unique bioclimatic regions of Morocco in 2011 [30]. However, other factors can influence seropositivity. The significant relationship between WNV seropositivity in a study in Northeast Algeria can be linked to the arrival of equids in a town bordering Tunisia where they seroconverted [38]; Tunisia already had WNV circulating in 2014 [48].

There was considerable heterogeneity in the design of the 16 reviewed studies. Although expensive to execute, probability sampling is more reliable with a representative population than non-probability sampling techniques. An increased sample size following an appropriate determination of the minimum sample size is often used to improve reliability of the latter. The non-calculation of the minimum sample size in most studies may explain the wide range (62–1189) in the total number of equids sampled across all studies.

All except two of the studies [37,39] employed ELISA as a preliminary screening assay. Mostly, the species-independent ID-Vet ELISA was used for the detection of the WNV anti-envelope (E) protein along with a few in-house ELISAs, even though some immunofluorescent-based tests are also commercially available. Despite the benefit of a quick and reproducible result, cross-reactivity between flaviviruses remains a serious limitation for using ELISAs or immunofluorescent tests alone. ELISA alone often results in false positives due to co-circulation of diverse flaviviruses [49]. Neutralization tests can resolve discrepancies that often occur with other assays due to cross-reactivity. Confirmatory neutralization tests were performed in most studies after initial screening with ELISA, microsphere immunoassay [26] or Western blot [31]. Neutralization tests performed included MNT, VNT and PRNT. Not paying attention to the cross-reactivity of flaviviruses among African equids could result in an erroneous representation of the seroprevalence of the disease. For instance, equids positive for USUV, WNV and undetermined flavivirus based on MIA were all confirmed as WNV positive by virus neutralization test [26]. On the other hand, one of the studies showed that seroprevalence determined by neutralization test varied only slightly from that determined by ELISA (30.4% vs. 33.7%, respectively) among equids in Morocco [26]. The survey in Tunisia [28] might have used ELISA alone based on the fact that two WNV epidemics had been reported before the survey [12,50]. This, however, does not fully account for co-circulation of other flaviviruses. Another approach that provides some reassurance that antibodies are specific to WNV is to screen vectors for the presence of virus in parallel. A recently published article screened 368 horses in Nigeria using ELISA alone along with 775 adult female *Culex* mosquitoes (pooled in 31 groups) from the stables of sampled horses. The resultant 89.9% seroprevalence in horses was in stark contrast to detection of the presence of WNV antigen in only one pool of mosquitoes using the VectorTest® WNV Antigen Assay [42]. Similarly, Assaid [26] found that 33.7% (31/92) of horses were antibody positive, but only one out of 146 mosquito pools was positive for WNV. This differed from the study in Egypt included in this review in which 500 horses and 5 pools of *Culex* mosquitoes (100 per pool) were sampled with an equine seroprevalence of 19.4% and the detection of the NS5 gene by RT-PCR in one pool (i.e., 20%) [40]. A recent study in Tunisia where a sheep serum sample was confirmed to be TBEV seropositive [51] together with reports of undetermined flavivirus antibodies in some of the studies from North Africa [26,29,36] suggest that antibodies to TBEV also need to be differentiated, at least in North Africa.

Demographic data (age, sex, breed, species) of the equids sampled are important in determining specific seroprevalence for different strata in the population and the risk factors associated with seropositivity. Although most studies reported the age of the equids, a few had no age description making it difficult to compare age outcomes across all studies. Two studies [27,38] described equids as ‘adults’ without defining this category, making assessment across age groups not possible. Nonetheless, several studies identified a significant increasing risk of seropositivity with age. The rise in age-specific seroprevalence of WNV observed as equids increase in age might be indicative of endemic transmission of WNV. The breeds of equids sampled were seldom mentioned [33,37,39,41] and any breed-specific differences in seroprevalence were difficult to determine. This challenge might be because of the diversity of horse breeds in Africa coupled with importation of several breeds that are often crossed with indigenous breeds. This probably influenced the description ‘different’ and ‘various’ breeds by some authors [26,30]. Categorizing breed types either as draft horses (used for work), light horses (for riding), crosses or ponies where specific breeds cannot be determined could reveal a general trend in disposition to

WNV seropositivity. Polymorphisms in the innate immune Oas1b gene have been identified as a determining factor for resistance to WNV [52]. However, to date, no difference in susceptibility to WNV infection between major equine breeds in the United States has been reported. Not all the studies described the sex of the equids sampled. Being a stallion was a protective factor [36], but this could be related to stallions being housed differently (e.g., more likely to be stabled).

There are four WNV vaccines available commercially for equids, three in the United States and one in Europe [53,54]. Vaccination of horses may not be common in Africa as it is probably limited to valuable sporting horses, but horses imported as adults could be previously vaccinated. It is therefore paramount that the WNV vaccination status of horses in Africa be declared when enrolled in seroprevalence studies. When this status is unknown, it should be stated clearly with the origin of the horse mentioned. In the study in this review where all sampled equids had been vaccinated against WNV (>400 days before sampling and most had received a single dose in 2010), it was presumed that vaccine antibodies waned (detected up to 300 days post injection). An ELISA to detect anti-NS1 antibodies was used in this study to differentiate infected from vaccinated horses, with 79% of the cELISA positive sera also positive for WNV NS1 antibodies indicative of infection. Conflicting results between cELISA and NS1 could suggest infection with a different flavivirus as two NS1 negative horses were found to have antibodies to USUV [36].

In one of the studies reviewed here, military working dogs in Morocco showed similar seroprevalence (62%) to that seen in horses (60%) leading to the suggestion that they could be an alternative sentinel species [36]. In contrast, another reviewed study [35] found that seroprevalence in dogs was less than half that in horses (27.3% vs. 68.7%). This study also found that seroprevalence was low in goats (6.9%) and no antibodies were detected in sheep or cattle. This agrees with a report from southern France where some animals including cows, goats, camels and zebra, tested following an outbreak among horses, were all negative [55]. Similarly, on the island of Corsica, France, sheep sera tested negative while seroprevalence in horses and dogs was 9.4% and 8.4%, respectively [56]. There have also been reports in China of WNV-neutralizing antibodies circulating in dogs and even cats [57] and 24 of 183 hunting dogs (13.11%) sampled in Italy recently showed seroneutralizing antibodies to USUV, but no antibodies to WNV were detected [58]. Thus, the horse remains the most relevant species to monitor circulation of WNV, despite the potential complication of vaccine-induced antibodies. Interestingly, in one article [29], it was reported that the presence of cattle is a protective factor. Seroprevalence of WNV among resident and migrating species of bird in northern Senegal [59] corroborates the high seroprevalence observed in equids in the region [33]. Despite several reports of WNV epidemics in Sudan [60–62] and a recent detection of the virus from wild mosquitoes in Khartoum, there are no prevalence reports among equids. In Nigeria, where the virus was isolated from humans in 1973 [63], it has become an increasingly-recognized public health issue across this most populous African nation [64,65].

In this scoping review, we observed the relative dearth of WNV seroprevalence studies in African equids (5746 equids screened in 16 studies published between 1937 and 2020) across all regions of the continent despite the early WNV outbreaks and seropositivity in humans and animals [66–70]. The low numbers of clinical cases of WNV among both human and veterinary species that have been reported could be blamed on misdiagnoses and lack of funding in the region [71]. Only a few seroprevalence studies of WNV among equids were reported before 2010 [31,33,37,39] and, apart from the report by Cabré et al. [31], were geographically limited. Although the number of publications increased from 2010 onward, and two studies published after the literature search date were conducted in West Africa [42,43], this was still minimal compared to a review of WNV seroprevalence studies in Europe [72] that identified 39 studies published between 2001 and 2018 involving a total of 14,327 equids.

Despite speculation that climate change is leading to increased circulation of vector-borne viruses such as WNV, there was no discernible trend in seroprevalence across the

years covered by the studies reviewed. Levels of seroprevalence were likely to be strongly influenced by location, timing of sampling, and other factors. For example, for two studies conducted in the same year, a seroprevalence of 17.4% was reported in Algeria [38] and 74.2% in Senegal [35]. The occurrence of a WNV outbreak may prompt subsequent seroprevalence studies (e.g., the study by Benjelloun et al. [30] was conducted the year after an outbreak in 2010).

Some studies were evidently performed in the rainy (wet) season when mosquito vectors are more abundant, while some were conducted over a longer period. The abundance of mosquito vectors suggests increased transmission and more infection. This might be the reason for the very high seroprevalence observed in equids sampled in the wet season in Senegal (78.3% and 85%) and Nigeria (90.3%) [33,34,41]. A high seroprevalence, 74.2%, was also observed among equids sampled at the end of the dry season [35]. It is challenging to describe the climate of the sampling period for studies that reported sampling period based on calendar month (January–December). With climate change, it is often difficult to describe the climate based on the calendar month, especially for Africa.

As equids are often used as draft animals and also in sport or leisure, the seroprevalence rate may indicate human exposure to WNV. The publication date of some of the studies was much later than the period the research/investigation was conducted. For instance, two studies conducted in 2010 and 2011 were both published in 2017 [30,32] while another conducted in 2012 was published in 2016 [35]. This time lag impacts the real-time comprehension of the status of WNV as well as the risk, thus impairing surveillance and public health responses. Regional and sub-regional collaborations could help reduce the period between field sampling and publication. Although a wide range of seroprevalence was reported, making it difficult to have a single reliable seroprevalence value for the different regions and individual countries in Africa, the high seroprevalence in some regions is a call to institute a workable regional or sub-regional surveillance protocol for Africa. One reason for the few numbers of studies across some regions could be the expense of the commercially available ELISA kit, coupled with the difficulty of confirmatory assays that need to be performed in CL3 laboratories, suggesting that development of cheaper but reliable and specific antibody tests are required. Furthermore, a regional collaborative research could ameliorate the deficit and encourage a One Health strategy to tackling the burden of WNV in Africa.

4. Materials and Methods

4.1. Search Strategy

The search strategy aimed to identify relevant published studies. Three electronic databases, PUBMED (Medline), OVID (via CAB Abstracts, Embase and Zoological records) and African Journal Online (AJOL) were searched to identify peer-reviewed articles on the topic. The literature search was performed using the key terms and Boolean operators ('AND' and 'OR') as follows: (Seroprevalence OR prevalence OR epidemiology OR frequency OR occurrence OR detection OR report OR identification OR isolation OR characterization OR investigation) AND (West Nile virus OR WNV OR Flavivirus) AND (Horses OR Equine OR Donkey OR Equid OR Mule OR Foal OR Zebra) AND (Africa OR South Africa OR West Africa OR East Africa OR North Africa). The search terms were applied to all fields with no language restrictions. Retrieved hits were entered into a Microsoft Excel (2019) file. The reference lists of all studies selected for critical appraisal were screened to expand the search.

4.2. Eligibility Criteria

Peer-reviewed studies published between 10 July 1937 and 10 July 2020 conducted in any of the 54 countries of the African continent were screened for eligibility. The following studies were considered eligible: primary research, descriptive and cross-sectional studies, and prospective and retrospective studies. Analytical observational studies, including longitudinal cohort studies and analytical cross-sectional studies, were considered for

inclusion. Case reports, case series and reviews were considered ineligible. This review considered prevalence determined by laboratory assessment of antibodies to WNV. The eligible study population was equids (horses, donkeys, mules and zebra) of all ages. Conference abstracts and reviews were not included in this scoping review.

4.3. Study Selection

Firstly, O.T.O. and J.M.D. screened all search results and removed duplicates before an independent screening of titles and abstracts of all retrieved studies for relevant potential studies. All articles with titles and abstracts not fulfilling the eligibility criteria above were excluded, along with studies that had missing or duplicated data published in included studies. Full texts were screened when eligibility could not be ascertained from the title and abstract. Secondly, all full texts retained were scanned independently in a standardized approach by O.T.O. and J.M.D. using the eligibility criteria. Only studies with accessible full texts were retained and subjected to eligibility checks.

Following completion of eligibility evaluation, the full text of selected citations were assessed in detail by two independent reviewers (O.T.O. and J.M.D.). Reasons for exclusion of full text studies that did not meet the inclusion criteria were recorded and reported.

4.4. Data Extraction

A custom data extraction form was created with data categorized into: general data (first author's name, year of publication, title, name of journal, language, country, region, study type, sampling protocol, etc.), diagnostic technique data (initial serological screening for immunoglobulin G, type of confirmatory test and other additional tests performed) and data related to the animal (species of equid, number of equids, sex, breed, WNV vaccination history, seroconversion, season of investigation and risk factors investigated). Disagreements that arose between the reviewers O.T.O. and J.M.D. were resolved through discussion, or by a third reviewer (M.B.).

5. Conclusions

This scoping review highlights the dearth of seroprevalence studies among African equids. WNV is endemic among equids in all regions of Africa and the risk of infection increases with age. Being good sentinels for WNV, there is a need for a subregional and regional collaboration targeted towards WNV among equids in Africa, thus curbing the threat of WNV to humans in Africa. ELISAs, Western blot and neutralization tests are established diagnostic methods in Africa. Researchers should employ specific serological tests to rule out possible cross-reactivity with other flaviviruses. For future research, detailed description of demographics of equids and associated risk factors to WNV and other related flaviviruses among African equids is encouraged. A 'One Health' initiative involving governmental and non-governmental parastatals along with other stakeholders in ministries, departments and agencies should be established to come up with a national, sub-regional and regional strategic plans for routine surveillance and reporting of WNV among equids and other animals as a public health preparedness plan to curb this zoonotic disease. These are very pressing issues that require a collaborative regional One Health approach [71].

Author Contributions: Conceptualization, J.M.D. and M.B.; methodology, J.M.D. and O.T.O.; extracting and charting the data, O.T.O. and J.M.D.; writing—original draft preparation, O.T.O.; writing—review and editing, J.M.D., M.B. and O.T.O. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding. O.T.O. is a recipient of a University of Nottingham Vice-Chancellor's Scholarship for Research Excellence (International).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. MacLachlan, N.J.; Dubovi, E.J. Chapter 29: Flaviviridae. In *Fenner's Veterinary Virology*, 5th ed.; MacLachlan, N.J., Dubovi, E.J., Eds.; Elsevier Academic Press: London, UK, 2017; pp. 525–545.
2. Smithburn, K.; Hughes, T.; Burke, A.; Paul, J. A neurotropic virus isolated from the blood of a native of Uganda. *Am. J. Trop. Med. Hyg.* **1940**, *1*, 471–492. [\[CrossRef\]](#)
3. Schmidt, J.R.; Mansoury, H.K.E. Natural and Experimental Infection of Egyptian Equines with West Nile Virus. *Ann. Trop. Med. Parasit.* **1963**, *57*, 415–427. [\[CrossRef\]](#)
4. Petersen, L.R.; Brault, A.C.; Nasci, R.S. West Nile Virus: Review of the Literature. *JAMA* **2013**, *310*, 308. [\[CrossRef\]](#)
5. Campbell, G.L.; Marfin, A.A.; Lanciotti, R.S.; Gubler, D.J. West Nile virus. *Lancet Infect. Dis.* **2002**, *2*, 519–529. [\[CrossRef\]](#)
6. Murgue, B.; Zeller, H.; Deubel, V. The ecology and epidemiology of West Nile virus in Africa, Europe and Asia. *Curr. Top. Microbiol. Immunol.* **2002**, *267*, 195–221. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Roehrig, J.T.; Layton, M.; Smith, P.; Campbell, G.L.; Nasci, R.; Lanciotti, R.S. The emergence of West Nile virus in North America: Ecology, epidemiology, and surveillance. *Curr. Top. Microbiol. Immunol.* **2002**, *267*, 223–240. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Venter, M.; Pretorius, M.; Fuller, J.A.; Botha, E.; Rakgotho, M.; Stivaktas, V.; Weyer, C.; Romito, M.; Williams, J. West Nile Virus Lineage 2 in Horses and Other Animals with Neurologic Disease, South Africa, 2008–2015. *Emerg. Infect. Dis.* **2017**, *23*, 2060–2064. [\[CrossRef\]](#) [\[PubMed\]](#)
9. Castillo-Olivares, J.; Wood, J. West Nile virus infection of horses. *Vet. Res.* **2004**, *35*, 467–483. [\[CrossRef\]](#)
10. May, F.J.; Davis, C.T.; Tesh, R.B.; Barrett, A.D. Phylogeography of West Nile virus: From the cradle of evolution in Africa to Eurasia, Australia, and the Americas. *J. Virol.* **2011**, *85*, 2964–2974. [\[CrossRef\]](#)
11. Schuffenecker, I.; Peyrefitte, C.N.; el Harrak, M.; Murri, S.; Leblond, A.; Zeller, H.G. West Nile virus in Morocco, 2003. *Emerg. Infect. Dis.* **2005**, *11*, 306–309. [\[CrossRef\]](#)
12. Triki, H.; Murri, S.; Le, B.G.; Bahri, O.; Hili, K.; Sidhom, M.; Dellagi, K. West Nile viral meningo-encephalitis in Tunisia. *Med. Trop.* **2001**, *61*, 487–490.
13. Garcia-Heras, M.-S.; Arroyo, B.; Mougeot, F.; Bildstein, K.; Therrien, J.-F.; Simmons, R.E. Migratory patterns and settlement areas revealed by remote sensing in an endangered intra-African migrant, the Black Harrier (*Circus maurus*). *PLoS ONE* **2019**, *14*, e0210756. [\[CrossRef\]](#)
14. Hahn, S.; Bauer, S.; Liechti, F. The Natural Link between Europe and Africa: 2.1 Billion Birds on Migration. *Oikos* **2009**, *118*, 624–626. [\[CrossRef\]](#)
15. Mackenzie, J.S.; Gubler, D.J.; Petersen, L.R. Emerging flaviviruses: The spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat. Med.* **2004**, *10*, 98–109. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Khasnis, A.A.; Nettleman, M.D. Global warming and infectious disease. *Arch. Med. Res.* **2005**, *36*, 689–696. [\[CrossRef\]](#)
17. Haines, A.; Kovats, R.S.; Campbell-Lendrum, D.; Corvalan, C. Climate change and human health: Impacts, vulnerability and public health. *Public Health* **2006**, *120*, 585–596. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Barzon, L.; Pacenti, M.; Franchin, E.; Pagni, S.; Martello, T.; Cattai, M.; Cusinato, R.; Palù, G. Excretion of West Nile virus in urine during acute infection. *J. Infect. Dis.* **2013**, *208*, 1086–1092. [\[CrossRef\]](#) [\[PubMed\]](#)
19. Busch, M.P.; Kleinman, S.H.; Tobler, L.H.; Kamel, H.T.; Norris, P.J.; Walsh, I.; Matud, J.L.; Prince, H.E.; Lanciotti, R.S.; Wright, D.J. Virus and antibody dynamics in acute West Nile virus infection. *J. Infect. Dis.* **2008**, *198*, 984–993. [\[CrossRef\]](#)
20. Busch, M.P.; Wright, D.J.; Custer, B.; Tobler, L.H.; Stramer, S.L.; Kleinman, S.H.; Prince, H.E.; Bianco, C.; Foster, G.; Petersen, L.R. West Nile virus infections projected from blood donor screening data, United States, 2003. *Emerg. Infect. Dis.* **2006**, *12*, 395. [\[CrossRef\]](#)
21. Lustig, Y.; Mannasse, B.; Koren, R.; Katz-Likvornik, S.; Hindiyeh, M.; Mandelboim, M.; Dovrat, S.; Sofer, D.; Mendelson, E. Superiority of West Nile virus RNA detection in whole blood for diagnosis of acute infection. *J. Clin. Microbiol.* **2016**, *54*, 2294–2297. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Niedrig, M.; Sonnenberg, K.; Steinhagen, K.; Paweska, J.T. Comparison of ELISA and immunoassays for measurement of IgG and IgM antibody to West Nile virus in human sera against virus neutralisation. *J. Virol. Meth.* **2007**, *139*, 103–105. [\[CrossRef\]](#)
23. Calisher, C.H.; Karabatsos, N.; Dalrymple, J.M.; Shope, R.E.; Porterfield, J.S.; Westaway, E.G.; Brandt, W.E. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J. Gen. Virol.* **1989**, *70*, 37–43. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Kuno, G. Serodiagnosis of flaviviral infections and vaccinations in humans. *Adv. Virus Res.* **2003**, *61*, 3–66. [\[PubMed\]](#)
25. Nelson, S.; Jost, C.A.; Xu, Q.; Ess, J.; Martin, J.E.; Oliphant, T.; Whitehead, S.S.; Durbin, A.P.; Graham, B.S.; Diamond, M.S. Maturation of West Nile virus modulates sensitivity to antibody-mediated neutralization. *PLoS Pathog.* **2008**, *4*, e1000060. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Assaid, N.; Mousson, L.; Moutailler, S.; Arich, S.; Akarid, K.; Monier, M.; Beck, C.; Lecollinet, S.; Failloux, A.B.; Sarih, M. Evidence of circulation of West Nile virus in *Culex pipiens* mosquitoes and horses in Morocco. *Acta Trop.* **2020**, *205*, 105414. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Bargaoui, R.; Lecollinet, S.; Lancelot, R. Mapping the serological prevalence rate of West Nile fever in equids, Tunisia. *Transbound. Emerg. Dis.* **2015**, *62*, 55–66. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Ben Hassine, T.; Hammami, S.; Elghoul, H.; Ghram, A. Détection de la circulation de virus West Nile chez les Équidés dans le nord-ouest de la Tunisie. *Bull. Soc. Pathol. Exot.* **2011**, *104*, 266–271. [\[CrossRef\]](#)

29. Ben Hassine, T.; De Massis, F.; Calistri, P.; Savini, G.; Belhaj Mohamed, B.; Ranen, A.; Di Gennaro, A.; Sghaier, S.; Hammami, S. First Detection of Co-circulation of West Nile and Usutu Viruses in Equids in the South-west of Tunisia. *Transbound. Emerg. Dis.* **2014**, *61*, 385–389. [\[CrossRef\]](#)
30. Benjelloun, A.; El Harrak, M.; Calistri, P.; Loutfi, C.; Kabbaj, H.; Conte, A.; Ippoliti, C.; Danzetta, M.L.; Belkadi, B. Seroprevalence of West Nile virus in horses in different Moroccan regions. *Vet. Med. Sci.* **2017**, *3*, 198–207. [\[CrossRef\]](#)
31. Cabre, O.; Grandada, M.; Marie, J.-L.; Gravier, P.; Prange, A.; Santinelli, Y.; Rous, V.; Bourry, O.; Durand, J.-P.; Tolou, H.; et al. West Nile Virus in Horses, sub-Saharan Africa. *Emerg. Infect. Dis.* **2006**, *12*, 1958–1960. [\[CrossRef\]](#)
32. Cardinale, E.; Bernard, C.; Lecollinet, S.; Rakotoharinome, V.M.; Ravaomanana, J.; Roger, M.; Olive, M.M.; Meenowa, D.; Jaumally, M.R.; Melanie, J.; et al. West Nile virus infection in horses, Indian ocean. *Comp. Immunol. Microbiol. Infect. Dis.* **2017**, *53*, 45–49. [\[CrossRef\]](#)
33. Chevalier, V.; Lancelot, R.; Diaité, A.; Mondet, B.; Sall, B.; De Lamballerie, X. Serological Assessment of West Nile Fever Virus Activity in the Pastoral System of Ferlo, Senegal. *Ann. N. Y. Acad. Sci.* **2006**, *1081*, 216–225. [\[CrossRef\]](#)
34. Chevalier, V.; Dupressoir, A.; Tran, A.; Diop, O.M.; Gottland, C.; Diallo, M.; Etter, E.; Ndiaye, M.; Grosbois, V.; Dia, M.; et al. Environmental risk factors of West Nile virus infection of horses in the Senegal River basin. *Epidemiol. Infect.* **2010**, *138*, 1601–1609. [\[CrossRef\]](#)
35. Davoust, B.; Maquart, M.; Roqueplo, C.; Gravier, P.; Sambou, M.; Mediannikov, O.; Leparac-Goffart, I. Serological Survey of West Nile Virus in Domestic Animals from Northwest Senegal. *Vector Borne Zoonotic Dis.* **2016**, *16*, 359–361. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Durand, B.; Haskouri, H.; Lowenski, S.; Vachier, N.; Beck, C.; Lecollinet, S. Seroprevalence of West Nile and Usutu viruses in military working horses and dogs, Morocco, 2012: Dog as an alternative WNV sentinel species? *Epidemiol. Infect.* **2016**, *144*, 1857–1864. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Guthrie, A.J.; Howell, P.G.; Gardner, I.A.; Swanepoel, R.E.; Nurton, J.P.; Harper, C.K.; Pardini, A.; Groenewald, D.; Visage, C.W.; Hedges, J.F.; et al. West Nile virus infection of Thoroughbred horses in South Africa (2000–2001). *Equine Vet. J.* **2003**, *35*, 601–605. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Lafri, I.; Prat, C.M.; Bitam, I.; Gravier, P.; Besbaci, M.; Zeroual, F.; Ben-Mahdi, M.H.; Davoust, B.; Leparac-Goffart, I. Seroprevalence of West Nile virus antibodies in equids in the North-East of Algeria and detection of virus circulation in 2014. *Comp. Immunol. Microbiol. Infect. Dis.* **2017**, *50*, 8–12. [\[CrossRef\]](#) [\[PubMed\]](#)
39. Olaleye, O.D.; Oladosu, L.A.; Omilabu, S.A.; Baba, S.S.; Fagbami, A.H. Complement fixing antibodies against arboviruses in horses at Lagos, Nigeria. *Rev. Elev. Med. Vet. Pays Trop.* **1989**, *42*, 321–325.
40. Selim, A.; Radwan, A.; Arnaout, F. Seroprevalence and molecular characterization of West Nile Virus in Egypt. *Comp. Immunol. Microbiol. Infect. Dis.* **2020**, *71*, 101473. [\[CrossRef\]](#)
41. Sule, W.F.; Oluwayelu, D.O.; Adedokun, R.A.; Rufai, N.; McCracken, F.; Mansfield, K.L.; Johnson, N. High seroprevalence of West Nile virus antibodies observed in horses from southwestern Nigeria. *Vector Borne Zoonotic Dis.* **2015**, *15*, 218–220. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Atadiose, E.O.; Kabir, J.; Adamu, S.G.; Umoh, J.U. Serosurvey of West Nile virus in horses and detection of West Nile virus antigen in mosquitoes in Kaduna State, Nigeria. *J. Equine Sci.* **2020**, *31*, 61–66. [\[CrossRef\]](#)
43. Idoko, I.S.; Schvartz, G.; Tirosch-Levy, S.; Erster, O.; Jibril, J.Y.; Adamu, A.M.; Enem, S.I.; Omeje, J.N.; Nafarnda, W.D.; Steinman, A. West Nile virus neutralizing antibody prevalence in donkeys from northern Nigeria. *Trans. R. Soc. Trop.* **2020**. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Baba, S.S.; NNnadi, O.D.; Hamman, K.D.; Saidu, A.; El Yuguda, A.; Oderinde, B.S. Preliminary study on the prevalence of West Nile virus antibody among horses, donkeys and camels in Borno State, Nigeria. *J. Appl. Virol.* **2014**, *3*, 39–45. [\[CrossRef\]](#)
45. Maquart, M.; Boyer, S.; Rakotoharinome, V.M.; Ravaomanana, J.; Tantely, M.L.; Heraud, J.-M.; Cardinale, E. High Prevalence of West Nile Virus in Domestic Birds and Detection in 2 New Mosquito Species in Madagascar. *PLoS ONE* **2016**, *11*, e0147589. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Tantely, L.M.; Cêtre-Sossah, C.; Rakotondranaivo, T.; Cardinale, E.; Boyer, S. Population dynamics of mosquito species in a West Nile virus endemic area in Madagascar. *Parasite* **2017**, *24*, 3. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Tantely, M.L.; Goodman, S.M.; Rakotondranaivo, T.; Boyer, S. Review of West Nile virus circulation and outbreak risk in Madagascar: Entomological and ornithological perspectives. *Parasite* **2016**, *23*, 49. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Hammami, S.; Hassine, T.B.; Conte, A.; Amdouni, J.; De Massis, F.; Sghaier, S.; Hassen, S.B. West Nile disease in Tunisia: An overview of 60 years. *Vet. Ital.* **2017**, *53*, 225–234. [\[CrossRef\]](#)
49. Beck, C.; Jimenez-Clavero, M.A.; Leblond, A.; Durand, B.; Nowotny, N.; Leparac-Goffart, I.; Zientara, S.; Jourdain, E.; Lecollinet, S. Flaviviruses in Europe: Complex circulation patterns and their consequences for the diagnosis and control of West Nile disease. *Int. J. Environ. Res. Public Health* **2013**, *10*, 6049–6083. [\[CrossRef\]](#)
50. Feki, I.; Marrakchi, C.; Ben Hmida, M.; Belahsen, F.; Ben Jemaa, M.; Maaloul, I.; Kanoun, F.; Ben Hamed, S.; Mhiri, C. Epidemic West Nile virus encephalitis in Tunisia. *Neuroepidemiology* **2005**, *24*, 1–7. [\[CrossRef\]](#)
51. Khamassi Khbou, M.; Romdhane, R.; Foughali, A.A.; Sassi, L.; Suin, V.; Rekik, M.; Benzarti, M.h. Presence of antibodies against tick-borne encephalitis virus in sheep in Tunisia, North Africa. *BMC Vet. Res.* **2020**, *16*, 441. [\[CrossRef\]](#) [\[PubMed\]](#)
52. Rios, J.J.; Fleming, J.G.W.; Bryant, U.K.; Carter, C.N.; Huber, J.C., Jr.; Long, M.T.; Spencer, T.E.; Adelson, D.L. OAS1 Polymorphisms Are Associated with Susceptibility to West Nile Encephalitis in Horses. *PLoS ONE* **2010**, *5*, e10537. [\[CrossRef\]](#) [\[PubMed\]](#)

53. El Garch, H.; Minke, J.M.; Rehder, J.; Richard, S.; Edlund Toulemonde, C.; Dinic, S.; Andreoni, C.; Audonnet, J.C.; Nordgren, R.; Juillard, V. A West Nile virus (WNV) recombinant canarypox virus vaccine elicits WNV-specific neutralizing antibodies and cell-mediated immune responses in the horse. *Vet. Immunol. Immunopathol.* **2008**, *123*, 230–239. [[CrossRef](#)] [[PubMed](#)]
54. Granwehr, B.P.; Lillibridge, K.M.; Higgs, S.; Mason, P.W.; Aronson, J.M.; Campbell, G.A.; Barrett, A.D.T. West Nile virus: Where are we now? *Lancet Infect. Dis.* **2004**, *4*, 547–556. [[CrossRef](#)]
55. Murgue, B.; Murri, S.; Zientara, S.; Durand, B.; Durand, J.-P.; Zeller, H. West Nile Outbreak in Horses in Southern France, 2000: The Return after 35 Years. *Emerg. Infect. Dis.* **2001**, *7*, 692. [[CrossRef](#)] [[PubMed](#)]
56. Maquart, M.; Dahmani, M.; Marié, J.L.; Gravier, P.; Leparç-Goffart, I.; Davoust, B. First Serological Evidence of West Nile Virus in Horses and Dogs from Corsica Island, France. *Vector Borne Zoonotic Dis.* **2017**, *17*, 275–277. [[CrossRef](#)]
57. Lan, D.; Ji, W.; Yu, D.; Chu, J.; Wang, C.; Yang, Z.; Hua, X. Serological evidence of West Nile virus in dogs and cats in China. *Arch. Virol.* **2011**, *156*, 893–895. [[CrossRef](#)]
58. Montagnaro, S.; Piantedosi, D.; Ciarcia, R.; Loponte, R.; Veneziano, V.; Fusco, G.; Amoroso, M.G.; Ferrara, G.; Damiano, S.; Iovane, G.; et al. Serological Evidence of Mosquito-Borne Flaviviruses Circulation in Hunting Dogs in Campania Region, Italy. *Vector Borne Zoonotic Dis.* **2019**, *19*, 142–147. [[CrossRef](#)] [[PubMed](#)]
59. Chevalier, V.; Reynaud, P.; Lefrançois, T.; Durand, B.; Baillon, F.; Balança, G.; Gaidet, N.; Mondet, B.; Lancelot, R. Predicting West Nile virus seroprevalence in wild birds in Senegal. *Vector Borne Zoonotic Dis.* **2009**, *9*, 589–596. [[CrossRef](#)]
60. Depoortere, E.; Kavle, J.; Keus, K.; Zeller, H.; Murri, S.; Legros, D. Outbreak of West Nile virus causing severe neurological involvement in children, Nuba Mountains, Sudan, 2002. *Trop. Med. Int. Health* **2004**, *9*, 730–736. [[CrossRef](#)] [[PubMed](#)]
61. McCarthy, M.C.; Haberberger, R.L.; Salib, A.W.; Soliman, B.A.; El-Tigani, A.; Khalid, I.O.; Watts, D.M. Evaluation of arthropod-borne viruses and other infectious disease pathogens as the causes of febrile illnesses in the Khartoum Province of Sudan. *J. Med. Virol.* **1996**, *48*, 141–146. [[CrossRef](#)]
62. Watts, D.M.; el-Tigani, A.; Botros, B.A.; Salib, A.W.; Olson, J.G.; McCarthy, M.; Ksiazek, T.G. Arthropod-borne viral infections associated with a fever outbreak in the northern province of Sudan. *J. Trop. Med. Hyg.* **1994**, *97*, 228–230. [[PubMed](#)]
63. Tomori, O.; Fagbami, A.; Fabiyi, A. Isolations of West Nile virus from man in Nigeria. *Trans. R. Soc. Trop.* **1978**, *72*, 103–104. [[CrossRef](#)]
64. Abdullahi, I.N.; Emeribe, A.U.; Ghamba, P.E.; Omosigho, P.O.; Bello, Z.M.; Oderinde, B.S.; Fasogbon, S.A.; Olayemi, L.; Daneji, I.M.; Musa, M.H.; et al. Distribution pattern and prevalence of West Nile virus infection in Nigeria from 1950 to 2020: A systematic review. *Epidemiol. Health* **2020**, *42*, e2020071. [[CrossRef](#)] [[PubMed](#)]
65. Oderinde, B.S.; Mora-Cárdenas, E.; Carletti, T.; Baba, M.M.; Marcello, A. Prevalence of locally undetected acute infections of Flaviviruses in North-Eastern Nigeria. *Virus Res.* **2020**, *286*, 198060. [[CrossRef](#)]
66. Guenno, B.L.; Bougermouh, A.; Azzam, T.; Bouakaz, R. West Nile: A deadly virus? *Lancet* **1996**, *348*, 1315. [[CrossRef](#)]
67. Kokernot, R.H.; Smithburn, K.C.; Weinbren, M.P. Neutralizing Antibodies to Arthropod-Borne Viruses in Human Beings and Animals in the Union of South Africa. *J. Immunol.* **1956**, *77*, 313.
68. Macnamara, F.N.; Horn, D.W.; Porterfield, J.S. Yellow fever and other arthropod-borne viruses: A consideration of two serological surveys made in South Western Nigeria. *Trans. R. Soc. Trop.* **1959**, *53*, 202–212. [[CrossRef](#)]
69. Nur, Y.A.; Groen, J.; Heuvelmans, H.; Tuynman, W.; Copra, C.; Osterhaus, A.D. An outbreak of West Nile fever among migrants in Kisangani, Democratic Republic of Congo. *Am. J. Trop. Med. Hyg.* **1999**, *61*, 885–888. [[CrossRef](#)]
70. Smithburn, K.C.; Jacobs, R.H. Neutralization-Tests against Neurotropic Viruses with Sera Collected in Central Africa. *J. Immunol.* **1942**, *44*, 9–23.
71. Fall, A.G.; Lo, M.M.; Diouf, N.D.; Ciss, M.; Bitèye, B.; Bakhoun, M.T.; Seck, M.T. West Nile Fever: A Challenge in Sahelian Africa. In *Transboundary Animal Diseases in Sahelian Africa and Connected Regions*, 1st ed.; Kardjadj, M., Diallo, A., Lancelot, R., Eds.; Springer International Publishing: Cham, Switzerland, 2019; pp. 149–177.
72. Metz, M.B.C.; Olufemi, O.T.; Daly, J.M.; Barba, M. Systematic review and meta-analysis of seroprevalence studies of West Nile virus in equids in Europe between 2001 and 2018. *Transbound. Emerg. Dis.* **2020**. [[CrossRef](#)]

CHAPTER 5

Serosurveillance of West Nile virus and other flaviviruses among horses in northwest Nigeria

Note: This chapter is ready to be submitted as a research article to a special issue (Emerging equine infectious diseases: control and biosecurity). ISSN: 2076-2615)

The journal grants authors rights to reuse their articles as part of a thesis.

Author contributions: Olufemi OT (46.4%)*, J. Richard Newton (17.8%), Daly JM (35.7%).

*OTO: Field sampling, sample processing, data curation, formal analysis, methodology, Writing- original draft and Writing - reviewing and editing.

Serosurveillance of West Nile virus and other flaviviruses among horses in northwest Nigeria

Olaolu Tosin Olufemi ^{1,2}, J. Richard Newton ³ and Janet M. Daly ^{1*}

¹ One Virology – Wolfson Centre for Global Virus Research, School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, LE12 5RD, United Kingdom; Olaolu.Olufemi@nottingham.ac.uk

² Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, University of Jos, Nigeria

³ Department of Veterinary Medicine, University of Cambridge, CB3 0ES, United Kingdom; rn428@cam.ac.uk

* Correspondence: janet.Daly@nottingham.ac.uk

Simple Summary: West Nile virus (WNV) is globally recognized as causing a notifiable zoonotic disease. There is little knowledge about the spread of WNV in northwest Nigeria. Seromonitoring of West Nile virus (WNV) and related viruses in equine sentinels are efficient tools for early warning and early detection of emerging viruses in humans. Enzyme Linked Immunosorbent Assay (ELISA) are sensitive and often used to detect the presence of these viruses while confirmatory tests requiring high containment laboratory are limited in developing nations. The work presents a surveillance for antibodies against West Nile virus and related viruses and the risk factors associated with infections among horses in northwest Nigeria. The study demonstrates the abundance of flaviviruses (94.3%) and emphasizes age and location as risk factors associated with seropositivity to WNV and related viruses. The high seroprevalence suggests a cocirculation of flaviviruses, the burden of flaviviruses and a zoonotic potential to humans in northwest Nigeria. Veterinarians and all stakeholders could further include WNV and other flaviviruses in their differential diagnoses. Overall, detecting antibodies to WNV and related flavivirus bids investigation for specific flavivirus research and definitive screening assay for WNV and specific related viruses to forestall a public health emergency in humans and animals.

Abstract: West Nile virus (WNV) disease is an emerging mosquito-borne viral zoonosis infecting birds and other mammals globally. Horses are sentinels for human WNV. In this cross-sectional study, 830 healthy equids were sampled by convenience from four states in northwest Nigeria to determine prevalence of WNV and related flaviviruses. Overall seroprevalence was 94.3% (95%CI: 92.5–95.7) using ID Screen® West Nile competition multi-species ELISA. Age and state remained significant in the final multivariable analysis. In comparison to young horses (<5 years), both adult horses (5–14 years) and older horses (>14 years) had higher odds of seropositivity (OR 5.24; 95% CI: 2.26–12.13; $p < 0.0001$ and OR 3.13; 95% CI: 1.03–9.57; $p < 0.0001$, respectively). Compared to horses located in Kaduna, ORs were significantly increased among horses located in Kano (OR 3.31; 95% CI: 1.69–6.45; $p = 0.0001$), Katsina (OR 17.3; 95% CI: 2.33–128.1; $p = 0.005$) and Sokoto (OR 19.9; 95% CI: 2.24–144.9, $p = 0.004$), respectively. Kaduna uniquely differs with a tropical wet-dry climate in contrast to Sokoto, Katsina and Kano. WNV and related flaviviruses are abundant in northwest Nigeria, suggestive of a probable public health threat. Age and location are risk factors for flavivirus infection. There is need for a collaborative one-health surveillance system with definitive diagnostic assays to prevent and control WNV.

Keywords: 1; Equine 2; West Nile virus 3; Flavivirus 4; Enzyme-Linked immunosorbent Assay (ELISA) 5; Seroprevalence

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Last-name

Received: date

Revised: date

Accepted: date

Published: date



Copyright: © 2023 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Northwest Nigeria has a large population of equids out the 1,445,093 in Nigeria, but there is little published on viruses, such as West Nile virus, affecting horses in the region. West Nile virus (WNV) is a reportable zoonotic arthropod-borne virus that belongs to the family Flaviviridae (genus Flavivirus); member of the Japanese encephalitis serocomplex [1]. Since 1937, when it was first reported in the West province of Uganda, WNV has become a public health concern with various sporadic cases described in different climes and the disease spread globally [2–6]. Primarily, *Culex* species of mosquitoes serve as vectors for WNV while wild birds are the amplifying hosts [7]. Humans and horses, which occasionally get infected due to spill over from the enzootic cycle of the disease, are ‘dead-end’ hosts; not developing sufficient viraemia for viral transmission [8]. Although the majority of WNV infections are asymptomatic (in horses and humans), some individuals may exhibit clinical signs ranging from flu-like symptoms to nervous manifestations and death [9–12]. Horses being more susceptible to WNV, with a likelihood of developing neurological signs in about 10% of infected horses, are ideal sentinels for predicting outbreaks of the disease in human populations [13, 14]. There are eight genetic lineages (1–8) of WNV; lineages 1 and 2, both circulating in Africa, are pathogenic with public health significance globally [15–17]. The exact epidemiological status of this notifiable disease in most African countries however remains unknown [18, 19].

Despite the lack of a treatment protocol for WNV disease in horses, several vaccines are licensed in Europe [20] and the United States [21]. These are not approved nor available for vaccination of horses in Nigeria, which means that vaccine-induced antibodies will not interfere with the identification of antibodies resulting from infection. There are currently no WNV vaccines available for human use. Surveillance of WNV among horses would be a preparedness tool that provides an early warning and response system for the prevention and control of WNV. A scoping review of seroprevalence studies among African equids revealed an overall 35% seroprevalence for WNV [22] where most studies used the ID Screen® West Nile Competition Multispecies (cELISA). There were two reports on WNV antibodies in Nigerian equids; an earlier report recorded 71% seroprevalence using the complement fixation test [23] while the more recent report recorded 90.3% using the cELISA [24]. More recently published studies also employed cELISA either solely or as a preliminary screening assay [25, 26].

The commercial cELISA is based on the prM-E proteins of WNV, which has a high level of antigenic similarity with other members of the Japanese encephalitis (JEV) serogroup complex. In fact, the manufacturer lists detection of antibodies against a wide range of flaviviruses, including tick-borne encephalitis (TBEV), Usutu (USUV), Zika (ZIKV) and dengue (DENV), as an advantage of the ELISA. Therefore, tests for neutralizing antibodies such as virus neutralization (VNT), microneutralization (MNT) or plaque reduction neutralization (PVNT) are often used to confirm the specificity of the antibodies for WNV [22]. However, although neutralization tests are more specific than ELISA, the need for higher level containment and cell culture is expensive and not practicable for routine serosurveillance. Furthermore, in some studies, antibodies against other flaviviruses including TBEV, USUV, JEV, Potiskum virus (POTV), Wesselsbron virus (WESSV) and yellow fever virus (YFV) were screened for and detected in horses in Africa [22]. Screening for all these additional viruses would be prohibitively expensive. Nonetheless, as all the co-circulating viruses identified can potentially cause human disease, there is value in conducting initial serosurveillance studies using the WNV cELISA. In this study, we aimed to determine the seroprevalence of WNV and other flavivirus and determine the factors associated with seropositivity among horses in northwest Nigeria.

2. Materials and Methods

Serum samples were obtained from 830 apparently healthy horses in four of the seven states of northwest Nigeria between October and December 2020 as previously described [22]. In addition, data were collected on sex (male; female), breed (“exotic” = Argentine/Sudan/South African; “mixed” = Talon; “local” = West African Dongola/Arewa), age (“young” = <5 years; “adult” = 5–14 years; “old” = ≥15 years), coat color (“basic” = bay/chestnut/black/brown; “bright” = white/golden/pinto/roan; grey) and location (i.e. state, Kaduna; Kano; Katsina; Sokoto) and use (polo; durbar festival; mixed = more than one use including racing).

All samples were screened with the commercially available competition ELISA (cELISA), ID Screen® West Nile Competition Multispecies (Innovative Diagnostics, Montpellier, France), according to the manufacturer’s instructions. Plates were read at 450 nm and samples with a sample-to-positive (S/N) ratio greater than 50% were considered negative while those less than or equal to 40% were positive; values between 40 and 50% were deemed doubtful.

All analyses were performed in StataSE (17.1, StataCorp LLC, College Station, TX, USA). Using the Pearson chi-square, the associations between explanatory variables and antibodies to WNV were tested. Binomial models were developed to predict the risk factors to WNV seropositivity with odds ratio and equivalent 95% confidence intervals (CI) calculated for each variable. A multinomial model was developed for all variables with $p \leq 0.2$ by conducting an ordinary logistic regression through a forward stepwise approach. The model fit was enhanced by adding more variables evaluated using likelihood ratio test with significance for variable retention set at $p = \leq 0.05$. The performance of the final model was assessed using Hosmer–Lemeshow goodness-of-fit test.

This study received approval (reference 2635181112) from the School of Veterinary Medicine and Science Ethics Clinical Review Panel, University of Nottingham, United Kingdom.

3. Results

A total of 783 horses (94.3%, 95% confidence interval (CI): 93–96) tested positive for WNV antibodies by cELISA. Although the age range of WNV positive equids was 4 months to 30 years, the highest percentage of WNV antibody was seen in adult horses; 95.7%. There were significant differences in WNV seropositivity in horses across age, sex, breed, location (states) and purpose categories. Though not statistically significant, horses with grey coat color had the most 97.3% antibodies in comparison to those with basic (94.1%) and bright (92.5%) coat-colored horses. Males had higher 96.1% (95% CI, 94–97) antibodies to WNV (and related viruses) than females 91.2% (95% CI, 87–94). Local and exotic breeds had a similar seroprevalence compared to exotic breeds which was slightly lower 91% (95% CI, 87–94). Also, horses sampled in Sokoto and Katsina states had the same seroprevalence (99%) which was significantly higher than those from Kaduna and Kano. Horses used for mixed purposes had the highest seroprevalence 98.6% (95% CI, 95–100) in contrast to those used for sporting and traditional leisure (Table 1).

Table 1. Factors associated with West Nile virus seropositivity determined by WNV cELISA among horses in northwest Nigeria.

FACTOR	WNV cELISA			P value
	N	Positive	% Prevalence (95% CI)	
AGE				
Young (<5 years)	113	103	91.2 (84–95)	0.002
Adult (5–14 years)	662	633	95.7 (94–97)	
Old (≥ 15 years)	55	47	87 (73–93)	
SEX				
Female	295	269	91.2 (87–94)	0.004
Male	535	514	96.1 (94–97)	
BREED				
Exotic	268	244	91 (87–94)	0.018
Local	321	308	96 (93–98)	
Mixed	241	231	95.9 (92–98)	
LOCATION				
Kaduna	256	224	87.5 (83–91)	0.0001
Kano	314	301	95.8 (93–97)	
Katsina	122	121	99.2 (94–100)	
Sokoto	138	137	99.3 (95–100)	
USE				
Polo	331	299	90.3 (87–93)	0.0001
Traditional Durbar	350	337	96.3 (93–97)	
Mixed	149	147	98.6 (95–100)	
COAT COLOR				
Basic	640	602	94.1 (92–96)	0.299
Bright	80	74	92.5 (84–97)	
Grey	110	107	97.3 (92–100)	
TOTAL	830	783	94.3 (93–96)	

Values of $p \leq 0.05$ is significant.

A univariate logistic regression (Table 2) showed significant association between a few variables and WNV seropositivity. Adult horses had an OR of 2.12 (95% CI, 1–4.47) while male horses had 2.36 (95% CI, 1.3–4.28). The odds of WNV seropositivity were highest in Sokoto state 19.6 (95% CI, 2.64–144.9) and declined from Katsina 17.3 (95% CI, 2.33–128.1) to Kano states 3.31 (1.69–6.45). Exotic breeds had a lower OR 0.43 (95% CI 0.21–0.86) compared to the local breed of horses. Old (≥ 15 years) horses as well as the mixed breed and those used for traditional durbar were retained as confounders in the univariate model.

Table 2. Univariate logistic regression analyses of West Nile virus seropositivity determined by cELISA among horses in northwest Nigeria.

FACTOR	ODDS RATIO (95% CI)	P value
AGE		
Young (< 5 years)	1.00	-
Adult (5–14 years)	2.12 (1–4.47)	0.049
Old (≥ 15 years)	0.57 (0.21–1.54)	0.267
SEX		
Female	1.00	-
Male	2.36 (1.3–4.28)	0.004
BREED		
Local	1.00	-
Exotic	0.43 (0.21–0.86)	0.017
Mixed	0.97 (0.42–2.26)	0.95
LOCATION		
Kaduna	1.00	-
Kano	3.31 (1.69–6.45)	<0.0001
Katsina	17.3 (2.33–128.1)	0.005
Sokoto	19.6 (2.64–144.9)	0.004
PURPOSE		
Mixed	1.00	-
Polo	0.13 (0.03–0.54)	0.005
Traditional Durbar	0.35 (0.07–1.58)	0.17
COAT COLOR		
Basic	1.00	-
Bright	0.77 (0.32–1.90)	0.58
Grey	2.25 (0.68–7.42)	0.18

* Values of $p \leq 0.05$ is significant.

In the final multiple regression model, age and location of horses remained significantly associated with presence of antibodies to WNV and related viruses in northwestern Nigeria. Although the odds were higher for adult horses 5.24 (2.26–12.13) to be seropositive to WNV than older horses 3.13 (1.03–9.57); it decreased from Sokoto 19.9 (2.64–144.9) to Katsina 17.3 (2.33–128.1) and Kano 3.31 (1.69–6.45) states. The Homer–Lemeshow test indicated the adequate fit of the model ($p = 0.909$) (Table 3).

Table 3. Multivariate logistic regression analysis of West Nile virus seropositivity determined by cELISA among horses in northwest Nigeria.

FACTOR	ODDS RATIO (95% CI)	P value
AGE		
Young (< 5 years)	1	-
Adult (5–14 years)	5.24 (2.26–12.13)	<0.0001
Old (≥ 15 years)	3.13 (1.03–9.57)	<0.0001
LOCATION		
Kaduna	1	-
Kano	3.31 (1.69–6.45)	0.0001
Katsina	17.3 (2.33–128.1)	0.005
Sokoto	19.9 (2.64–144.9)	0.004

Values of $p \leq 0.05$ is significant.

4. Discussion

In this study, the overall prevalence of flavivirus antibodies among horses in northwest Nigeria determined by the cELISA was high (94.3%). However, this high seroprevalence is comparable to a previous report that revealed 90.3% seropositivity in horses in southwest Nigeria using the cELISA [24]. Furthermore, a recent study reported a seroprevalence of 89.9% using the cELISA among horses in Kaduna State [25]; in this study, a very similar seropositivity of 87.5% was observed among horses in the same State.

Location was one of the two variables that remained significant in the final multivariate logistic regression model. Data collected in this study revealed the highest proportion of seropositive horses in Sokoto and Katsina states with OR of 19.9 (95% CI: 2.64–144.9) and 17.3 (95% CI: 2.33–128.1), respectively, with lower seroprevalence amongst horses in Kano state and lowest in Kaduna, the referent category. Sokoto is the most westerly state from which samples were obtained and Kaduna the most southerly. The drivers of WNV distribution have been associated with climate. Although the climate in Nigeria is popularly described in relation to Köppen model (Figure 2), with northern Nigeria being dry and dusty combined with marginal rainfall expressed in amount, frequency, intensity and period [27–29], more recent climatic classification has augmented the Köppen model in Nigeria by focusing on temperature and humidity [30]. Kaduna falls entirely within the ‘temperate-dry’ climate while the other northwest states fall mainly within the ‘hot-dry’ climate. These warm semiarid areas are bordered by a warm desert climate in Niger. The very brief rainfall in northern Nigeria accompanied with warm climate and savannah vegetation may not present an ideal environment for the mosquito vectors. However, the main vector of WNV, *Culex pipiens*, has been described in northwest Nigeria [31]. Furthermore, horses are probably tethered longer in the open, under tall trees in these hot-dry climatic areas in a bid to reduce solar radiation and provide cool air. This creates an environment habitable to birds, thus potentially establishing an enzootic transmission cycle and expose equids to mosquito bites.

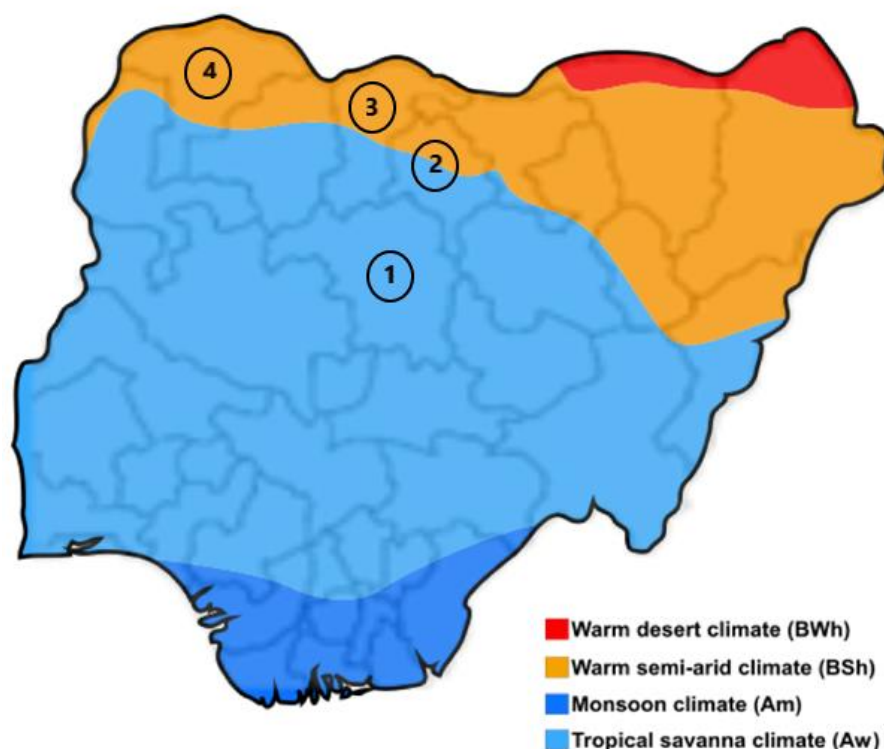


Figure 1. Köppen's classification of Nigerian climate overlapping states (Adapted from Mobolade and Pourvahidi, 2020).

* 1-4 indicates Kaduna, Kano, Katsina and Sokoto respectively.

The slightly lower seroprevalence in exotic breeds, which are also the breeds mostly used for polo, which also had the lowest seroprevalence compared to horses used for other purposes, might imply lower risk of being bitten by mosquitoes as they would mostly be stabled with intensive management. Similarly, Sule *et al.* [24] found that local-breed horses (Dongola in their study) had a significantly higher OR of 2.51 (95% CI: 0.78–8.16) of being seropositive than the 'exotic' Argentine Criollo horses. In a study of horses in Egypt, Selim *et al.* [32] found that age, sex and breed remained significant risk factors for seropositivity to WNV (cELISA) in the final logistic regression model with mixed breed horses having an OR of 1.9 (95% CI: 1.2–2.8) compared to the referent Arabian breed.

The high seroprevalence across all age category of horses is indicative of WNV and related viruses circulating actively in northwest Nigeria. Some of the "young" horses might have maternally derived antibodies as well as being exposed to bites from infected mosquitoes. The seroprevalence observed in the adult horses is consistent with the increased risk of exposure over time. The lower antibodies observed in the "old" horses could be due to reduced activity translating to reduced exposure to mosquito vectors and waning immunity to the virus. In the Egyptian study, the probability of being seropositive increased with age with horses 15 years and older having an OR of 4.3 (95% CI: 3.0–6.2) compared to horses <2.5 years old [32].

Although not retained in the final model, the OR was higher for male than for female horses. Sule *et al.* [24] did not find any difference in OR between male and female horses screened by cELISA in Kaduna state in their study; this may be due to their population

being more homogenous as all were kept for polo. In contrast, in a study by Baba et al. (2014), all 11 of the 96 horses that were positive for WNV antibodies in a microneutralisation test were male. These horses were from small stables owned by individuals in Borno state (northeast), Nigeria. Selim et al. [32] also found that the OR for being seropositive in the cELISA was higher in stallions (2.4) than in geldings (1.3) and mares (Ref).

No confirmatory tests to detect neutralizing antibodies were performed in this study. Other studies have demonstrated the cocirculation of JEV, POTV, TBEV, USUV, WESSV, YFV among horses in Nigeria and Africa, but none have screened for all of these [22]. Where alternative assays to the cELISA were performed, the seroprevalence was generally found to be lower. A study demonstrated 71% complement fixing antibodies to WNV among horses in Lagos state (southwest Nigeria) [23], although this study was conducted over 30 years ago. A study of donkeys in Kaduna and Katsina states with animals sampled majorly from the Mai'adua International Livestock market revealed 27.8% anti-WNV neutralizing antibodies [26], although it could be argued that donkeys may be less susceptible to WNV infection. Nonetheless, the finding by Sule et al. [24] that although the overall seroprevalence determined by the cELISA was 90.3%, only 35.7% of the sera were positive for neutralizing antibodies against WNV measured by PRNT provides more convincing evidence that a substantial portion of the sera found to be positive in the cELISA may have antibodies to other flaviviruses. A study from Burkina Faso reported a 90.1% prevalence (73 of 81 samples tested using the cELISA) in horses, but when MNTs were performed on the positive samples, 12 were positive for WNV, 3 for USUV and 2 for both WNV and USUV. In the same study, 77.64% of human serum samples were positive by cELISA but only 19.16% were positive for WNV, 14.17% for USUV and 10.38% for both WNV and USUV in MNTs [33].

5. Conclusions

Our finding supports the widespread distribution of flaviviruses among equids in northwest Nigeria with a seroprevalence of 94.3% which was clearly abundant across all States inferring serious public health implications. Age and location of horses in northwest Nigeria were significantly associated with seropositivity to WNV and other flaviviruses.

It is well established that the WNV cELISA used in this study is highly cross-reactive with antibodies against related flaviviruses and it cannot, therefore, be assumed that WNV alone is responsible for the high seroprevalence found in this study. However, the closely related viruses that co-circulate also have zoonotic potential. Therefore, it is recommended that affordable diagnostic tests that are specific for a wider range of viruses in the flavivirus genus are developed in order to better understand their epidemiology and potential disease burden in both equine and human populations.

Author Contributions: Conceptualization, J.M.D. and O.T.O.; methodology, J.M.D., O.T.O. and J.R.N.; formal analysis, J.M.D., O.T.O. and J.R.N.; investigation, J.M.D., O.T.O. and J.R.N.; resources, J.M.D., O.T.O. and J.R.N.; data curation, O.T.O.; writing—original draft preparation, O.T.O.; writing—review and editing, J.M.D., O.T.O. and J.R.N.; visualization, J.M.D., O.T.O. and J.R.N.; supervision, J.M.D.; project administration, J.M.D. and O.T.O.; funding acquisition, J.M.D. and O.T.O. All authors have read and agreed to the published version of the manuscript.

Funding: O.T.O is an awardee of the University of Nottingham Vice-Chancellor's Scholarship for Research Excellence (International).

Institutional Review Board Statement: This study received approval (reference 2635181112) from the School of Veterinary Medicine and Science Ethics Clinical Review Panel, University of Nottingham, United Kingdom.

Data Availability Statement: Not applicable

Acknowledgments: The authors thank the Emirate council of Kano, Sokoto and Zazzau for their support. Also, the staff of the Ministry of Agriculture in Katsina State and the Hon. Commissioner

Ministry of Animal Health and Fisheries Development Sokoto State, Professor Abdulkadir Usman Junaidu.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Maclachlan, N.J.; Dubovi, E.J., Chapter 29: *Flaviviridae*. In: *Fenner's veterinary virology / edited by N. James MacLachlan and Edward J. Dubovi*. Veterinary virology. 2016: 5th ed.; Elsevier Academic Press: Amsterdam, London, 2016.
2. Chowdhury, P.; Khan, S.A., Global emergence of West Nile virus: Threat & preparedness in special perspective to India. *Indian J Med Res*, **2021**. 154(1): p. 36-50.
3. Huang, C.; Slater, B.; Rudd, R.; Parchuri, N.; Hull, R.; Dupuis, M.; Hindenburg, A., First Isolation of West Nile virus from a patient with encephalitis in the United States. *Emerg Infect Dis*, **2002**. 8(12): p. 1367-1371.
4. Murgue, B.; Zeller, H.; Deubel, V., *The Ecology and Epidemiology of West Nile Virus in Africa, Europe and Asia*, in *Japanese Encephalitis and West Nile Viruses*, Mackenzie, J.S.; Barrett, A.D.T.; Deubel, V., Editors. 2002, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 195-221.
5. Roehrig, J.T.; Layton, M.; Smith, P.; Campbell, G.L.; Nasci, R.; Lanciotti, R.S., The emergence of West Nile virus in North America: ecology, epidemiology, and surveillance. *Curr Top Microbiol Immunol*, **2002**. 267: p. 223-240.
6. Smithburn, K.; Hughes, T.; Burke, A.; Paul, J., A neurotropic virus isolated from the blood of a native of Uganda. *American journal of tropical medicine*, **1940**. 20: p. 471-472.
7. Campbell, G.L.; Marfin, A.A.; Lanciotti, R.S.; Gubler, D.J., West Nile virus. *Lancet Infect Dis*, **2002**. 2(9): p. 519-529.
8. Brault, A.C., Changing patterns of West Nile virus transmission: altered vector competence and host susceptibility. *Vet Res*, **2009**. 40(2): p. 43.
9. Bunning, M.L.; Bowen, R.A.; Cropp, C.B.; Sullivan, K.G.; Davis, B.S.; Komar, N.; Godsey, M.S.; Baker, D. et al., Experimental infection of horses with West Nile virus. *Emerg Infect Dis*, **2002**. 8(4): p. 380-386.
10. Cantile, C.; Di Guardo, G.; Eleni, C.; Arispici, M., Clinical and neuropathological features of West Nile virus equine encephalomyelitis in Italy. *Equine Vet J*, **2000**. 32(1): p. 31-35.
11. Monaco, F.; Savini, G.; Calistri, P.; Polci, A.; Pinoni, C.; Bruno, R.; Lelli, R., 2009 West Nile disease epidemic in Italy: First evidence of overwintering in Western Europe? *Research in Veterinary Science*, **2011**. 91(2): p. 321-326.
12. Petersen, L.R.; Roehrig, J.T., West Nile virus: a reemerging global pathogen. *Emerg Infect Dis*, **2001**. 7(4): p. 611-614.
13. Castillo-Olivares, J.; Wood, J., West Nile virus infection of horses. *Vet Res*, **2004**. 35(4): p. 467-483.
14. Venter, M.; Pretorius, M.; Fuller, J.A.; Botha, E.; Rakgotho, M.; Stivaktas, V.; Weyer, C.; Romito, M. et al., West Nile Virus Lineage 2 in Horses and Other Animals with Neurologic Disease, South Africa, 2008-2015. *Emerging infectious diseases*, **2017**. 23(12): p. 2060-2064.
15. Beck, C.; Leparac Goffart, I.; Franke, F.; Gonzalez, G.; Dumarest, M.; Lowenski, S.; Blanchard, Y.; Lucas, P. et al., Contrasted Epidemiological Patterns of West Nile Virus Lineages 1 and 2 Infections in France from 2015 to 2019. *Pathogens*, **2020**. 9(11): p. 908.
16. Fall, G.; Di Paola, N.; Faye, M.; Dia, M.; Freire, C.C.M.; Loucoubar, C.; Zannotto, P.M.A.; Faye, O. et al., Biological and phylogenetic characteristics of West African lineages of West Nile virus. *PLoS Negl Trop Dis*, **2017**. 11(11): p. e0006078.
17. Sule, W.F.; Oluwayelu, D.O.; Hernández-Triana, L.M.; Fooks, A.R.; Venter, M.; Johnson, N., Epidemiology and ecology of West Nile virus in sub-Saharan Africa. *Parasites & Vectors*, **2018**. 11(1): p. 414.
18. de Heus, P.; Kolodziejek, J.; Camp, J.V.; Dimmel, K.; Bagó, Z.; Hubálek, Z.; van den Hoven, R.; Cavalleri, J.V. et al., Emergence of West Nile virus lineage 2 in Europe: Characteristics of the first seven cases of West Nile neuroinvasive disease in horses in Austria. *Transbound Emerg Dis*, **2020**. 67(3): p. 1189-1197.

19. Mencattelli, G.; Ndione, M.H.D.; Rosà, R.; Marini, G.; Diagne, C.T.; Diagne, M.M.; Fall, G.; Faye, O. et al., Epidemiology of West Nile virus in Africa: An underestimated threat. *PLoS Negl Trop Dis*, **2022**. 16(1): p. e0010075.
20. Rebollo, B.; Sarraseca, J.; Lecollinet, S.; Abouchoaib, N.; Alonso, J.; García-Bocanegra, I.; Sanz, A.J.; Venteo, Á. et al., Monitoring Anti-NS1 Antibodies in West Nile Virus-Infected and Vaccinated Horses. *BioMed research international*, **2018**. 2018: p. 8309816-8309816.
21. Castro-Jorge, L.A.; Siconelli, M.J.L.; Ribeiro, B.D.S.; Moraes, F.M.; Moraes, J.B.; Agostinho, M.R.; Klein, T.M.; Floriano, V.G. et al., West Nile virus infections are here! Are we prepared to face another flavivirus epidemic? *Rev Soc Bras Med Trop*, **2019**. 52: p. e20190089.
22. Olufemi, O.T.; Barba, M.; Daly, J.M., A Scoping Review of West Nile Virus Seroprevalence Studies among African Equids. *Pathogens*, **2021**. 10(7).
23. Olaleye, O.D.; Oladosu, L.A.; Omilabu, S.A.; Baba, S.S.; Fagbami, A.H., Complement fixing antibodies against arboviruses in horses at Lagos, Nigeria. *Rev Elev Med Vet Pays Trop*, **1989**. 42(3): p. 321-325.
24. Sule, W.F.; Oluwayelu, D.O.; Adedokun, R.A.; Rufai, N.; McCracken, F.; Mansfield, K.L.; Johnson, N., High seroprevalence of West Nile virus antibodies observed in horses from southwestern Nigeria. *Vector Borne Zoonotic Dis*, **2015**. 15(3): p. 218-220.
25. Atadiose, E.O.; Kabir, J.; Adamu, S.G.; Umoh, J.U., Serosurvey of West Nile virus in horses and detection of West Nile virus antigen in mosquitoes in Kaduna State, Nigeria. *Journal of Equine Science*, **2020**. 31(3): p. 61-66.
26. Idoko, I.S.; Schwartz, G.; Tirosh-Levy, S.; Erster, O.; Jibril, J.Y.; Adamu, A.M.; Enem, S.I.; Omeje, J.N. et al., West Nile virus neutralizing antibody prevalence in donkeys from northern Nigeria. *Transactions of The Royal Society of Tropical Medicine and Hygiene*, **2020**. 115(5): p. 566-568.
27. Akande, A.; Costa, A.C.; Mateu, J.; Henriques, R., Geospatial Analysis of Extreme Weather Events in Nigeria (1985–2015) Using Self-Organizing Maps. *Advances in Meteorology*, **2017**. 2017: p. 8576150.
28. Kottek, M.; Grieser, J.; Beck, C.; Rudolf, B.; Rubel, F., World map of the Köppen-Geiger climate classification updated. **2006**.
29. Peel, M.C.; Finlayson, B.L.; McMahon, T.A., Updated world map of the Köppen-Geiger climate classification. *Hydrology and earth system sciences*, **2007**. 11(5): p. 1633-1644.
30. Mobolade, T.D.; Pourvahidi, P., Bioclimatic Approach for Climate Classification of Nigeria. *Sustainability*, **2020**. 12(10): p. 4192.
31. Mohammed, B.R.; Yayo, A.M.; Ajanusi, O.J.; Lawal, I.A., Relative abundance and molecular identification of *Culex pipiens* complex (Diptera: Culicidae), in Kura Local Government Area, North-western Nigeria. *Parasite epidemiology and control*, **2021**. 14: p. e00213-e00213.
32. Selim, A.; Megahed, A.; Kandeel, S.; Alouffi, A.; Almutairi, M.M., West Nile virus seroprevalence and associated risk factors among horses in Egypt. *Sci Rep*, **2021**. 11(1): p. 20932.
33. Tinto, B.; Kaboré, D.P.A.; Kagoné, T.S.; Constant, O.; Barthelemy, J.; Kiba-Koumaré, A.; Van de Perre, P.; Dabiré, R.K. et al., Screening of Circulation of Usutu and West Nile Viruses: A One Health Approach in Humans, Domestic Animals and Mosquitoes in Burkina Faso, West Africa. *Microorganisms*, **2022**. 10(10).

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

CHAPTER 6

Serosurveillance of West Nile virus and hepatitis A among horses in northwest Nigeria using ELISA based on plant-expressed proteins

Note: This chapter is ready to be submitted as a research article to a *Pathogens* (ISSN: 2076-0817), a peer-reviewed open access journal published by MDPI

Pathogens grants authors rights to reuse their articles as part of a thesis.

Author contributions: Olufemi OT* (42.2%), Radley MA (4.4%), Tarr AW (7.7%), Kydd JH (4.4%), J. Richard Newton (12.2%), Daly JM (28.8%).

*OTO: Field sampling, sample processing, data curation, formal analysis, methodology, Writing- original draft and Writing - reviewing and editing.

Serosurveillance of West Nile virus and Hepacivirus A among horses in northwest Nigeria

Olaolu Tosin Olufemi^{1,2}, Mirelle A. Radley¹, Alexander W. Tarr³, Julia H. Kydd¹, J. Richard Newton⁴ and Janet M. Daly^{1*}

¹ One Virology – Wolfson Centre for Global Virus Research, School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, LE12 5RD, United Kingdom

² Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, University of Jos, Nigeria

³ Wolfson Centre for Global Virus Research, School of Life Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, United Kingdom

⁴ Department of Veterinary Medicine, University of Cambridge, CB3 0ES, United Kingdom

* Correspondence: janet.daly@nottingham.ac.uk

Abstract: West Nile virus (WNV) and Hepacivirus A (EqHV) are members of the family Flaviviridae, both having a global spread. Commercially available enzyme-linked immunosorbent assays (ELISAs) are expensive for surveillance studies in developing nations, and there is no commercial ELISA available for EqHV. Use of plant-expressed proteins in indirect ELISAs to detect antibodies to WNV and EqHV among horses in northwest Nigeria was explored in this study. The WNV non-structural 1 (NS1) and envelope protein mutated to enhance specificity (Equad) and EqHV core (C) proteins were transiently expressed in *Nicotiana benthamiana*. Out of the 691 horses screened, antibodies to EqHV were detected in 12% (83/691, 95% CI; 10–15%). In a final multivariable logistic regression, location and purpose of horses remained significantly associated with the detection of EqHV antibodies. Antibodies against WNV NS1 and Equad were detected in 33.9% (234/691) and 12.6% (89/691) horses, respectively, which are much lower values than detected using a commercial competitive WNV ELISA that detects cross-reactive antibodies. This is the first study, to the best of our knowledge, to report the circulation of EqHV among equids in Nigeria and demonstrates the potential for plant based ELISAs to be used for antibody surveillance in developing countries.

Keywords: West Nile virus; Hepacivirus A; plant-based ELISA; equine; Flaviviruses; Nigeria

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Last-name

Received: date

Revised: date

Accepted: date

Published: date



Copyright: © 2023 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Northwest Nigeria has a large population of horses, many of which are used for traditional Durbar festivals, polo and horseracing and are therefore highly mobile. The vulnerability of this population to emerging and re-emerging viruses was demonstrated by the outbreak of equine influenza in 2018/2019 [1]. We subsequently conducted a cross-sectional study to determine the seroprevalence of equine influenza and its associated risk factors, for which serum samples were obtained from 830 horses in Northwest Nigeria between October 2019 and January 2020. The same serum samples were also screened for antibodies against West Nile virus (WNV) using a commercially available competitive ELISA (cELISA), the ID Screen® West Nile Competition Multispecies.

West Nile virus (WNV) is a member of the Japanese encephalitis virus (JEV) serocomplex of the Flavivirus genus in the Flaviviridae family [2]. It is well established that there is extensive cross-reactivity between different flavivirus species in serological assays (reviewed by [3]), particularly in ELISA detecting immunoglobulin G antibodies against the envelope protein such as the cELISA. Therefore, the high seroprevalence previously

identified among horses in northwest Nigeria (Olufemi et al. submitted) using the cELISA might include antibodies to multiple other flaviviruses, including dengue virus (DENV), Japanese encephalitis virus (JEV), tick borne encephalitis (TBEV), Usutu virus (USUV), West Nile virus (WNV), Zika virus (ZIKV) and more.

Although the plaque reduction neutralization tests (PRNT) are considered the gold standard for detecting flavivirus species-specific antibodies, they can be subject to cross-reactivity [3]. There are several studies suggesting that ELISAs based on detection of antibodies to non-structural 1 (NS1) protein flaviviruses are more species specific than the commercially available ELISA based on the envelope protein [4-9]. For example, an NS1-based ELISA revealed a 93% specificity for DENV and reliably differentiated DENV positive sera from closely related ZIKV [10]. Although structural protein-based ELISAs are limited by cross-reactivity among members of the flavivirus group, mutating the envelope protein at four amino acid positions ('Equad'), which are the main contributors to cross reactivity, shows promise for improving specificity of these assays. The novel approach has been used for DENV, ZIKA, USUV, TBEV and WNV assays. Specifically, a WNV Equad ELISA yielded 100% specificity and about 90% sensitivity [11-14].

Serological assays using plant-expressed antigens potentially provide an effective and cheap surveillance tool for use in developing nations with limited resources. To date, use of plant-expressed proteins for viral vaccines has been extensively explored but there are fewer publications describing their use in serological assays. The potential to use African horse sickness virus proteins (VP2) expressed in plants in an ELISA to detect serotype-specific antibodies is briefly described in a recent paper [15]. Antibodies to recombinant SARS-CoV-2 proteins have also been detected in COVID-19 patients using a plant-based ELISA Makatsa 2021 [16]. We therefore developed indirect ELISAs to detect antibodies to WNV NS1 and Equad proteins expressed in plants.

Equine Hepacivirus (EqHV; recently defined as species *Hepacivirus equi*), formerly non-primate hepacivirus (NPHV)/ canine hepacivirus (CHV) is also a member of the family *Flaviviridae* (genus *Hepacivirus*) that is closely related to *Hepacivirus hominis* (hepatitis C virus; HCV) and naturally infects horses [17, 18]. Transmission of EqHV remains unclear requiring more investigation. However, there is evidence of vertical, horizontal, and parenteral transmission routes [19]. Following the first report of EqHV in 2012 from dogs with respiratory disease in the USA [20], infection in equids have been reported globally with evidence from Africa (South African and Morocco), America (Brazil and United States), Asia (China, Japan, Korea and Mongolia) Oceania (Australia) and Europe (France, Germany, Hungary Italy, and United Kingdom) [19, 21]. While few studies applied *Gaussia* luciferase immunoprecipitation system (GLIPS) [22, 23] and western blotting [24, 25] for EqHV serological investigations, most reports preferred luciferase immunoprecipitation system (LIPS) [17, 26-30] to detect antibodies against EqHV protease/helicase NS3 antigen; which although effective requires laborious cell-based techniques and expensive reagent. The LIPS assay has produced reliable results comparable to a commercial ELISA for detecting antibodies to African swine fever [31]. However, there remains no commercial ELISA for EqHV screening. In order to improve specificity, one study developed a biphasic ELISA with recombinant NS3 and core proteins in which only sera reactive to both ELISAs were confirmed positive [28]. Also, a recent study [32] demonstrated the efficacy of plant-based ELISA in serodiagnosis. We therefore also developed a cheap, straight forward ELISA to detect anti-EqHV antibodies in equine sera using plant-expressed core protein.

2. Materials and Methods

Serum samples were obtained from 830 apparently healthy horses in four of the seven states of Northwest Nigeria between October and December 2019 as previously described [33]. In addition, data were collected on sex, breed, age, coat color (categorised as basic/bright/grey) and location (Kaduna, Kano, Katsina and Sokoto states). A total of 691

of the 830 horses previously screened by cELISA (Olufemi et al., submitted) were randomly selected to be further screened in this study. Serum from a horse vaccinated with the Ixiaro JEV vaccine and sera previously identified as positive or negative with the cELISA were used as control sera.

Expression of viral proteins in *Nicotiana benthamiana*

The core protein (C) of EqHV was expressed from a consensus strain generated from sequences obtained from GenBank. For WNV ELISAs, viral antigens were generated based on the sequence of a recent WNV-lineage 1 isolate circulating in Africa (accession number; LC489409/isolate Croc1102019ZM) [34]. A quadruple mutant E-protein comprising of 1-400 amino acid residues with point mutations (T76A, M77G, W101R, L107R) in the conserved fusion loop domain was synthesized as previously described [14]. A modified Kozak sequence (GCTACT), was added to the start of WNV mutant E-protein (Equad), NS1 protein and EqHV core sequences, a C-terminal hex histidine (6XHis) tag and an endoplasmic retention signal (SEKDEL) were added to the end of each gene with *AgeI* and *XhoI* restriction sites flanking either end of the gene. The sequences were codon optimized for *Nicotiana benthamiana* and synthesized by GeneArt (Life Technologies). Each gene was separately cloned into an *AgeI/XhoI* digested pEAQ-HT plasmid [35] to generate pEAQ-HT-Equad, pEAQ-HT-NS1 and pEAQ-HT-C, and subsequently transformed into *Agrobacterium tumefaciens* (LBA4404) by electroporation. After *Agrobacterium tumefaciens* was grown in LB agar at 28°C for 72 h, the bacteria separately harboring the Equad and NS1 constructs were adjusted to OD600 = 2. Transient expression of the genes was achieved by infiltrating the bacterial suspension into 5- to 6-week-old plants grown in a greenhouse as earlier described [36].

Purification of proteins

Leaves of *N. benthamiana* infiltrated with pEAQ-HT-Equad and pEAQ-HT-NS1 were homogenized in two volumes of ice-cold extraction buffer pH 7.2, using a prechilled handheld waring blender. Leaf extract was centrifuged at 10,000 g for 15 min at 4°C. A sterile 0.45 µm filter (Millipore) was used to filter the supernatant. To purify the proteins, filtered supernatants (for Equad and NS1) were passed through designated Ni NTA agarose columns before a sequential graded imidazole wash with 25 ml of 5 mM, 10 mM, 20 mM, and 30 mM. The purified protein was eluted with 6 x 1 mL at a time of the elution buffer with each ml collected into labelled Eppendorf tubes. Eluted proteins were detected by western blot.

Indirect ELISAs for detection of antibodies using proteins expressed in plants

NUNC Maxisorp plates (Thermo Fisher Scientific, Waltham, MA USA) were coated with 2 µg immobilized metal affinity column (IMAC) purified proteins and incubated overnight at 4 °C. Plates were saturated with blocking/dilution buffer (1 % bovine serum albumin plus 3 % skimmed milk in PBS 0.1 Tween 20) and incubated at 4 °C for 48 h. The blocking buffer was tipped off with excess tapped onto a paper towel. After the blocking step, plates were washed three times with 300 µL/well of phosphate buffered saline with 0.1% Tween 20 (PBST) washing buffer. Equine and control sera were diluted in blocking/dilution buffer (1:100) before adding 50 µL/well to plates and incubating at room temperature for 1 h. After three washes in 300 µL/well of Tris buffered saline with 0.1 % Tween 20 (TBST), plates were incubated for 1 h at room temperature with 50 µL/well of goat anti-horse IgG horse radish peroxidase conjugate (SeraCare Life Sciences Inc, Milford, MA United States) at 1:1000 dilution in blocking/dilution buffer for the WNV ELISAs. At this stage, the method differed. For the EqHV Core ELISA which used anti-horse alkaline phosphatase conjugate and p-nitrophenyl phosphate, disodium salt (PNPP)

(Thermo Fisher Scientific, Waltham, MA USA). Lastly, after three washing steps in 300 μ L/well of TBST, 100 μ L/well of Ultra-fast 3,3', 5,5'-Tetramethylbenzidine substrate (Thermo Fisher Scientific, Waltham, MA USA) was added and incubated in the dark (at room temperature) for 10–15 min. The reaction was stopped with 100 μ L/well of 2M H₂SO₄ and read at 450 nm optical density (OD).

For the EqHV C ELISA, OD values higher than the mean OD obtained with the negative control plus 3x the standard deviation were regarded as positive. For the WNV ELISAs, the results of each plate were independently interpreted by change-point analysis [37]. The change-point was validated by comparison with control sera: serum from a horse vaccinated with JEV, and sera that tested positive and negative with the cELISA.

Statistical Analyses

As previously described [33], with all analyses performed in StataSE (17.1, StataCorp LLC, College Station, TX, USA). Associations between explanatory variables and antibodies to EqHV were tested using the Pearson chi-square. Binomial models were developed to predict the risk factors to EqHV seropositivity with odds ratio and equivalent 95% confidence intervals (CI) calculated for each variable. A multinomial model was developed for all variables with $P \leq .2$ by conducting an ordinary logistic regression through a forward stepwise approach. The model fit was enhanced by adding with the addition further variables evaluated using likelihood ratio test, with significance for variable retention set at $p = \leq 0.05$. The performance of the final model was assessed using Hosmer–Lemeshow goodness-of-fit test.

3. Results

Equine hepatitis virus seropositivity among horses in Northwest Nigeria determined by plant based Indirect ELISA.

A total of 691 horses were screened for antibodies to EqHV: 83 (12%, 95% CI; 10–15) tested positive for EqHV-C antibodies by ELISA. Significant differences in seropositivity were recorded in all variable category except coat color, where bright-colored horses had the most antibodies, 15.4% (95% CI; 8–26) to EqHV as compared to basic (12.5%, 95% CI; 10–16) and grey (6.67%, 95% CI; 3–14) horses. Old horses had higher antibodies (27.7%, 95% CI; 7–42) than adult (11.42%, 95% CI; 9–14) and young horses (6.67%, 95% CI; 3–15). Antibodies to EqHV were significantly higher in female horses (18.1%, 95% CI; 14–23) than their male counterpart (8.71%, 95% CI; 6–12) and exotic horses (18.1%, 95% CI; 14–24) revealed more antibodies to EqHV than mixed (10.2%, 95% CI; 7–15) and local (8.2%, 95% CI; 5–12) breeds. Horses sampled in Kaduna had the most antibodies (22.7% 95% CI; 17–28) to EqHV while those in Kano and Katsina had 7.4% (95% CI; 5–11) and 8.33%, 95% CI; 4–16) respectively while Sokoto had the least (7.1% 95% CI; 3–14). Horses used for polo also had the most antibodies; 18.64% (95% CI; 87–93) while those for traditional durbar and mixed purposes had 9.21% (95% CI; 93–97) and 2.78% (95% CI; 95–100) respectively (Table 1).

Table 1. Factors associated with Hepacivirus seropositivity determined by in-house indirect EqHV-C ELISA among horses in Northwest Nigeria.

Variable	EqHV-C ELISA			
	N	Positive	% Prevalence (95% CI)	p-value
AGE				
Young (<5 years)	75	5	6.67 (3–15)	0.001
Adult (5–14 years)	569	65	11.42 (9–14)	
Old (≥ 15 years)	47	13	27.66 (17–42)	
SEX				
Male	448	39	8.71 (6–12)	<0.0001
Female	243	44	18.1 (14–23)	
BREED				
Exotic	227	41	18.06 (14–24)	0.002
Local	268	22	8.21 (5–12)	
Mixed	196	20	10.2 (7–15)	
LOCATION				
Kaduna	213	47	22.07 (17–28)	<0.0001
Kano	283	21	7.42 (5–11)	
Katsina	96	8	8.33 (4–16)	
Sokoto	99	7	7.07 (3 –14)	
PURPOSE				
Polo	279	52	18.64 (87–93)	<0.0001*
Traditional Durbar	304	28	9.21 (93–97)	
Mixed	108	3	2.78 (95–100)	
COAT COLOUR				
Basic	536	67	12.5 (10–16)	0.197
Bright	65	10	15.38 (8–26)	
Grey	90	6	6.67 (3–14)	
TOTAL	691	83	12.01 (10–15)	

In the univariate logistic regression comparing variables and EqHV seropositivity, old (≥ 15 years) horses significantly had a higher OR of 5.35 (95% CI, 1.76–16.23) while male horses had lower odds of 0.43 (95% CI; 0.27–0.68) EqHV seropositivity. The odds ratio was significantly higher for exotic breeds (OR 2.46, 95% CI; 1.42–4.28) and not-significantly higher in mixed breeds (OR 1.27, 95% CI; 0.67–2.39), both compared local breeds of horses. The odds were significantly lower in all locations compared to Kaduna: Katsina, 0.32 (95% CI, 0.14–0.71); Kano, 0.28 (95% CI, 0.16–0.49) and Sokoto, 0.26 (95% CI, 0.12–0.62). In comparison to horses used for mixed purposes, those used for polo (OR 8.02, 95% CI; 2.45–26.3) and traditional durbar (OR 3.55, 95% CI, 1.06–11.9) were significantly at a higher odds of being seropositive for EqHV. Adult horses (5–14 years) as well as bright and grey colored horses were maintained as confounders in the univariate analyses (Table 2).

Table 2. Univariate logistic regression analyses of Hepacivirus seropositivity determined by in-house indirect EqHV-C ELISA among horses in Northwest Nigeria.

Variable	ODDS RATIO (95% CI)	p-value
AGE		
Young (< 5 years)	1.00	-
Adult (5–14 years)	1.81 (0.70–4.63)	0.22
Old (≥ 15 years)	5.35 (1.76–16.23)	0.003
SEX		
Female	1.00	-
Male	0.43 (0.27–0.68)	<0.0001
BREED		
Local	1.00	-
Exotic	2.46 (1.42–4.28)	0.001
Mixed	1.27 (0.67–2.39)	0.46
LOCATION		
Kaduna	1.00	-
Kano	0.28 (0.16–0.49)	<0.0001
Katsina	0.32 (0.14–0.709)	0.005
Sokoto	0.26 (0.12–0.618)	0.002
PURPOSE		
Mixed	1.00	-
Polo	8.02 (2.45–26.3)	0.001
Traditional Durbar	3.55 (1.06–11.9)	0.04
COAT COLOUR		
Basic	1.00	-
Bright	1.27 (0.62 – 2.62)	0.512
Grey	2.25 (0.68–7.42)	0.117

The purpose and location of horses remained significantly associated with EqHV seropositivity in the final multivariate regression model. Both polo (OR 6.18, 95% CI, 1.41–27.2) and traditional durbar 5.18 (95% CI, 1.32–20.3) horses were significantly at higher odds of EqHV seropositivity. Horses in Kano 0.35 (95% CI, 0.16–0.75) and Katsina 0.41 (95% CI, 0.18–0.96) were however at a lower odd significantly. Horses in Sokoto were retained as confounders. The Homer–Lemeshow test indicated an adequate fit model ($p = 0.888$) (Table 3).

Table 3. Multivariate logistic regression analysis of Hepacivirus seropositivity determined by in-house indirect EqHV-C ELISA among horses in Northwest Nigeria.

Variable	ODDS RATIO (95% CI)	p-value
PURPOSE		
Mixed	1.00	-
Polo	6.18 (1.41–27.2)	0.016
Traditional Durbar	5.18 (1.32–20.3)	0.019
LOCATION		
Kaduna	1.00	-
Kano	0.35 (0.16–0.75)	0.007
Katsina	0.41 (0.18–0.96)	0.039
Sokoto	0.75 (0.24–2.34)	0.62

Comparison of results obtained with WNV iELISAs using plant-expressed proteins and cELISA

There were 662 samples in the subset of 691 samples that were positive in the cELISA (95.8%). When these were screened using the specific WNV NS1 and Equad indirect ELISAs, 33.8% (95% CI; 30.4–37.4) and 12.6% (95% CI; 10.3–15.3) were positive, respectively. In total, 664 horses were positive in at least one ELISA but only 72 were positive in all three assays (Figure 1).

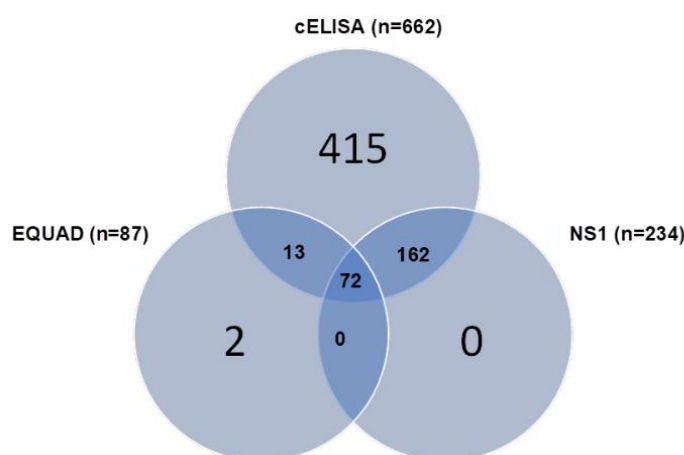


Figure 1. Venn diagram comparing screening for WNV among representative horses in northwest Nigeria using competitive, Equad and NS1 ELISAs.

The NS1 ELISA identified 234 of the 415 samples (56%) that were positive by the cELISA and did not identify any additional samples as positive. The Equad ELISA, on the other hand, only identified 85 (20.5%) of the 415 samples positive by cELISA but did identify 2 samples as positive that were negative in both the cELISA and NS1 ELISA (Figure 1).

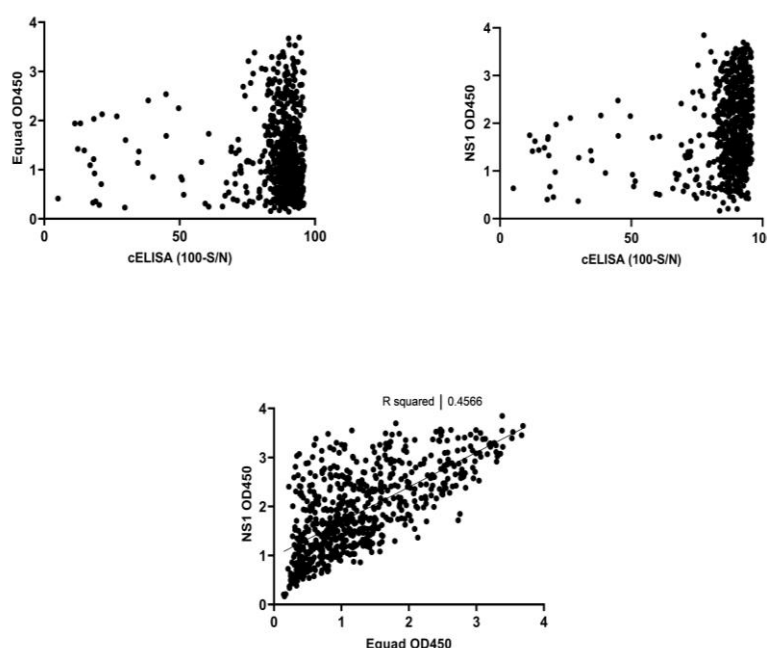


Figure 2. Simple regression of optical densities of WNV equad and NS1 ELISAs against commercial cELISA at 450nm.

4. Discussion

Our findings provide evidence that EqHV is circulating in northwest Nigeria and that the risk factors for EqHV seropositivity differ from those for WNV. Horses in Kaduna state are less at risk of WNV infection but higher risk of EqHV infection. Plant-expressed protein-based ELISAs provide a cheap option for screening for the presence of emerging viruses and could be considered as a surveillance assay where resources are limited, with little requirement for advanced assays and facilities. There is need for collaborative studies with continual routine surveillance of sentinel animals for emerging zoonotic viruses to safeguard human and animal health.

This is the first report to demonstrate the circulation of EqHV in West Africa. Detecting antibodies to EqHV among horses in northwest Nigeria implies an exposure to the emerging virus. Our study also reveals the value of plant expressed proteins in development of ELISAs and their potential in surveillance of emerging viruses like WNV and hepaciviruses.

All variables examined except for coat color were significantly associated with EqHV seropositivity in univariate analysis, but in the final multivariate analysis, only purpose and location were associated with EqHV seropositivity. Polo horses had the highest odds ratio and while the relationship between the use of the horses and EqHV seropositivity is unclear, this strongly relates to a report on EqHV seroprevalence among horses in Brazil [38] where competition horses revealed proportionately higher antibodies (19.1%) to EqHV than cart (6.2%) or farm horses (6.3%). Although the authors suggested nonsterile needle sharing as a possible reason, a lot remains unknown about the transmission of EqHV. Nevertheless, if fomites are involved in EqHV transmission, closely associated polo horses with poor biosecurity would be more likely to become infected. Kaduna State had the highest seropositivity to EqHV with Kano and Katsina having similar and relatively lower odds ratios. Kaduna is the focus for equestrian events in northwest Nigeria. It is notable that in our analysis of risk factors for WNV seropositivity in the same population (Olufemi et al., 2023 submitted), Kaduna, as the reference category, had the lowest odds ratio while Sokoto had the highest. The routes of transmission of EqHV are not yet fully identified, and this contrast with the mosquito borne WNV would appear to support a previous study that found no evidence for transmission of EqHV by mosquitoes [26].

The seroprevalence of EqHV obtained in this study is low (12%) compared to the seroprevalence obtained in Morocco (65.7%) [22] and South Africa (83.7%) [27]. Studies from Austria [26], Germany [29, 30], Japan [23-25], United Kingdom [28] and United States of America [17] also had higher antibodies to EqHV in horses. The low seroprevalence observed in our study in comparison to other studies in Africa and other continents is more likely to be due to differences in sensitivity of the assays used, as other studies mostly used LIPS/GLIPS. A higher seroprevalence of 48% was observed among horses using an ELISA developed with recombinant NS3 and core EqHV proteins [28]. In relation to prevalence of EqHV RNA positive horses in Africa (8.89%), the pooled was similar to a general global prevalence of 7.88% [21]; investigations into EqHV RNA among Nigerian horses would be valuable.

In our previous study, the overall seroprevalence of WNV among horses in northwest Nigeria determined by the cELISA was high (94.3%). In the subset of 691 samples analyzed in this study, the seroprevalence determined by cELISA was similar (95.8%). As previously reported, this high seroprevalence is comparable to findings in other studies that used the cELISA. The lower seroprevalence obtained using the NS1 and Equad ELISAs suggest that these assays are more specific than the cELISA, although it could also be due to reduced sensitivity. In a study, where Equad ELISA was used to specifically detect and differentiate between WNV and TBEV IgG antibodies, a specificity of 100% was recorded with a sensitivity of 87.9% for WNV [14].

In comparison to neutralization assays, the cELISA used is often more sensitive with lower specificity [39]. In detecting neutralizing antibodies to WNV, a study reported 5.8% prevalence following an 10.9% prevalence for flavivirus specific antibody. Although three

horses revealed neutralizing antibodies to TBEV (2 WNV+TBEV) and USUV (1 WNV+USUV), six horses had no neutralizing antibodies against any of the viruses tested despite being positive by cELISA [40]. In another study, although the overall seroprevalence by cELISA was 90.1% (73/81), by MNT, only 6.2% and 17.3% were positive for USUV and WNV respectively [41]. The two horses positive by our EQUAD ELISA but negative by cELISA could probably be due to varying antibody isotype. The WNV strain Croc1102019ZM used in this study belongs WNV lineage 1a, which is phylogenetically related to WNV isolates from Senegal and the United Arab Emirates [34]. Interestingly, a recent study in Algeria demonstrated the cELISA less sensitive than the more specific VNT. The authors observed a seropositivity of 25.2% and 30.2 in horses detected by cELISA and VNT respectively [42].

Although some studies have reported reduced sensitivity of NS1 based assays in re-infections for DENV associated with IgG antibodies against NS1 [43–45], others have also shown similarities in sensitivities of the primary and secondary infections. Interestingly, our WNV NS1 and Equad ELISAs did not only produce positive results for samples that were most strongly positive in the cELISA but also for samples that had high S/N ratios (i.e. were less strongly positive), which suggests they are not simply less sensitive (Figure 2). However, these assays were not fully validated. A selection of the samples strongly positive in the cELISA and either positive or negative in the NS1 and Equad ELISAs would be necessary before they are used for serosurveillance.

5. Conclusions

Our findings provide evidence that EqHV is circulating in northwest Nigeria and that the risk factors for EqHV seropositivity differ from those for WNV with horses in Kaduna state are less at risk of WNV infection but higher risk of EqHV infection. Plant-based ELISAs provide a cheap option for screening emerging viruses and could be considered as a surveillance assay where resources are limited with little or no access to advanced assays and facilities. There is need for collaborative studies with continual routine surveillance of sentinel animals for emerging zoonotic viruses to safeguard human and animal health.

Author Contributions: Conceptualization, O.T.O. and J.M.D.; methodology, O.T.O., M.A.R., A.W.T., J.H.K., J.R.N. and J.M.D.; formal analysis, O.T.O., J.R.N. and J.M.D.; investigation, J.M.D., O.T.O. and J.R.N.; resources, O.T.O., M.A.R., A.W.T., J.H.K., J.R.N. and J.M.D.; data curation, O.T.O.; writing—original draft preparation, O.T.O.; writing—review and editing, O.T.O., M.A.R., A.W.T., J.H.K., J.R.N. and J.M.D.; formal analysis, O.T.O., J.R.N. and J.M.D.; visualization, O.T.O. and J.M.D.; supervision, A.W.T. and J.M.D.; project administration, O.T.O. and J.M.D.; funding acquisition, O.T.O. and J.M.D. All authors have read and agreed to the published version of the manuscript.

Funding: O.T.O. is a recipient of the University of Nottingham Vice-Chancellor’s Scholarship for Research Excellence (International).

Institutional Review Board Statement: This study received approval (reference 2635181112) from the School of Veterinary Medicine and Science Ethics Clinical Review Panel, University of Nottingham, United Kingdom.

Data Availability Statement: Not applicable

Acknowledgments: We appreciate Kano, Sokoto and Zazzau Emirate councils of for their support and also staff of the Ministry of Agriculture in Katsina State and the Hon. Commissioner Ministry of Animal Health and Fisheries Development Sokoto State, Professor Abdulkadir Usman Junaidu.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Shittu, I.; Meseko, C.A.; Sulaiman, L.P.; Inuwa, B.; Mustapha, M.; Zakariya, P.S.; Muhammad, A.A.; Muhammad, U. et al., Fatal multiple outbreaks of equine influenza H3N8 in Nigeria, 2019: The first introduction of Florida clade 1 to West Africa. *Veterinary Microbiology*, **2020**. 248: p. 108820.
2. Maclachlan, N.J.; Dubovi, E.J., Chapter 29: Flaviviridae. In: *Fenner's veterinary virology / edited by N. James MacLachlan and Edward J. Dubovi*. Veterinary virology. 2016: 5th ed.; Elsevier Academic Press: Amsterdam, London, 2016.
3. Endale, A.; Medhin, G.; Darfiro, K.; Kebede, N.; Legesse, M., Magnitude of Antibody Cross-Reactivity in Medically Important Mosquito-Borne Flaviviruses: A Systematic Review. *Infect Drug Resist*, **2021**. 14: p. 4291-4299.
4. Cleton, N.B.; Godeke, G.J.; Reimerink, J.; Beersma, M.F.; Doorn, H.R.; Franco, L.; Goeijenbier, M.; Jimenez-Clavero, M.A. et al., Spot the difference-development of a syndrome based protein microarray for specific serological detection of multiple flavivirus infections in travelers. *PLoS Negl Trop Dis*, **2015**. 9(3): p. e0003580.
5. Mora-Cárdenas, E.; Aloise, C.; Faoro, V.; Knap Gašper, N.; Korva, M.; Caracciolo, I.; D'Agaro, P.; Avšič-Županc, T. et al., Comparative specificity and sensitivity of NS1-based serological assays for the detection of flavivirus immune response. *PLoS Negl Trop Dis*, **2020**. 14(1): p. e0008039.
6. Nascimento, E.J.M.; George, J.K.; Velasco, M.; Bonaparte, M.I.; Zheng, L.; DiazGranados, C.A.; Marques, E.T.A.; Huleatt, J.W., Development of an anti-dengue NS1 IgG ELISA to evaluate exposure to dengue virus. *J Virol Methods*, **2018**. 257: p. 48-57.
7. Steinhagen, K.; Probst, C.; Radzimski, C.; Schmidt-Chanasit, J.; Emmerich, P.; van Esbroeck, M.; Schinkel, J.; Grobusch, M.P. et al., Serodiagnosis of Zika virus (ZIKV) infections by a novel NS1-based ELISA devoid of cross-reactivity with dengue virus antibodies: a multicohort study of assay performance, 2015 to 2016. *Euro Surveill*, **2016**. 21(50).
8. Warnecke, J.M.; Lattwein, E.; Saschenbrecker, S.; Stöcker, W.; Schlumberger, W.; Steinhagen, K., Added value of IgA antibodies against Zika virus non-structural protein 1 in the diagnosis of acute Zika virus infections. *J Virol Methods*, **2019**. 267: p. 8-15.
9. Zhang, H.; Li, W.; Wang, J.; Peng, H.; Che, X.; Chen, X.; Zhou, Y., NS1-based tests with diagnostic utility for confirming dengue infection: a meta-analysis. *Int J Infect Dis*, **2014**. 26: p. 57-66.
10. Pereira, S.S.; Andreato-Santos, R.; Pereira, L.R.; Soares, C.P.; Félix, A.C.; de Andrade, P.; Durigon, E.L.; Romano, C.M. et al., NS1-based ELISA test efficiently detects dengue infections without cross-reactivity with Zika virus. *Int J Infect Dis*, **2021**. 112: p. 202-204.
11. Berneck, B.S.; Rockstroh, A.; Barzon, L.; Sinigaglia, A.; Vocale, C.; Landini, M.P.; Rabenau, H.F.; Schmidt-Chanasit, J. et al., Serological differentiation of West Nile virus- and Usutu virus-induced antibodies by envelope proteins with modified cross-reactive epitopes. *Transbound Emerg Dis*, **2022**. 69(5): p. 2779-2787.
12. Rockstroh, A.; Barzon, L.; Pacenti, M.; Palù, G.; Niedrig, M.; Ulbert, S., Recombinant Envelope-Proteins with Mutations in the Conserved Fusion Loop Allow Specific Serological Diagnosis of Dengue-Infections. *PLOS Neglected Tropical Diseases*, **2015**. 9(11): p. e0004218.
13. Rockstroh, A.; Moges, B.; Barzon, L.; Sinigaglia, A.; Palù, G.; Kumbukgolla, W.; Schmidt-Chanasit, J.; Sarno, M. et al., Specific detection of dengue and Zika virus antibodies using envelope proteins with mutations in the conserved fusion loop. *Emerging microbes & infections*, **2017**. 6(11): p. e99-e99.
14. Rockstroh, A.; Moges, B.; Berneck, B.S.; Sattler, T.; Revilla-Fernández, S.; Schmoll, F.; Pacenti, M.; Sinigaglia, A. et al., Specific detection and differentiation of tick-borne encephalitis and West Nile virus induced IgG antibodies in humans and horses. *Transboundary and Emerging Diseases*, **2019**. 66(4): p. 1701-1708.

15. O'Kennedy, M.M.; Coetzee, P.; Koekemoer, O.; du Plessis, L.; Lourens, C.W.; Kwezi, L.; du Preez, I.; Mamputha, S. et al., Protective immunity of plant-produced African horse sickness virus serotype 5 chimaeric virus-like particles (VLPs) and viral protein 2 (VP2) vaccines in IFNAR^{-/-} mice. *Vaccine*, **2022**. *40*(35): p. 5160-5169. 375 376 377
16. Makatsa, M.S.; Tincho, M.B.; Wendoh, J.M.; Ismail, S.D.; Nesamari, R.; Pera, F.; de Beer, S.; David, A. et al., SARS-CoV-2 Antigens Expressed in Plants Detect Antibody Responses in COVID-19 Patients. *Front Plant Sci*, **2021**. *12*: p. 589940. 378 379
17. Burbelo, P.D.; Dubovi, E.J.; Simmonds, P.; Medina, J.L.; Henriquez, J.A.; Mishra, N.; Wagner, J.; Tokarz, R. et al., Serology-enabled discovery of genetically diverse hepaciviruses in a new host. *J Virol*, **2012**. *86*(11): p. 6171-6178. 380 381
18. Simmonds, P.; Becher, P.; Bukh, J.; Gould, E.A.; Meyers, G.; Monath, T.; Muerhoff, S.; Pletnev, A. et al., ICTV Virus Taxonomy Profile: Flaviviridae. *J Gen Virol*, **2017**. *98*(1): p. 2-3. 382 383
19. Pacchiarotti, G.; Nardini, R.; Scicluna, M.T., Equine Hepacivirus: A Systematic Review and a Meta-Analysis of Serological and Biomolecular Prevalence and a Phylogenetic Update. *Animals (Basel)*, **2022**. *12*(19). 384 385
20. Kapoor, A.; Simmonds, P.; Gerold, G.; Qaisar, N.; Jain, K.; Henriquez, J.A.; Firth, C.; Hirschberg, D.L. et al., Characterization of a canine homolog of hepatitis C virus. *Proc Natl Acad Sci U S A*, **2011**. *108*(28): p. 11608-11613. 386 387
21. Bezerra, C.S.; Limeira, C.H.; Monteiro Dos Anjos, D.; Nogueira, D.B.; Morais, D.A.; Falcão, B.M.R.; Alves, C.J.; Santos, C. et al., Global Prevalence of RNA-Positive Horses for Hepacivirus (EqHV): Systematic Review and Meta-Analysis. *J Equine Vet Sci*, **2022**. *114*: p. 104003. 388 389 390
22. Abbadi, I.; Lkhider, M.; Kitab, B.; Jabboua, K.; Zaidane, I.; Haddaji, A.; Nacer, S.; Matsuu, A. et al., Non-primate hepacivirus transmission and prevalence: Novel findings of virus circulation in horses and dogs in Morocco. *Infection, Genetics and Evolution*, **2021**. *93*: p. 104975. 391 392 393
23. Matsuu, A.; Hobo, S.; Ando, K.; Sanekata, T.; Sato, F.; Endo, Y.; Amaya, T.; Osaki, T. et al., Genetic and serological surveillance for non-primate hepacivirus in horses in Japan. *Vet Microbiol*, **2015**. *179*(3-4): p. 219-227. 394 395
24. Hayashi, S.; Tanaka, T.; Moriishi, K.; Hirayama, K.; Yamada, A.; Hotta, K., Seroepidemiology of non-primate hepacivirus (NPHV) in Japanese native horses. *J Vet Med Sci*, **2018**. *80*(1): p. 186-189. 396 397
25. Tanaka, T.; Kasai, H.; Yamashita, A.; Okuyama-Dobashi, K.; Yasumoto, J.; Maekawa, S.; Enomoto, N.; Okamoto, T. et al., Hallmarks of hepatitis C virus in equine hepacivirus. *J Virol*, **2014**. *88*(22): p. 13352-13366. 398 399
26. Badenhorst, M.; de Heus, P.; Auer, A.; Rümenapf, T.; Tegtmeyer, B.; Kolodziejek, J.; Nowotny, N.; Steinmann, E. et al., No Evidence of Mosquito Involvement in the Transmission of Equine Hepacivirus (Flaviviridae) in an Epidemiological Survey of Austrian Horses. *Viruses*, **2019**. *11*(11). 400 401 402
27. Badenhorst, M.; Tegtmeyer, B.; Todt, D.; Guthrie, A.; Feige, K.; Campe, A.; Steinmann, E.; Cavalleri, J.M.V., First detection and frequent occurrence of Equine Hepacivirus in horses on the African continent. *Vet Microbiol*, **2018**. *223*: p. 51-58. 403 404
28. Lyons, S.; Kapoor, A.; Schneider, B.S.; Wolfe, N.D.; Culshaw, G.; Corcoran, B.; Durham, A.E.; Burden, F. et al., Viraemic frequencies and seroprevalence of non-primate hepacivirus and equine pegiviruses in horses and other mammalian species. *J Gen Virol*, **2014**. *95*(Pt 8): p. 1701-1711. 405 406 407
29. Pfaender, S.; Cavalleri, J.M.; Walter, S.; Doerrbecker, J.; Campana, B.; Brown, R.J.; Burbelo, P.D.; Postel, A. et al., Clinical course of infection and viral tissue tropism of hepatitis C virus-like nonprimate hepaciviruses in horses. *Hepatology*, **2015**. *61*(2): p. 447-459. 408 409 410
30. Reichert, C.; Campe, A.; Walter, S.; Pfaender, S.; Welsch, K.; Ruddat, I.; Sieme, H.; Feige, K. et al., Frequent occurrence of nonprimate hepacivirus infections in Thoroughbred breeding horses - A cross-sectional study for the occurrence of infections and potential risk factors. *Vet Microbiol*, **2017**. *203*: p. 315-322. 411 412 413
31. Ding, J.; Yang, J.; Jiang, D.; Zhou, Y.; Li, C.; Li, Y., Development of a highly sensitive Gaussia luciferase immunoprecipitation assay for the detection of antibodies against African swine fever virus. *Front Cell Infect Microbiol*, **2022**. *12*: p. 988355. 414 415

32. Tariq, M.; Hur, J.; Seo, J.W.; Kim, D.Y.; Yun, N.R.; Lee, Y.M.; Bang, M.S.; Hwang, S.Y. et al., Usefulness of ELISA Using Total Antibody against Plant-Expressed Recombinant Nucleocapsid Protein of SARS-CoV-2. *Microbiol Spectr*, **2021**. 9(3): p. e0067221.
33. Olufemi, O.T.; Edeh, E.R.; Isyaku, M.S.; Haliru, M.; Samaila, S.; Mshelia, P.W.; Owolodun, O.A.; Newton, J.R. et al., Seroprevalence of Equine Influenza and Its Associated Risk Factors in Northwest Nigeria. *Pathogens*, **2022**. 11(11).
34. Simulundu, E.; Ndashe, K.; Chambaro, H.M.; Squarre, D.; Reilly, P.M.; Chitanga, S.; Changula, K.; Mukubesa, A.N. et al., West Nile Virus in Farmed Crocodiles, Zambia, 2019. *Emerging infectious diseases*, **2020**. 26(4): p. 811-814.
35. Sainsbury, F.; Thuenemann, E.C.; Lomonossoff, G.P., pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnol J*, **2009**. 7(7): p. 682-693.
36. Thuenemann, E.C.; Meyers, A.E.; Verwey, J.; Rybicki, E.P.; Lomonossoff, G.P., A method for rapid production of heteromultimeric protein complexes in plants: assembly of protective bluetongue virus-like particles. *Plant Biotechnol J*, **2013**. 11(7): p. 839-846.
37. Lardeux, F.; Torrico, G.; Aliaga, C., Calculation of the ELISA's cut-off based on the change-point analysis method for detection of Trypanosoma cruzi infection in Bolivian dogs in the absence of controls. *Memorias do Instituto Oswaldo Cruz*, **2016**. 111(8): p. 501-504.
38. Gemaque, B.S.; Junior Souza de Souza, A.; do Carmo Pereira Soares, M.; Malheiros, A.P.; Silva, A.L.; Alves, M.M.; Gomes-Gouvêa, M.S.; Pinho, J.R. et al., Hepacivirus infection in domestic horses, Brazil, 2011-2013. *Emerg Infect Dis*, **2014**. 20(12): p. 2180-2182.
39. Beck, C.; Lowenski, S.; Durand, B.; Bahuon, C.; Zientara, S.; Lecollinet, S., Improved reliability of serological tools for the diagnosis of West Nile fever in horses within Europe. *PLoS Negl Trop Dis*, **2017**. 11(9): p. e0005936.
40. Ganzenberg, S.; Sieg, M.; Ziegler, U.; Pfeffer, M.; Vahlenkamp, T.W.; Hörügel, U.; Groschup, M.H.; Lohmann, K.L., Seroprevalence and Risk Factors for Equine West Nile Virus Infections in Eastern Germany, 2020. *Viruses*, **2022**. 14(6).
41. Tinto, B.; Kaboré, D.P.A.; Kagoné, T.S.; Constant, O.; Barthelemy, J.; Kiba-Koumaré, A.; Van de Perre, P.; Dabiré, R.K. et al., Screening of Circulation of Usutu and West Nile Viruses: A One Health Approach in Humans, Domestic Animals and Mosquitoes in Burkina Faso, West Africa. *Microorganisms*, **2022**. 10(10).
42. Laabassi, F.; Dheilily, N.; Beck, C.; Amaral, R.; Gonzalez, G.; Gaudaire, D.; Madeline, A.; Lecouturier, F. et al., Serological evidence of circulation of West Nile virus in equids in Algerian eastern drylands and its epidemiological risk factors. *Comp Immunol Microbiol Infect Dis*, **2023**. 94: p. 101947.
43. Blessmann, J.; Winkelmann, Y.; Keoviengkhone, L.; Sopraseuth, V.; Kann, S.; Hansen, J.; El Halas, H.; Emmerich, P. et al., Assessment of diagnostic and analytic performance of the SD Bioline Dengue Duo test for dengue virus (DENV) infections in an endemic area (Savannakhet province, Lao People's Democratic Republic). *PLoS One*, **2020**. 15(3): p. e0230337.
44. Duong, V.; Ly, S.; Lorn Try, P.; Tuiskunen, A.; Ong, S.; Chroeung, N.; Lundkvist, A.; Leparç-Goffart, I. et al., Clinical and virological factors influencing the performance of a NS1 antigen-capture assay and potential use as a marker of dengue disease severity. *PLoS Negl Trop Dis*, **2011**. 5(7): p. e1244.
45. Fuchs, I.; Bin, H.; Schlezinger, S.; Schwartz, E., NS1 antigen testing for the diagnosis of dengue in returned Israeli travelers. *J Med Virol*, **2014**. 86(12): p. 2005-2010.

CHAPTER 7

Summary

This study aimed to provide epidemiological data on some emerging RNA viruses among horses in northwest Nigeria. In this cross-sectional study the sero-epidemiology of equine influenza virus (EIV), West Nile virus (WNV) and *Equine Hepacivirus* (EqHV) was investigated. A total of 830 horses bled on a single occasion revealed antibodies to influenza A/equine/Richmond/2007 (H3N8) and A/equine/Worcestershire/2019 (H3N8) using single radial haemolysis (SRH). The burden of EI in northwest Nigeria is abundant with a 71.3% exposure among horses. Age of horses was significantly associated with seropositivity for EI and only 9% of the horses had sufficient antibodies (SRH value > 150 mm²) protective against clinical disease or virus shedding.

A systematic review and meta-analysis of WNV seroprevalence studies among equids in Europe (2001 – 2018) conducted followed by a scoping review on WNV antibodies among African equids revealed the global burden of flaviviruses and the paucity of seroprevalence studies among African equids despite the

endemicity of WNV in the region. Despite an observed seroprevalence of 9.8% (2765/28089) and 35% (1998/5746) in Europe and Africa, respectively, WNV among European equids (39 studies) was extensively more investigated than in African equids (16 studies). This is evidenced by the varying geographic widespread and number of studies in the two regions. Although both regions mainly used cELISA, only 37.5% of studies conducted on African samples probed further for specific flaviviruses (compared with 66.7% of studies conducted in Europe).

With an overall WNV burden of 94.3% (783/830) among equids in northwest Nigeria obtained, age and state were risk factors for flavivirus infection. Antibodies to WNV and other flaviviruses were detected with the commercial ID screen® West Nile competition multi-species ELISA (cELISA). Also, plant-based ELISAs using WNV NS1, WNV mutant envelope - 'Equad' and EqHV-core proteins were used to screen a subset of 691 sera for antibodies against WNV and EqHV. WNV NS1 and Equad ELISAs revealed 33.9% (234/691) and 12.6% (89/691) seroprevalence, respectively suggestive of more specificity than the cELISA. The seroprevalence ranging between 12.6%–94.3% revealed by the ELISAs establishes the presence of WNV and related flaviviruses in the study area and

suggests a potential zoonotic threat. Plant-based ELISAs are cheap options to be considered for serosurveillance of WNV and related viruses among sentinel hosts. Antibodies to EqHV (*Hepacivirus equi*) were detected in 12% (83/691, 95% CI; 10–15%) of the horses screened. State and purpose of horses were associated with EqHV seropositivity. This project to the best of my knowledge is the first to describe the circulation of EqHV in Nigeria demonstrating the potential of plant-based ELISA for the serosurveillance of emerging viruses in developing countries. EI, WNV and EqHV are abundant among horses in northwest Nigeria.

Limitations of the programme of work

The convenience sampling approach together with inaccessibility to horses in Jigawa, Kebbi and Zamfara States due to insecurity, banditry and administrative bottlenecks, the disease burden across the whole region could not be estimated. Although not practicable in the field, random sampling would better represent the study population. The non practicability of random sampling in the field is mainly due to the lack of information on the total number of horses in each location. This limitation made random sampling

practically impossible. Screening every horse in the study area provides the true seroprevalence of the diseases. Seroprevalence can only be estimated with associated intricacies. These intricacies, however, have been accommodated in the confidence interval and statistical analyses. Seroprevalence studies only reveal exposure to virus but not necessarily whether animals got sick. There can be cross-reactivity. Also, seroprevalence studies do not provide virus isolates or sequences for inclusion in vaccines.

A further limitation of the study design is that it only provides a timepoint assessment of the disease burden. The seroprevalence of any infectious disease is influenced by the timing of sampling in relation to the occurrence of an outbreak. For example, the seroprevalence to EI was most likely lower before the widespread 2018/19 outbreak and it is likely that the antibody levels will decay over time making more of the population susceptible to future outbreaks without vaccination. Whether or not the high WNV seroprevalence antibodies was also because of a recent outbreak is unknown. There were no reports of outbreaks of West Nile fever or paralysis in equids. This might indicate that this virus is endemic in the region and that high levels of antibodies provide protection to the equine population.

Impact of the work for the equine industry in Nigeria

Surveillance of diseases among susceptible host populations is paramount in precisely predicting the virulence and transmissibility of emerging viruses (Howard, 2012), thus forestalling public health emergencies with potential global impact. Being the first study to investigate some emerging RNA viruses among horses in northwest Nigeria, it provides a surveillance bedrock for private and governmental animal health institutions to collaborate and routinely investigate sentinel animals, sharing disease data and intervention strategies. Providing an up-to-date data on the surveillance status of horses in northwest Nigeria regarding important diseases of economic (EI) and zoonotic (WNV) importance: the serostatus of EqHV in these horses, which is the first in Nigeria along with the risk analyses to these emerging diseases are public health resources of utmost importance in strategic planning for early detection, prevention and control of infectious diseases.

The EI chapter reveals the horses are poorly protected against the disease especially the Florida clade 2-like EI viruses. Horse owners should ensure stringent quarantine and biosecurity measures

within and across stables. The policy makers and stakeholders should consider adopting a vaccination protocol with routine monitoring and surveillance programs to forestall an outbreak of EI. Unlike previous studies that used HA and ELISA to determine the seroprevalence of EI among horses parts of Nigeria, using SRH in this study provides reliable information on the protection level of horses in northwestern Nigeria. The small proportion of horses (9%) with protective antibody against Florida clade 2 reveals the urgent need to commence vaccination campaigns against EI using vaccines representative of the circulating EIVs.

The systematic and scoping reviews showed that antibodies to WNV are more investigated among equids in Europe than in Africa where the disease was first reported. The availability of funding bodies and laboratory facilities for the monitoring and control of WNV is a limitation in African climes. Regional collaborative studies targeted at the control and prevention of emerging viral zoonoses would maximize resources and establish linkages for future collaborations.

The experiments described in this thesis investigated the seroepidemiology of WNV and EqHV in equids with a focus on

northwest Nigeria resulting in the development of cheap novel WNV ELISA assays from plant-expressed proteins as an alternative to routine commercial ELISAs which cost about £1,120 to screen only half of the samples obtained in this study. The developed assay would provide a cheap source for routine surveillance and monitoring.

Sample size determination and decision is often based on varying factors ranging from study design and funding to the precision of variable estimation. Also, the final sample size obtained for any proposed study can vary depending on the criteria used by the investigator. These criteria according to Dendukuri et al. (2004) are summarized either as average coverage criterion, average length criterion and a conservative worst outcome criterion (WOC). A combination of these criteria - modified WOC which considers the normal distribution of the data set averts a highly improbable sample size.

Several approaches based on either achieving a pre-specified confidence level or a pre-specified power at a chosen significance level; can be used in determining the sample size for a prevalence study (Qiu et al., 2016). This study determined a minimum sample

size using the formula by Niang et al. (2006) with a 95% confidence level and a significance level set at $p \leq 0.05$. The Epi-Info computer package is a software maintained by the Centres for Disease Control and Prevention (CDC) available for researchers to compute sample size. The sample size for every study is unique and impacts is often impacted by the choice of the investigators considering earlier mentioned factors that often result in trade-offs. The formula presented by Naing et al. (2006) was chosen because they describe why they recommend selecting a desired precision (d) of 5% (0.05) if the prevalence of disease is expected to fall between 10 and 90%, whereas if the prevalence is extremely low or high, the 95% confidence intervals will have meaningless negative lower-bound values or upper bound values greater than 1.

They also describe with relevant illustrations the best choice of the expected prevalence, although we were able to obtain this for equine influenza from a previous study, giving us confidence that the sample size was appropriate for seroprevalence against this disease even if it might not be correct for other diseases we studied. As we found a very high seroprevalence for WNV, the sample size was more than adequate.

Although the data obtained was normally distributed, this study is limited by its design and necessitates the need for future research to consider cohort study which was practically impossible at the time due to cost and time constraint.

Horses in the northwest are under threat by emerging RNA viruses and remain vulnerable to emerging diseases, especially without a sustained vaccination campaign against vaccine preventable diseases like EI. Vaccinations of highly mobile horses with appropriate vaccines (including the circulating strain(s) of equine influenza) would ameliorate the burden of emerging viral diseases. The EI outbreak in 2018 spread across countries in West Africa caused mortalities among equids in 7 states in northern Nigeria including Sokoto, Kano and Kaduna. Had there being a routine surveillance and a vaccination programme, the economic impact would have been minimal. The said outbreak was the first report of Florida clade 1 strain of EI and this report also establishes that Florida clade 1 is circulation in Nigeria and therefore should be included in the recommended vaccines in the region. Considering the growing influx of horses into Nigeria from neighbouring countries and across the globe, border-related studies to investigate new arrivals for antibody levels against EI should be

instituted. Monitoring these animals over specific timelines in comparison to locally bred animals could inform stakeholders of the potential risk and exposure of equids to EI and possibly the need to vaccinate against EI.

Future work

Future research with robust funding and grants could undertake a similar study with a longitudinal study design. This will provide emerging disease information for particular equine populations over a prolonged time frame.

Perhaps, conducting a cohort study on the EI vaccines used in neighbouring countries and other countries from which horses are imported into Nigeria would reveal the protection conferred on these animals and the probable need to vaccinate Nigerian equids.

Antibodies to EqHV evidenced among equids in northwest Nigeria should be further investigated across other regions of the country.

This study provides a preliminary report on the potential threat of emerging equine viruses. The innovation of a plant-based ELISA to screen for WNV and EqHV could serve as an option to routinely screen for other cocirculating flavivirus with a low budget should

the method be modified. We could have retested for some possible cocirculating viruses but the limitation of time and funding was constraining. Sero-neutralization test was initially considered as a confirmatory assay. However, accessibility to live WNV and the long wait time to get trained for a category level 3 facility hampers this plan. However, Binjari virus, a dual-host laboratory adapted insect-specific flavivirus is an explorable insight for future studies that could be compared to our current finding; it is also confirmatory, replacing the need for a category level 3 laboratory. The development of such methods for other flaviviruses should be encouraged as there remains a gap in the serosurveillance of flavivirus closely related to WNV in Africa as there are no cheap reliable assays available. The ability of the developed ELISA to detect antibodies to WNV and EqHV with apparently more specificity indicates these assays could serve as a contingency option in developing nations with scarce resources or as screening assays in surveillance programmes before using more advanced techniques. This would allow investigation of target populations with prompt detection and early warning to promote public health.

In the future, spatial mapping of EI, WNV and EqHV among equids in northwest Nigeria along with an in-depth investigation on the impact of climatic variables of the spread of these diseases would help horse owners, veterinarians, para-veterinarians and other health workers to prioritize efforts in disease monitoring, prevention and control of these emerging diseases. Including genetics epidemiology as well as seroprevalence would provide more specific information on any mutations on the virus that could inform including specific sequences in vaccines.

The same problem of cost of surveillance for diseases like EI and WNV is true for the cost of vaccination. Despite several options for vaccinating against EI in Nigeria and the availability of vaccines against WNV used in developed countries, very few equids in Nigeria are vaccinated. Plant expression of virus proteins in the same way that they were produced to make the ELISAs used in this study can be used to make vaccines. In particular, expression of the haemagglutinin protein of EIV, which will assemble into virus-like particles is a potential cheap alternative for vaccine production.

Establishing an equine disease surveillance team with a focus on equine mobility and border patrol would help monitor equine health and provide stakeholders with tool for early detection and warning for emerging equine diseases in north western Nigeria.

Appendix A

Sampling and data collection

Purposive sampling technique was employed along with the 'gatekeeper' approach to access the horse population in the study. A blend of snowball and convenience sampling methods were used alongside medical and management incentives. Apparently healthy horses were sampled based on availability and willingness of horse owners to participate in the study. All horses included in this study were sampled with consent from relevant authorities and community inhabitants.

With an expected EI seroprevalence of 60.9% (Meseko et al., 2016), a precision (maximum acceptable error) of $\pm 5\%$ and a confidence level of 95%; the number of minimum samples to be collected was determined using the formula by Naing et al. (2006).

$$N = \frac{Z^2 P_{exp}(1 - P_{exp})}{d^2}$$

Where:

N = Sample size

Z = Standard normal deviation for 95% confidence interval (1.96)

Pexp = Expected prevalence (Using 60.9% prevalence)

d = Desired precision (0.05)

$$\frac{(1.96)^2 \times 0.609 \times 0.391}{(0.05)^2}$$
$$\frac{3.8416 \times 0.609 \times 0.391}{0.0025}$$
$$= 365$$

A total of 830 samples were obtained.

The sex, breed and vaccination history were determined. The animal was also aged using dentition, following adequate restraint. Then, 5 ml of blood was collected aseptically by jugular venipuncture using 21-gauge needle. The blood was then dispensed into labelled 8 ml serum clot activator tubes (Medline USA) and kept on an ice pack temporarily after which the serum was harvested and decanted into centrifuge tubes.

All centrifuge tubes were appropriately labelled and transported on ice to the Biotechnology laboratory, National Veterinary Research Institute, Vom, Plateau State Nigeria where it was stored at -80°C in preparation for shipping.

Each sample was identified with a unique sample number and all data obtained about each horse sampled (state, sex, breed,

source, vaccination history and use of horse) was entered into an Excel spread sheet and archived in the Nottingham research repository (Olufemi and Daly, 2024)

Appendix A.1

Propagation of A/equine/Richmond/07 (H3N8) and A/equine/Worchestershire/2019 (H3N8)

Stock of A/equine/Richmond/07 (H3N8) and A/equine/Worcester/2019/ (H3N8) influenza virus was grown separately in 10-day-old embryonated hens' eggs.

Fertile eggs commercially purchased were carefully sorted and set in trays of egg incubator at 37°C (humid with fan), with the pointed end downwards. At day 10 of incubation, eggs were removed and examined with an egg candler before discarding infertile eggs. Air sacs of suitable eggs were marked off and an 'x' mark was placed on the allantoic cavity area just 2-3 mm below the line demarcating the air sac and away from the embryo's eye. Eggs were then held at 37°C until inoculation with A/equine/Richmond/07 (H3N8) and A/equine/Worcester/2019/ (H3N8) influenza viruses.

Seed of the viruses grown were diluted (1:100) in virus dilution medium to minimize the production of defective interfering virus particles. Just before inoculation, eggs were sprayed with 70% industrial methylated spirit (IMS) and allowed to dry, then two holes were pierced in the eggshell; firstly, at the demarcated air sac and secondly at the marked site of inoculation. A 1 ml syringe and 25G needle was used to inoculate 0.1 ml of the each influenza virus per egg by carefully injecting the needle about 2 mm into the allantoic cavity through the hole pierced at the marked inoculation site. Molten paraffin wax was used to seal hole pieced the inoculation site first before that at the air sac.

Following inoculation, eggs were appropriately labelled and incubated at 34°C (humid) for 72 hours after which the eggs were wrapped in autoclave bags and placed in 4°C cold room overnight to kill the embryo and contract the blood vessel thus minimizing contamination of fluid with blood when harvesting.

In order to harvest the allantoic fluid, the area of shell over the air sac was removed with a pair of scissors to allow access to the allantoic cavity. With the membranes pressed down with the handle end of a pair of forceps, allantoic fluid was gently pipetted

out with a 10 ml serological pipette. Allantoic fluid from all eggs was pooled into 15 ml centrifuge tube and a benchtop centrifuge was used to centrifuged at 2500rpm for 5 minutes to clarify the fluid before aliquoting and storing at -80°C.

Egg inoculation and allantoic fluid harvest were both performed in a Class II safety cabinet (Thermo Electron Corporation, Germany).

Appendix A.2

Hemagglutination assay

Haemagglutination titration was carried out to ascertain the propagation of the viruses (A/equine/Richmond/07 (H3N8) and A/equine/Worchester/2019/ (H3N8)) grown in embryonated hens' eggs.

Chicken red blood cell (RBC) in Alsever's solution was commercially purchased (TCS Biosciences Ltd, United Kingdom) and washed in phosphate buffered saline (PBS) at 2500 rpm for 5 minutes using a benchtop centrifuge. The packed cell volume (PCV) of the washed chicken RBC was then determined by haematocrit before preparing a 1% chicken RBC solution, which was temporarily stored at 4°C until needed for titration.

Twenty-five microlitre (25 uL) of PBS was first dispensed into wells A2–A12 of a V-bottomed 96-well microtiter plate; then 50 uL of the grown A/equine/Richmond/07 (H3N8) influenza virus was dispensed into well A1, before titrating 25 uL (A/equine/Richmond/07 (H3N8)) from well A1 across wells A2–A11, leaving well A12 as control. Gently mixing the 1% chicken RBC, 25 uL was dispensed into wells A1–A12 before incubating the

microtiter plate at 22°C for 30 minutes. The plate was then observed to distinguish agglutinated wells from non-agglutinated ones, then the titre was determined.

Appendix B

Single radial haemolysis assay (SRH)

Single radial haemolysis (SRH) assay is an immunodiffusion assay based on virus-coupled erythrocytes. The assay is sensitive, reliable and reproducible within and between laboratories with concrete correlation between antibody and protection from challenge (Daly et al., 2007, Mumford, 2000, Wood et al., 1994). A/equine/Richmond/07 (H3N8) was coupled with sheep red blood cells and assay plates were prepared before performing the assay (Figure 1). The assay was performed according to standard procedures (OIE, 2019).

Sheep red blood cell (RBC) in Alsever's solution was washed in saline/HEPES buffer by centrifugating at 2500 rpm for 5 minutes using a benchtop centrifuge. This was repeated until a clear supernatant was obtained. The washed sheep blood was then spined in a haematocrit centrifuge for 1.5 minutes to determine the packed cell volume with a micro-haematocrit reader before preparing an 8% suspension of sheep RBC in saline/HEPES. One millilitre of 8% sheep RBC was allowed per plate of SRH to be

prepared. The prepared 8% suspension of sheep RBC in saline/HEPES was used fresh or stored at 4°C for up to 24 hours.

Sensitization of sheep RBC with A/equine/Richmond/07 (H3N8)

The 8% sheep RBC was sensitised with an appropriate volume of A/equine/Richmond/07 (H3N8) predetermined by titration at 0.3, 0.6 and 0.9 ml per 1 ml of sheep RBC for optimal results (clear zone of lysis).

The mixture was held on ice for 10–15 minutes with intermittent gentle occasional mixing. Then freshly diluted chromium chloride (CrCl₃) solution (1:400) in 0.85% sodium chloride (NaCl) was slowly added and held for 5–10 minutes at room temperature with occasional gentle mixing. The sensitised RBC were then sedimented at 1000 rpm for about 5–10 minutes using a benchtop centrifuge.

Sedimented A/equine/Richmond/07 (H3N8)-RBC mixture was then resuspended in saline/HEPES/BSA buffer to its original volume by gentle pipetting. This was again sedimented at 1000 rpm for about 10 minutes using a benchtop centrifuge.

Finally, the sensitised sheep RBC was resuspended to an 8% suspension in London PBS (the initial volume of cells – (the initial volume of cells x 0.08) = ml of London PBS to be added).

Preparation of Assay plates

Prepared 1 % agarose (36°C gelling temp) in London PBS was melted and 7.8 ml was pipetted into universal bottles pre-warmed at 42°C in a water bath. One universal bottle is needed for each assay plate being poured.

A total of 0.9 ml of virus sensitised sheep RBC and 0.3 ml undiluted Guinea-pig complement was quickly added to the agarose at 42°C. The mixture was quickly and gently mixed before pouring into immunoplates on a level surface. Plates were clearly labelled on the left-hand side with plate number, virus strain and the date poured. The plates were allowed to air dry for a minimum of 5 minutes before covering with a lid.

Performing the SRH assay

With each plate allowing for a positive control serum and 16 test sera, a supplied template and a 3 mm hole punch was used to punch wells into the set gel.

Ten microlitre of heat inactivated test or control sera were pipetted into the appropriate wells. Plates were then covered with lid and incubated in humidified plastic 'luncheon box' at 34°C for 20 hours.

A digital calliper was used to read the radial zone of lysis. The area of the punched well was calculated from the diameter measured by the digital callipers.

The SRH assay as described for A/equine/Richmond/07 (Figure B-1) was similarly performed for A/equine/Worchester/2019/ (H3N8).

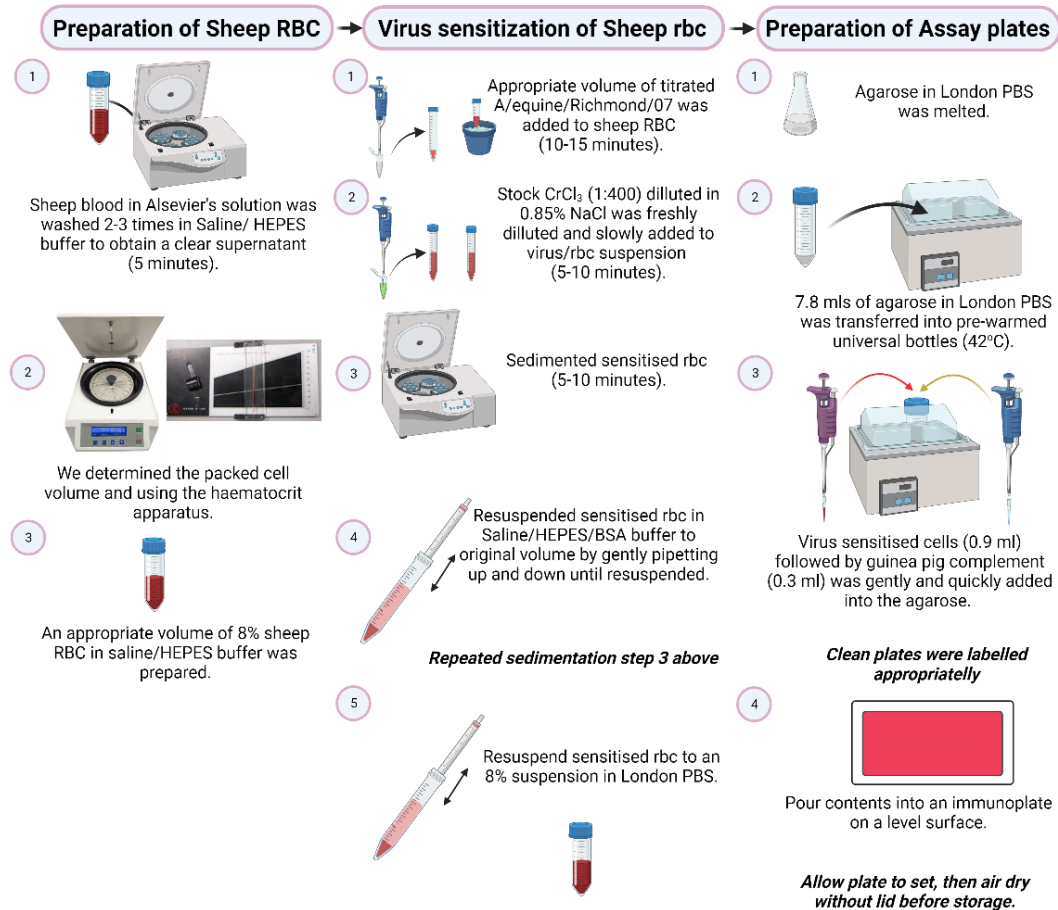


Figure B-1: Schematic representation of single radial haemolysis

Appendix C

Plant expression of WNV recombinant proteins (Mutant E (equad) and NS1)

Sequences of WNV- NS1 and WNV-E was obtained from GenBank database (LC489409) (Simulundu et al., 2020). A mutant was obtained by inserting a four-point mutation (Berneck et al., 2022) in the WNV-E sequence. The mutant WNV-E protein (Equad) and the WNV-NS1 proteins were first synthesized (codon optimized) by GeneArt Gene Synthesis (Thermo Fisher Scientific, Regensburg, Germany) before cloning the constructs separately into plant expression plasmids – pEAQ-HT. The resultant pEAQ-HT-Equad and pEAQ-HT-NS1 were transformed with *Agrobacterium tumefaciens* (strain LBA4404) and infiltrated into *Nicotiana benthamiana* leaves. *Agrobacterium tumefaciens* transformed with pEAQ-HT-Equad and pEAQ-HT-NS1 were streaked out on LB Agar containing relevant antibiotics (rifampicin at 50 µg/ml for *Agrobacterium tumefaciens* and kanamycin at 50 µg/ml for pEAQ-HT) and then incubated at 28°C for three days. Then three overnight cultures (at 28°C) of the pEAQ-HT-Equad, pEAQ-HT-NS1 and an empty vector (as a negative control) colonies were prepared and pooled together. The optical density (OD) of a 1:5

dilution of the pooled culture was measured at 600nm and used to determine the dilution to make 10ml of infiltration solution at 0.4 OD (i.e. $0.4 \div OD \times 10$).

Infiltration of *Nicotiana benthamiana* with pEAQ-HT-Equad and pEAQ-HT-NS1

Infiltration of *Nicotiana benthamiana* plants performed in the green house was achieved by applying the infiltration solution (Figure C-1) on to the underside of the leaves directly using a 1 ml syringe to force the solution through the stroma without damaging the leaves. The plants were identified with construct name and date clearly written on plastic labelled buried in the plant pot. The plants are often infiltrated at 5-6 weeks of age with only three middle leaves (not too young and not too old) infiltrated per construct of interest. Around 7 – 8 and 2 plants are sufficient for each construct of interest and negative control respectively. The leaves were harvested one week after infiltration; by tearing off with fingers. The leaves were placed into pre-labelled Ziploc bags and weighed before temporary storage at -20°C until needed for extraction and purification.

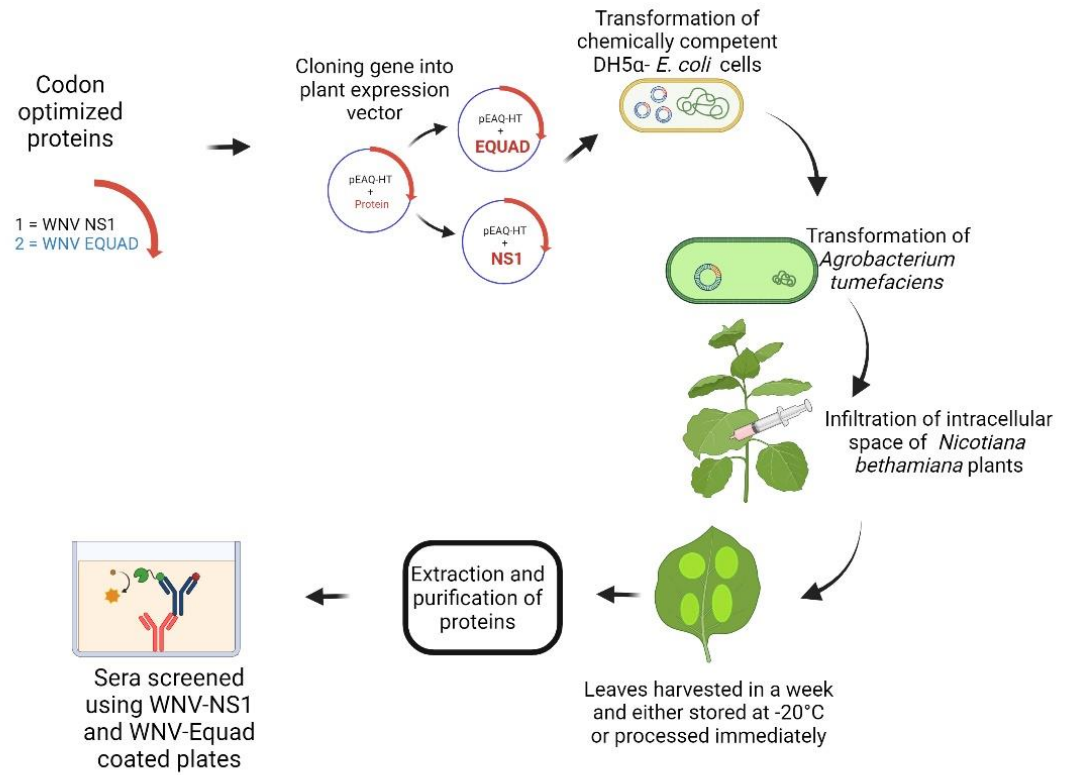


Figure C-1: Diagrammatic representation of plant expression of recombinant protein

Extraction and Purification of *Nicotiana benthamiana* leaves infiltrated with pEAQ-HT-Equad and pEAQ-HT-NS1

Leaves infiltrated with pEAQ-HT-Equad and pEAQ-HT-NS1 were separately weighed and transferred into 1 L blender jug and then homogenized in two volumes of ice-cold extraction buffer (500 ml pH 7.2: 25 ml 1 M Tris, 50 ml 5 M NaCl, 1.24 g Na₂S₂O₃·5H₂O (final conc. 10 mM), 0.5 ml Triton-X-100 (final conc. 0.1% v/v) 425 ml sterile H₂O,) with protease inhibitor buffer using a handheld waring blender (Braun MQ5235). After homogenizing for around 1 minute, the homogenate was filtered through a piece of Mira cloth (VWR International Ltd. Cat. No. 475855-1) into a 500 ml beaker squeezing out the juice. The unfiltered leaf matter was discarded while the juice was transferred into a 250 ml centrifuge bottle (for SS34 roto at JIC) and centrifuged at 13,000 *g* for 20 minutes at 4°C.

A 5 ml polypropylene column (QIAGEN Cat. No. 34964) was charged by placing a filter at the bottom of the column before adding 3–4 ml of Ni NTA agarose (QIAGEN) and add 2–3 ml of binding buffer (500 ml pH 8.0: 25 ml 1 M Tris, 50 ml 5 M NaCl, 0.5 ml Triton-X-100, 425 ml sterile H₂O). The charged column was placed on a retort stand allowing the buffer to completely flow

through. A stopper was then placed at the open end of the column to prevent the filter from drying up.

The supernatant extract juice was filtered through a sterile EO GF + CA 0.45 μ M filter using a 50 ml syringe. A sample of this filtered plant extract was saved and labelled S1 before adding to the charged column and allowed to drip through. A sample of the column flow through was saved and labelled S2. After adding all supernatant extract juice to the column, it was then sequentially washed with 25 ml of 5 mM, 10 mM, 20 mM and 30 mM imidazole with a sample of the 30 mM imidazole wash saved and labelled S3. Elution buffer (2.5 ml 1 M Tris, 5 ml 5 M NaCl, 25 ml 500 mM imidazole and 17.5 ml sterile H₂O), 6 x 1 ml at a time was added to the column, collecting each ml into separate Eppendorf tubes labelled E1–E6. These samples were ran on 12% Mini-PROTEAN TGX™ Precast Protein Gels under reducing conditions at 200 V for 50 minutes in MOPS-SDS running buffer. The gel was then stained with instant Blue® Coomassie protein stain (ABCAM, ISB1L) for 1 hour and de-stained for 1 hour before viewing with iBright™ FL1500 Imaging System. Western blotting was performed to confirm protein expression and protein concentration was

determined with Pierce Bradford Assay Kit (Thermo Fisher Scientific, Waltham, MA USA).

Appendix D

Development and Optimization of WNV equad and NS1 Indirect ELISAs

Antigen Coating/Coating buffer

Purified equad protein expressed in plants were first quantified using Bradford assay before coating 96 well micro titre plates (Thermo Fisher Scientific, Waltham, MA USA). Phosphate buffered saline (PBS) and carbonate-Bicarbonate buffers were compared to determine the optimal coating ability of the buffers. Checkerboard was used to coat designated wells equad protein at 1ug, 2ug and 4ug/ml.

For the optimization of the amount of antigen for coating the wells, 1ug, 2ug and 4ug per well of WNV equad protein diluted in PBS and carbonate-Bicarbonate were evaluated. Coated plates were incubated at 4°C overnight.

Blocking Buffers

Three blocking buffers designated SMT (3% skimmed milk in PBS 0.1 Tween 20), BSASMAT (1% bovine serum albumin plus 3% skimmed milk in PBS 0.1 Tween 20) and EB (ELISA blocker

(Thermo Fisher Scientific, Waltham, MA USA)) were compared to determine the best blocking condition for the indirect ELISA.

Wells blocked with EB and BSASMAT resulted in comparative lower background compared to those blocked with SMT.

Sera/Sera dilutions

A positive and negative pool of heat inactivated sera field screened with WNV ID vet Screen were used as test sera. Also, a commercial pathogen free horse sera (Thermo Fisher Scientific, Waltham, MA USA) and a WNV positive sera were used as positive and negative control sera respectively.

Sera (positive and negative) were compared at 1:50 and 1:100 dilutions, incubated for 1 and 2 hours. Sera at 1:50 had higher signal and background when compared to 1:100. However, the signal to positive ratio was same for both dilutions (Trial 3 AP 27 June.) This was consistent with all conjugates used. Surprisingly, the commercial IDVET positive control gave a higher signal in wells not coated with equad in comparison to pool of positive sera used in uncoated wells (Trial 13 AP buffer 1 and 2).

Conjugates/Substrate

Anti-Goat Horse radish peroxidase (HRP) and Alkaline phosphatase (AP) were both optimized at 1:1000 and 1:5000 dilution. Using the blocking buffer as the diluent, the 1:5000 dilution did not have any difference in signal with the 1:1000 dilution when using the AP (trial 3 AP 27 June). With HRP, 1:1000 conjugate dilution had a higher signal and a corresponding higher signal to positive ratio when compared to 1:5000 dilution (trial 3 HRP 27 June22, also trial 2 June HRP 22). Summarily, 1:100 sera dilution with HRP at 1:1000 gave optimal signal with minimal background.

Each conjugate was used with corresponding conventional substrate; TMB (Thermo Fisher Scientific Waltham, MA USA) and ppNP were used with Anti-Goat Horse radish peroxidase and Alkaline phosphatase respectively. The plates with HRP TMB were stopped with 2M H₂SO₄.

Controls

Positives controls used in the optimization of these assays study include: WNV positive sera (ID Vet Diagnostics) and pooled sera (from the study), positive with the commercial WNV competitive ELISA. The negative controls are: a commercial equine serum and

pooled sera (from the study), negative with the commercial WNV competitive ELISA.

		Conjugate dilution 1:1000						Conjugate dilution 1:5000					
		Wells coated with EQUAD 2ug/ml						Wells coated with EQUAD 2ug/ml					
		1	2	3	4	5	6	7	8	9	10	11	12
Sera dilution 1:50	A	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera
	B	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera
	C	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera
	D	NO EQUAD COATING +ve sera	NO EQUAD COATING -ve sera	NO EQUAD COATING No sera	NO EQUAD COATING -ve sera	NO EQUAD COATING +ve sera	NO EQUAD COATING No sera	NO EQUAD COATING +ve sera	NO EQUAD COATING -ve sera	NO EQUAD COATING No sera	NO EQUAD COATING -ve sera	NO EQUAD COATING +ve sera	NO EQUAD COATING No sera
Sera dilution 1:100	E	NO EQUAD COATING +ve sera	NO EQUAD COATING -ve sera	NO EQUAD COATING No sera	NO EQUAD COATING -ve sera	NO EQUAD COATING +ve sera	NO EQUAD COATING No sera	NO EQUAD COATING +ve sera	NO EQUAD COATING -ve sera	NO EQUAD COATING No sera	NO EQUAD COATING -ve sera	NO EQUAD COATING +ve sera	NO EQUAD COATING No sera
	F	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera
	G	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera
	H	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera
Results of Photometric1													
Plate:1 - Wavelength:405													
photometric1		3.433	1.993	0.115	1.547	3.22	0.095	1.945	1.092	0.096	1.018	1.985	0.083
		3.475	1.836	0.078	1.674	3.243	0.085	1.971	1.01	0.078	1.024	1.86	0.09
		3.342	1.733	0.076	1.507	3.216	0.084	1.815	0.952	0.086	1.008	1.782	0.083
		0.638	0.669	1.214	0.546	0.514	1.625	0.779	0.37	0.075	0.324	0.347	0.086
		0.398	0.377	0.079	0.356	0.35	0.08	0.198	0.231	0.081	0.23	0.198	0.1
		1.757	0.95	0.087	0.846	1.685	0.082	0.948	0.556	0.083	0.536	0.903	0.087
		1.596	0.989	0.08	0.88	1.559	0.089	0.916	0.51	0.077	0.565	0.859	0.086
		1.745	0.981	0.18	0.933	1.587	0.126	0.936	0.552	0.081	0.549	0.983	0.081

Plate layout and optical density (OD) values of trial 3 using alkaline phosphatase and different sera dilutions.

		Conjugate dilution 1:1000						Conjugate dilution 1:5000					
		Wells coated with EQUAD 2ug/ml						Wells coated with EQUAD 2ug/ml					
		1	2	3	4	5	6	7	8	9	10	11	12
Sera dilution 1:50	A	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera
	B	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera
	C	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera
	D	NO EQUAD COATING +ve sera	NO EQUAD COATING -ve sera	NO EQUAD COATING No sera	NO EQUAD COATING -ve sera	NO EQUAD COATING +ve sera	NO EQUAD COATING No sera	NO EQUAD COATING +ve sera	NO EQUAD COATING -ve sera	NO EQUAD COATING No sera	NO EQUAD COATING -ve sera	NO EQUAD COATING +ve sera	NO EQUAD COATING No sera
Sera dilution 1:100	E	NO EQUAD COATING +ve sera	NO EQUAD COATING -ve sera	NO EQUAD COATING No sera	NO EQUAD COATING -ve sera	NO EQUAD COATING +ve sera	NO EQUAD COATING No sera	NO EQUAD COATING +ve sera	NO EQUAD COATING -ve sera	NO EQUAD COATING No sera	NO EQUAD COATING -ve sera	NO EQUAD COATING +ve sera	NO EQUAD COATING No sera
	F	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera
	G	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera
	H	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera

Results of Photometric1

		Plate:1 - Wavelength:450											
		1	2	3	4	5	6	7	8	9	10	11	12
A		2.163	0.596	0.048	0.615	1.767	0.143	0.248	0.102	0.037	0.108	0.259	0.035
B		1.636	0.535	0.034	0.551	1.653	0.034	0.25	0.096	0.037	0.084	0.253	0.03
C		1.699	0.571	0.033	0.582	1.707	0.036	0.278	0.113	0.031	0.109	0.275	0.03
D		0.156	0.137	0.032	0.136	0.113	0.032	0.046	0.048	0.031	0.054	0.053	0.032
E		0.087	0.1	0.032	0.096	0.068	0.031	0.037	0.042	0.03	0.042	0.042	0.035
F		1.867	0.338	0.031	0.322	0.922	0.037	0.194	0.09	0.03	0.084	0.165	0.033
G		1.008	0.315	0.032	0.335	0.924	0.038	0.179	0.079	0.03	0.08	0.158	0.034
H		1.06	0.375	0.035	0.331	0.908	0.038	0.16	0.075	0.035	0.086	0.141	0.042

Plate layout and optical density (OD) values of trial 3 using horse radish peroxidase and different sera dilutions

WNV EQUAD HRP ELISA TRIAL 22 JUNE 22 photometric 1

HRP DIUTION: 1:5000

Sera dilution: 1:100

Results of Photometric1

Plate:1 - Wavelength:450

EQUAD DILUTED IN BICARBONATE BUFFER						EQUAD DILUTED IN PBS BUFFER					
1ug/ml		2ug/ml		4ug/ml		1ug/ml		2ug/ml		4ug/ml	
0.057	0.054	0.054	0.048	0.063	0.05	0.054	0.053	0.055	0.052	0.065	0.057
0.053	0.079	0.056	0.074	0.055	0.05	0.063	0.058	0.064	0.059	0.065	0.053
0.059	0.052	0.07	0.157	0.056	0.051	0.07	0.049	0.055	0.052	0.057	0.053
0.049	0.047	0.07	0.088	0.049	0.05	0.045	0.048	0.048	0.062	0.05	0.051

Plate layout and optical density values of trial 2 using horse radish peroxidase enzyme with two coating buffers.

	Wells coated with Buffer 1 EQUAD 2ug/ml			Wells coated with Buffer 1 EQUAD 2ug/ml			Wells coated with Buffer 2 EQUAD 2ug/ml			Wells coated with Buffer 2 EQUAD 2ug/ml			
	NORMAL EQUINE SERA and ID VET +ve control			Pooled equine +ve and - ve sera screened with ID VET competitive ELISA lit			NORMAL EQUINE SERA and ID VET +ve control			Pooled equine +ve and - ve sera screened with ID VET competitive ELISA lit			
	+ve seraN	-ve seraN	No sera	-ve sera	+ve sera	No sera	+ve sera	-ve sera	No sera	-ve sera	+ve sera	No sera	
	1	2	3	4	5	6	7	8	9	10	11	12	
A	1.2014	0.4628	0.1399	0.5061	1.5977	0.1371	1.2894	0.4635	0.1147	0.5323	1.6668	0.1158	EQUAD COATING
B	1.2075	0.4970	0.1202	0.5258	1.5620	0.1211	1.2791	0.4602	0.1111	0.5517	1.6710	0.1242	
C	1.0869	0.4856	0.0978	0.5126	1.5621	0.0973	1.2463	0.4502	0.1109	0.5134	1.6521	0.1150	
D	0.6525	0.2237	0.1040	0.2567	0.2465	0.1075	0.6227	0.2224	0.1091	0.2691	0.2675	0.1280	NO EQUAD COATING

Plate layout and optical density values of Trial 13 using alkaline phosphatase enzyme with different control sera.

Protocol for developed plant based ELISA

Following several trials in optimising the WNV ELISA at different steps, we decided to adopt the protocol itemised below;

1. Dilute equad protein to 2ug/ml in Phosphate Buffered saline (PBS) ELISA Coating Buffer. Add 50uL/well to a 96-well NUNC Maxisorp ELISA plate.
2. Incubate stationary overnight at 4°C.
3. Wash plate 3 times with Phosphate buffered saline 0.1% Tween 20 (PBST), tapping plate on paper towel in between washes.
4. Add **200uL** of blocking buffer: 1% bovine serum albumin + 3% skimmed milk in Phosphate buffered saline 0.1% Tween 20 (PBST).
5. Incubate for 48 hours at 4°C.
6. Tip off blocking buffer and wash plate 3 times with Phosphate buffered saline 0.1% Tween 20 (PBST), tapping plate on paper towel in between washes.
7. Tip off blocking buffer and tap out excess into paper towel.

8. Dilute test and control sera **1:100** in 1% bovine serum albumin + 3% skimmed milk in Phosphate buffered saline 0.1% Tween 20 (PBST) dilution buffer and add **50uL/well**.
9. Incubate for 1h at 37°C.
10. Wash plate five times with TBST wash Buffer using wash bottle, and tap out excess into paper towel.
11. Dilute Protein A/G-HRP conjugate (**1:1000**) in 1% bovine serum albumin + 3% skimmed milk in Phosphate buffered saline 0.1% Tween 20 (PBST) dilution and add 50uL/well.
12. Incubate for 1h at 37°C.
13. Wash plate five times with TBST wash buffer using wash bottle, and tap out excess into paper towel.
14. Add 100uL/well of Ultra fast TMB substrate (Thermo Fisher Scientific, Waltham, MA USA) and incubate in the dark at room temperature for 20 minutes.
15. Stop reaction with 100uL/well 2M H₂SO₄.
16. Read Optical Density at 450nm on ELISA Plate Reader.

Appendix E

Data Analyses (Regression analyses)

The data analyses involved a prior data cleansing followed by orderly stepwise analyses (Figure E-!). Using StataSE (17.1, StataCorp LLC, College Station, TX, USA). Firstly, Pearson's chi square was used to investigate the association between explanatory variables and seropositivity to EI, WNV and EqHV: ordinary logistic regression was then used to determine the risk factors predictive of the dependent variables with matched 95% confidence interval (CI) and odds ratio calculated for variables in the univariate analyses.

Using a forward-stepwise approach, non-collinear variables with $p \leq 0.2$ were chosen for multivariate analyses by ordinary logistic regression. The final model was assessed using a goodness of fit test.

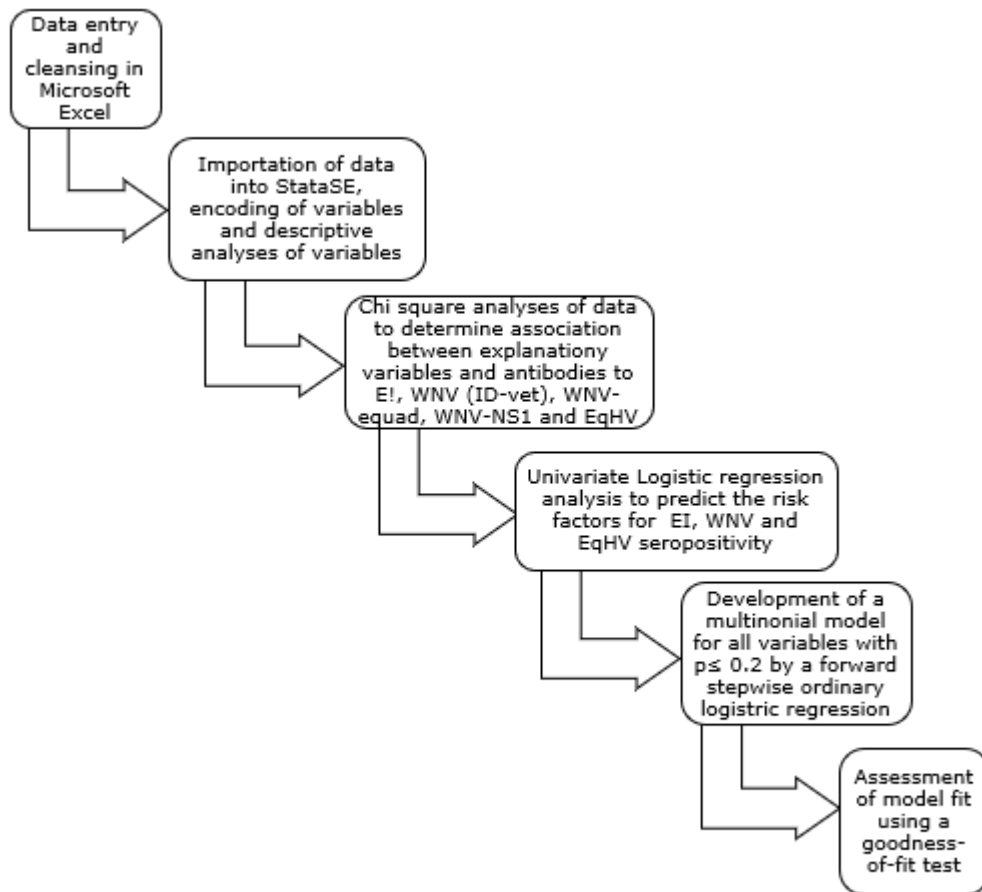


Figure E-1: Flow chart of data analyses

Bibliography

- ABBADI, I., LKHIDER, M., KITAB, B., JABBOUA, K., ZAIDANE, I., HADDAJI, A., NACER, S., MATSUU, A., PINEAU, P. & TSUKIYAMA-KOHARA, K. 2021. Non-primate hepacivirus transmission and prevalence: Novel findings of virus circulation in horses and dogs in Morocco. *Infection, Genetics and Evolution*, 93, 104975.
- ADEYEFA, C. A., HAMBLIN, C., CULLINANE, A. A. & MCCAULEY, J. W. 1996a. Nationwide serological survey of equine influenza in Nigeria. *Revue d'elevage et de medecine veterinaire des pays tropicaux*, 49, 24-27.
- ADEYEFA, C. A. & MCCAULEY, J. W. 1994. Outbreak of equine influenza in polo horses in Ibadan, Nigeria: virus isolation, clinical manifestation and diagnosis. *The Veterinary record*, 134, 683-684.
- ADEYEFA, C. A. O., JAMES, M. L. & MCCAULEY, J. W. 1996b. Antigenic and genetic analysis of equine influenza viruses from tropical Africa in 1991. *Epidemiology and Infection*, 117, 367-374.
- BADENHORST, M., TEGTMEYER, B., TODT, D., GUTHRIE, A., FEIGE, K., CAMPE, A., STEINMANN, E. & CAVALLERI, J. M. V. 2018. First detection and frequent occurrence of Equine Hepacivirus in horses on the African continent. *Vet Microbiol*, 223, 51-58.
- BENDER, J. B. & TSUKAYAMA, D. T. 2004. Horses and the risk of zoonotic infections. *Veterinary Clinics of North America: Equine Practice*, 20, 643-653.
- BERNECK, B. S., ROCKSTROH, A., BARZON, L., SINIGAGLIA, A., VOCALE, C., LANDINI, M. P., RABENAU, H. F., SCHMIDT-CHANASIT, J. & ULBERT, S. 2022. Serological differentiation of West Nile virus- and Usutu virus-induced antibodies by envelope proteins with modified cross-reactive epitopes. *Transbound Emerg Dis*, 69, 2779-2787.
- BEZERRA, C. S., LIMEIRA, C. H., MONTEIRO DOS ANJOS, D., NOGUEIRA, D. B., MORAIS, D. A., FALCÃO, B. M. R., ALVES, C. J., SANTOS, C., SILVA, M. & DE AZEVEDO, S. S. 2022. Global Prevalence of RNA-Positive Horses for Hepacivirus (EqHV): Systematic Review and Meta-Analysis. *J Equine Vet Sci*, 114, 104003.
- BUKAR, M., SADIQ, M. & GEIDAM, Y. 2007. A survey of cutaneous neoplasms among horses used for cultural festivals in Borno state, Nigeria. *Nigerian Veterinary Journal*, 28, 27-33.
- BURBELO, P. D., DUBOVI, E. J., SIMMONDS, P., MEDINA, J. L., HENRIQUEZ, J. A., MISHRA, N., WAGNER, J., TOKARZ, R., CULLEN, J. M., IADAROLA, M. J., RICE, C. M., LIPKIN, W. I. & KAPOOR, A. 2012. Serology-enabled discovery of genetically diverse hepaciviruses in a new host. *J Virol*, 86, 6171-8.
- CENTERS FOR DISEASE CONTROL AND PREVENTION 1998. Preventing Emerging Infectious Diseases: A Strategy for the 21st Century. Overview of the Updated CDC plan. *MMWR Recomm Rep*, 47, 1-14.
- DENDUKURI, N., RAHME, E., BÉLISLE, P. & JOSEPH, L. 2004. Bayesian sample size determination for prevalence and diagnostic test studies in the absence of a gold standard test. *Biometrics*, 60, 388-97.
- DURAND, B., HASKOURI, H., LOWENSKI, S., VACHIER, N., BECK, C. & LECOLLINET, S. 2016. Seroprevalence of West Nile and Usutu viruses in military

- working horses and dogs, Morocco, 2012: dog as an alternative WNV sentinel species? *Epidemiology and Infection*, 144, 1857-1864.
- EHIZIBOLO, D. O., GUSI, A. M., EHIZIBOLO, P. O., MBUK, E. U. & OCHOLI, R. A. 2011. Serologic Prevalence of Brucellosis in Horse Stables in Two Northern States of Nigeria. *Journal of Equine Science*, 22, 17-19.
- Longbottom, H. 1997. Emerging infectious diseases. *Commun Dis Intell*, 21, 89-93.
- MESEKO, C. A., EHIZIBOLO, D. O., NWOKIKE, E. C. & WUNGAK, Y. S. 2016. Serological evidence of equine influenza virus in horse stables in Kaduna, Nigeria. *Journal of Equine Science*, 27, 99-105.
- MORSE, S. S. 1993. *Examining the origins of emerging viruses*. In: Morse, S. S, ed. *Emerging Viruses*, New York, Oxford University Press.
- MORSE, S. S. & SCHLUEDERBERG, A. 1990. Emerging viruses: the evolution of viruses and viral diseases. *Journal of Infectious Diseases*, 162, 1-7.
- NAING, L., WINN, T. & RUSLI, B. 2006. Practical issues in calculating the sample size for prevalence studies. *Archives of orofacial Sciences*, 1, 9-14.
- OLUFEMI, O. T. & DALY, J. M. 2024. Variables of horses and serostatus to some emerging equine RNA viruses in northwest Nigeria. University of Nottingham, United Kingdom <http://doi.org/10.17639/nott.7342>.
- OLUSA, T. A. O., ADEGUNWA, A. K., ADERONMU, A. A. & ADEYEFA, C. A. O. 2010. Serologic evidence of Equine H7 Influenza Virus in polo horses in Nigeria. *Science World Journal*, 5, 17-19.
- ORLANDO, L. 2015. Equids. *Curr Biol*, 25, R973-8.
- PACCHIAROTTI, G., NARDINI, R. & SCICLUNA, M. T. 2022. Equine Hepacivirus: A Systematic Review and a Meta-Analysis of Serological and Biomolecular Prevalence and a Phylogenetic Update. *Animals (Basel)*, 12.
- PIERSON, T. C. & DIAMOND, M. S. 2020. The continued threat of emerging flaviviruses. *Nat Microbiol*, 5, 796-812.
- POLLARD, C. 1995. The emerging infectious diseases. *Physician Assist*, 19, 73, 77-8, 80-1.
- QIU, S. F., POON, W. Y. & TANG, M. L. 2016. Sample size determination for disease prevalence studies with partially validated data. *Stat Methods Med Res*, 25, 37-63.
- RESNICK, M. P., GRUNENWALD, P., BLACKMAR, D., HAILEY, C., BUENO, R. & MURRAY, K. O. 2008. Juvenile dogs as potential sentinels for West Nile virus surveillance. *Zoonoses Public Health*, 55, 443-7.
- SACK, A., CULLINANE, A., DARAMRAGCHAA, U., CHULUUNBAATAR, M., GONCHIGOO, B. & GRAY, G. C. 2019. Equine Influenza Virus—A Neglected, Reemergent Disease Threat. *Emerging Infectious Disease journal*, 25, 1185.
- SAIZ, J. C. 2020. Animal and Human Vaccines against West Nile Virus. *Pathogens*, 9.
- SCHERWITZL, I., MONGKOLSAPAJA, J. & SCREATON, G. 2017. Recent advances in human flavivirus vaccines. *Curr Opin Virol*, 23, 95-101.
- SHITTU, I., MESEKO, C. A., SULAIMAN, L. P., INUWA, B., MUSTAPHA, M., ZAKARIYA, P. S., MUHAMMAD, A. A., MUHAMMAD, U., ATUMAN, Y. J., BARDE, I. J., ZECCHIN, B., QUARANTA, E. G., SHAMAKI, D., ALABI, O., MONNE, I., FUSARO, A. & JOANNIS, T. M. 2020. Fatal multiple outbreaks of equine influenza H3N8 in Nigeria, 2019: The first introduction of Florida clade 1 to West Africa. *Veterinary Microbiology*, 248, 108820.

- SHOMAKER, T. S., GREEN, E. M. & YANDOW, S. M. 2013. Perspective: One Health: a compelling convergence. *Acad Med*, 88, 49-55.
- SIMULUNDU, E., NDASHE, K., CHAMBARO, H. M., SQUARRE, D., REILLY, P. M., CHITANGA, S., CHANGULA, K., MUKUBESA, A. N., NDEBE, J., TEMBO, J., KAPATA, N., BATES, M., SINKALA, Y., HANG'OMBE, B. M., NALUBAMBA, K. S., KAJIHARA, M., SASAKI, M., ORBA, Y., TAKADA, A. & SAWA, H. 2020. West Nile Virus in Farmed Crocodiles, Zambia, 2019. *Emerging infectious diseases*, 26, 811-814.
- WOAH, W. O. F. A. H. 2022a. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2022 Chapter 1.1.6 "Principle and Methods of Validation of Diagnostic Assays for Infectious Diseases". Available online: https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/1.01.06_VALIDATION.pdf (accessed on 25 March 2023).
- WOAH, W. O. F. A. H. 2022b. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2022 Chapter 2.2.3. "Development and Optimisation of Nucleic Acid Detection Assays" Available online: https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/2.02.03_NAD_ASSAYS.pdf (accessed on 25 March 2023).
- WORLD ORGANISATION FOR ANIMAL HEALTH. 2019. *OIE Expert Surveillance Panel on Equine Influenza Vaccine Composition. 2019 April 4. Conclusions and Recommendations* [Online]. Available: <https://www.oie.int/our-scientific-expertise/specific-information-and-recommendations/equine-influenza/> [Accessed 20 March 2020].