

**SURVEY FOR WEST NILE VIRUS INFECTION IN HORSES AND *CULEX*
MOSQUITOES IN HORSE STABLES IN SELECTED LOCAL GOVERNMENT
AREAS OF KADUNA STATE, NIGERIA**

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NIGERIA.**

JULY, 2017

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**A DESERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE
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MEDICINE**

**DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE
MEDICINE,**

AHMADU BELLO UNIVERSITY,

ZARIA, KADUNA STATE

JULY, 2017

DECLARATION

I, hereby declare that the work in this Dissertation titled “**Survey for West Nile Virus Infection in Horses and *Culex* Mosquitoes in Horse Stables in Selected Local Government Areas of Kaduna State, Nigeria**” was carried out by me in the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University Zaria, under the supervision of Prof. J. kabir and Prof. J.U. Umoh. The information derived from this literature has been duly acknowledged in the text and a list of references provided. No part of the Dissertation has been previously presented for another Degree or Diploma in this or any other Institution.

Everest Otakpobuno ATADIOSE
Name of Student

Signature

Date

CERTIFICATION

This Dissertation titled “SURVEY FOR WEST NILE VIRUS INFECTION IN HORSES AND *CULEX* MOSQUITOES IN HORSE STABLES IN SELECTED LOCAL GOVERNMENT AREAS OF KADUNA STATE, NIGERIA” by Everest Otakpobuno ATADIOSE meets the regulations governing the award of the degree of Masters of Science in Veterinary Public Health and Preventive Medicine of Ahmadu Bello University Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to God almighty for His grace and mercy upon my life; and to my dear husband Mr. Sunday Atadiose and lovely children Prince Efe and Isabella Ese Atadiose.

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ABSTRACT

West Nile virus (WNV) infection is mosquito-borne zoonoses involving birds, horses, humans and other species of animals. This study was designed to determine the seroprevalence and risk factors of WNV infection in horses and to detect for the viral antigen in mosquitoes in Kaduna State, Nigeria. A total of 368 horses and 31 pools of *Culex* species of mosquitoes were tested for anti WNV IgG antibodies and antigen using Competitive Enzyme-Linked Immunosorbent Assay and Vector test[®] respectively in four selected Local Government Areas (LGAs) of Kaduna State. A structured interviewer administered questionnaire was used to determine presence of factors in the stables that are associated with WNV infection in horses. Out of the 368 horses tested, 331(89.9%) were seropositive for WNV infection. Based on the LGA, a statistically significant higher seroprevalence ($P=0.003$) was found in Kaduna North LGA (98.9%) followed by Zaria, Igabi and Sabon Gari with prevalence of 88.7%, 86.0% and 78.3% respectively. There was no significant association of WNV seropositivity with age and sex of horses. Only 1(3.2%) pool from Zaria LGA out of the 31 mosquito pools, tested positive for WNV antigen. There was low level of awareness on WNV infection and its transmission among horse attendants and presence of wild birds, stagnant water, grasses, trees and waste bins around the stables. This study has identified high seroprevalence of WNV in apparently healthy horses in Kaduna State and *Culex* species of mosquito in stables at the time of study are carrying WNV. Conditions suitable for transmission of WNV infection are abundant in horse stable environments in the state. There is the need for dedicated surveillance for WNV infection in Nigeria. The inclusion of WNV in the differential diagnosis of fevers of unknown origin in medical hospitals, along-side public health education of the population on the disease is necessary.

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LIST OF ABBREVIATIONS

Abbreviations	Explanations
CDC-----	Centres for Disease Control
CSF-----	Cerebrospinal fluid
CNS-----	Central Nervous System
cELISA-----	Competitive Enzyme Linked Immunosorbent Assay
DNA-----	Deoxyribonucleic acid
ECDC----- Control	Eurosurveillance Centres for Disease Control
FRET-----	Fluorescent resonance energy transfer
HI-----	Heamagglutination inhibition test
IgG-----	Immunoglobuline G
IgM-----	Immunoglobuline M
JEV-----	Japanese encephalitis virus
KDSG-----	Kaduna State Government
LGAs-----	Local Government Areas
LAMP----- amplification	Loop-mediated isothermal amplification
Nm-----	Nanometer
NS-----	Non Structural
NAAT-----	Nucleic acid amplification test
OD-----	Optical density
OIE----- Health	World Organization for Animal Health (Office Internationale des Epizootics)
PBS-----	Phosphate-buffered saline
PRNT-----	Plague reduction neutralization test

PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
SLEV	Saint Louis encephalitis virus
USGCRP	US Global Change Research Program
USDA	United State department of Agriculture
Vec	Vector test
WHO	World Health Organization
WNV	West Nile virus
WN	West Nile
WNF	West Nile fever
WNND	West Nile neuroinvasive disease
χ^2	Chi square

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

West Nile virus (WNV) is a re-emerging mosquito-transmitted disease causing WNV disease in humans and animals (Cantile *et al.*, 2000; Durand *et al.*, 2002; Murgue *et al.*, 2001a; Autorino *et al.*, 2002; Charrel *et al.*, 2003 Castillo-Olivares and Wood, 2004). WNV was first isolated in 1937 from the blood of a febrile adult human in the West Nile District of Uganda (Smithburn *et al.*, 1940). The virus (WNV) is a member of the Japanese encephalitis virus complex which includes, Japanese encephalitis virus (JEV), Saint Louis encephalitis virus (SLEV), and Murray Valley encephalitis virus within the genus *Flavivirus* and family *Flaviviridae* (Heinz *et al.*, 2000). The genetic material of the WNV is a positive-sense, single stranded RNA that is between 11,000 and 12,000 nucleotides long, which encode seven non-structural proteins and three structural proteins. The RNA strand is held within a nucleocapsid formed from 12-kDa protein blocks, the capsid is contained within a host-derived membrane altered by two viral glycoproteins (Galli *et al.*, 2004).

The virus is transmitted in natural cycles mainly between mosquitoes and birds, with humans and horses serving as incidental hosts as well as dead-end host (Burke and Monath, 2001). In humans and equines, WNV infection is usually asymptomatic or characterized by a mild febrile illness to encephalitis with fatal outcome (Petersen and Reohrig, 2001; Bunning *et al.*, 2002; Garcia-Bocanegra *et al.*, 2011). There is no specific treatment for WNV disease and clinical management is supportive. Diagnosis is based on serology, viral isolation and characterization (CDC, 2015a).

WNV has since been reported in Africa, the Middle East, Asia, Southern Europe, Australia and North America (Campbell *et al.*, 2002; Roehrig *et al.*, 2002; Zeller and Schuffenecker, 2004; MacKenzie and Williams, 2009). The initial outbreak of the virus in North America was recognized in the fall of 1999 in New York City with reported death in humans, horses, and numerous species of birds, since then, there has been an increase in geographic distribution of WNV in North America (CDC, 2002a; Brien *et al.*, 2008).

WNV disease is considered a re-emerging pathogen affecting both humans and animals (Morens *et al.*, 2004). Multiple factors contributing to the emergence of the virus include human susceptibility to infections, climate and weather, breakdown of public health measures, economic development and land use, human demographics and behaviour, along with international travel and commerce, contribute to the emergence and re-emergence of the disease (Morse, 1995; Felissa, 2006).

WNV is globally distributed and the first case was reported in Western Hemisphere in 1999 in New York City (Nash *et al.*, 2001). The virus has also spread to Europe, beyond the Mediterranean basin, and a new strain of the virus was reported in Italy in 2012 (Barzon *et al.*, 2012). WNV is considered to be an endemic pathogen in Africa, Asia, Australia, Middle East, Europe and United States, of which there was an experience of one of its worst epidemics in the year 2012 resulting in death of 286 people in the United States of which Texas being mostly affected with the disease outbreak (Murray *et al.*, 2013), making the year (2012) the worst record for WNV disease outbreak in the United States (Fox, 2013).

WNV can occur in horses as West Nile encephalitis virus, resulting from mild febrile illness to encephalitis, the incubation period for equine West Nile encephalitis following

mosquito transmission is estimated to be 3–15 days (OIE, 2013). A transient viraemia of low virus load anticipate clinical onset of WNV infection (Schmidt and El Mansoury, 1963; Bunning *et al.*, 2002). In humans and equine, most WNV infections are asymptomatic; approximately 20 to 30% of infected individuals develop flu-like clinical manifestation characterized as West Nile fever (Perelygin *et al.*, 2002; Wang *et al.*, 2004a). In a subpopulation of individuals, at least 1 out of 150 develops a neuroinvasive or encephalitis disease caused by WNV. (Petersen *et al.*, 2001; Perelygin *et al.*, 2002; Pestka *et al.*, 2004).

The clinical features of severe WNV infection varies and it includes, severe headache, ocular manifestations, muscle weakness, vomiting, anorexia, cognitive impairment, tremors, and a poliomyelitis-like flaccid paralysis (Ceausu *et al.*, 1997; Perelygin *et al.*, 2002; Bakri and Kaiser, 2004; Sejvar, 2006). The mortality rate following neuroinvasive infection is approximately 3-15% (Nathanson and Cole, 1970; Petersen and Marfin, 2002) and long-term neurological sequelae are common (King and Kesson, 1988; Scholle and Mason, 2005). Neuronal damage is most prevalent in the brain stem and anterior-horn, neurons of the spinal cord; although in immunosuppressed individual's infection can spread throughout the central nervous system (CNS) (Guarner *et al.*, 2004; Klee *et al.*, 2004).

The most important risk factor for WNV transmission is through the bite of infected mosquitoes (Hayes *et al.*, 2005a; Winters *et al.*, 2008). Also, inappropriate personal protection measures could establish risk for WNV infection (Mandalakas *et al.*, 2005). The existence of mosquitoes breeding site, such as stagnant water body, old tires and bushy environment favours the spread of the virus (Han *et al.*, 1999). Risk factors that independently potentiate the development of neuroinvasive diseases rather than West Nile

fever in California in 2005 include old age, male sex, hypertension and diabetes mellitus (Jean *et al.*, 2007).

Successful isolation of the virus from dead birds has allowed the subsequent identification of WNV as the etiologic agent of both human and animal disease, (CDC, 1999; WHO, 2011).

Seropositivity of the virus was reported in patients in Western part of Nigeria, in 1951 and 1955 (Macnamara *et al.*, 1959). Recently, other researchers reported seroprevalence of WNV infection in camels, horses, domestic birds and febrile humans in some parts of Nigeria (Mishelia, 2014). High prevalence of WNV antibodies was reported in some State in South-western part of Nigeria (Olaleye *et al.*, 1990; Sule *et al.*, 2015). Evidence of WNV infection has also been reported in febrile individuals in South-eastern and North-eastern part of Nigeria (Ezeifeke *et al.*, 1986; Baba *et al.*, 2006). Seroprevalence to WNV infection was reported in domestic birds in Kaduna State, Nigeria (Mishelia, 2014).

1.2 Statement of the Research Problem

Most sicknesses in Africa, especially in Nigeria that present with high temperature are reported as fever of unknown origin, if they do not respond to anti-microbial treatment (Olaleye *et al.*, 1990). The knowledge and experience of medical practitioners and other medical specialists in Nigeria regarding the management of *Arboviral* diseases seem inadequate therefore; ignorance of doctors on *Arboviral* diseases could lead to misdiagnosis and thereby adopt inappropriate treatment (Baba *et al.*, 2006). This speculation is supported by a report that, due to the non-specific nature of WNV infection it often escapes medical attention (Monath *et al.*, 2001).

Since WNV outbreaks in animals precede human WNV cases, the establishment of an active animal health surveillance system to detect new cases in birds and horses is essential in providing early warning for veterinary and human public health authorities (WHO, 2011). The disease outbreak experienced in U.S. some years ago was evidence that the geographical distribution of WNV has expanded and the number of States in the U.S. where infected mosquitoes, birds and animals have been found increased from 4 in 1999 to 26 in 2001 (Senay, 2002). During the recent outbreak of the viral infection, horses were the mammalian species mostly affected in North America with high morbidity and mortality, (Ostlund *et al.*, 2001). Despite the availability of two commercial vaccines to prevent WNV infection and immense public notice on the devastating impact of WNV infection in horses, it still causes widespread morbidity and mortality in California horses, (Minke *et al.*, 2004).

There has been an expanding host range of WNV infection. Infection of the virus has been occasionally reported in reptiles and amphibians including Lake Frog (Jose *et al.*, 2006).

Several species of wild mammals have been reported to be commonly exposed to WNV, occasionally with high seroprevalence rates (Root, 2013). Dead and sick tree squirrels have been reported to be ubiquitous signs of WNV activity in some parts of the United State (Heinz-Thaheny *et al.*, 2004; Kiupel *et al.*, 2003; Padgett *et al.*, 2007).

Culex mosquitoes have been incriminated as the major primary transmission vector; the main mosquito vector of WNV in the western United States that feed on a variety of avian and mammalian species is *Culex tarsalis* (Kilpatrick *et al.*, 2005; Reisen *et al.*, 2005). Other

Culex mosquitoes vectors have shown to be competent for both infection and transmission of WNV includes; *C. quinquefasciatus*, *C. stigmatosoma*, *C. thriambus*, *C. pipiens*, and *C.*

Nigripalpus (Reisen *et al.*, 2006a; Hamer *et al.*, 2008; Vitek *et al.*, 2008). Vector competency studies have indicated that, *Aedes aegypti* is capable of transmitting WNV infection (Ecdc, 2014). The virus has also been isolated from this mosquito species in the field (Turell *et al.*, 2005). It has also been reportedly isolated from hard (ixodid) and soft (argasid) tick species in some parts of Europe, Africa, and Asia (Hoogstraal *et al.*, 1976; Platonov, 2001). However the vector (Ticks) has not been adequately investigated (Lawrie *et al.*, 2004). Non-vector transmission has also been reported among humans by blood transfusion, organ transplantation, transplacental transmission, and through breast milk (Pealer *et al.*, 2003).

Due to poor environmental conditions it is common to keep horses in shaded stables and under trees which serve as favourable locations for mosquitoes to breed. Fumigation of these places is usually rarely carried out in Kaduna State. Moreover, most of the environments have poor drainage system which favours mosquitoes breeding site in the State. Not much is known about WNV in Nigeria especially in Kaduna State. Horse owners and horse groomers in polo farms infested with mosquitoes are at risk of WNV infection and there is no outbreak investigations and surveillance of WNV in Kaduna State. Therefore, there is need to investigate the presence of the virus in horses in Nigeria. It is important to carry out study on the occurrence of the disease which will help to support the differential diagnosis of other mosquito transmitted diseases in Nigeria.

1.3 Justification of the Study

Many febrile illnesses in Nigeria are misdiagnosed as malaria and typhoid, because of absence of surveillance for *arboviral* infections. The study will further highlight the

importance of surveillance in order to bring out the extent of the circulation of the virus in Kaduna State. This will help fill the gap in surveillance for *arboviruses*.

Polo and race tournaments are common events in the State, which bring horses and humans together providing conducive environment for possible disease outbreak like, influenza and WNV infections. This study will provide an insight for public health and fill the gaps in biosecurity measures and zoonotic disease spread associated with horses and human congregations, including the need for more intensified environmental mosquito control. WNV provides a good platform for activating and implementing one health approach in disease control where humans and animals environmental health overlap strongly towards providing both animal and humans epidemics control.

Detecting the virus in mosquitoes is the major evidence needed to establish possibility of animal and human outbreaks or occurrence of undetected outbreaks. This will help convince medical authorities to include WNV infections as part of routine differential diagnosis of febrile illnesses in Nigeria, which will reduce misdiagnosis of the infection and the risk of further transmission of the disease through blood transfusion, organ transplantation in hospitals. Similarly providing evidence of WNV infection in horses and that of contemporary virus circulation will help justify vaccination of prized horses such as, thorough bred race and polo horses against the disease in Nigeria.

1.4 Aim of the Study

To determine the seroprevalence of West Nile virus infection in horses and to detect the virus antigen in mosquitoes in horse stables in Kaduna State, Nigeria.

1.5 Objectives of the Study

1. To determine the seroprevalence of West Nile virus infection in horses in selected LGAs in Kaduna State.
2. To detect West Nile virus antigen in mosquitoes from horse stables in selected LGAs in Kaduna State.
3. To determine demographic management and environmental factors associated with the occurrence of West Nile virus infection in horses in Kaduna State, such as, presence of mosquitoes, trees, grasses, stagnant water, waste bins wild birds.

1.6 Research Questions

1. What proportion of horses has West Nile virus antibodies in selected LGAs in Kaduna State?
2. Are the mosquitoes found in the stables in selected LGAs in Kaduna State infected with West Nile virus?
3. Are there demographic and environmental factors associated with the occurrence of West Nile Virus infection in horses in Kaduna State?

CHAPTER TWO

LITERATURE REVIEW

2.1 Brief History of West Nile Virus

West Nile virus (WNV) was first isolated and identified as a distinct pathogen from the blood of a febrile woman in the West Nile region of Uganda in 1937 (Smithburn *et al.*, 1940). The epidemiology and ecology of WNV was first shown during several outbreaks in the Mediterranean basin in early 1950s and 1960s (Murgue *et al.*, 2001a). The initial recognition of WNV epidemic was in Israel in 1951 in a small town with a total of 123 cases with no fatalities of WNV occurring among the 303 inhabitants (Bernkopf *et al.*, 1953). Several large outbreaks of WNV in Egypt between 1951 and 1954 led to understanding of its ecology, epidemiology, and clinical characteristics (Taylor *et al.*, 1956 and Murgue *et al.*, 2001b). The virus was also found to be infectious in a number of animals, particularly in equines, in which infection was frequently symptomatic and often fatal (James and Sejvar, 2003). Subsequent similar sporadic outbreaks occurred in Russia, Spain, South Africa, and India (Hubalek and Halouzka, 1999). Large outbreaks of WNV were so infrequent all through late 1970s and 1980s. Following the 1996 outbreak in Romania, several subsequent epidemics of the virus associated with relatively high rates of central nervous system (CNS) infection were reported throughout the Middle East and Europe, including Morocco in 1996, Tunisia in 1997 and large outbreaks in Italy and Israel in 1998 (Hubalek and Halouzka, 1999). West Nile virus was first reported in the Western Hemisphere in 1999 during an outbreak of encephalitis cases in New York City, which spread across the continental United States as well as northward into Canada and southward into the Caribbean Islands and Latin America over the next five years of its appearance (Dauphin *et al.*, 2004).

2.2 Aetiology of West Nile Virus

West Nile virus is an Arthropod-borne virus and it's a member of the Japanese encephalitis virus complex within the genus *Flavivirus*, family *Flaviviridae* (Heinz *et al.*, 2000). It is a single-stranded positive sense, enveloped RNA virus (Brinton *et al.*, 1986). It consists of a single open reading frame of approximately 11 kb, with no polyadenylation tail at the 3' end, both the 5' and 3' non-coding regions of the genome form stem-loop structures that aid in replication, transcription, translation, and packaging (Shi *et al.*, 1996; Khromykh *et al.*, 2001; Friebe and Harris, 2010).

2.3 Structure and Protein of West Nile Virus

West Nile virus RNA is translated as a single polyprotein that is post and co-translationally cleaved by both host and viral proteases, resulting in three structural (capsid, envelope, and premembrane) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (Rice, 1996).

The 5' end of the genome encodes the structural proteins, which are necessary for virus entry and fusion as well as encapsidation of the viral genome during assembly (Lindenbach, 2001). NS1 has both a “cellular” form and a secreted form and is highly immunogenic but has no described role in virion assembly, though it has been suggested to play a role in replication (Westaway *et al.*, 2002). NS3 is the viral protease responsible for cleaving other non-structural proteins from the viral polyprotein and encodes enzyme activities, and these functions have been widely characterized (Lindenbach, 2001). The NS5 protein serves as the viral polymerase and encodes a methyltransferase, and it is necessary for viral replication (Lindenbach and Rice, 2003). Several of the non-structural proteins, including

NS2A, NS2B, NS4A, and NS4B, have been shown to inhibit one or more components of the innate immune response against viral infection (Liu *et al.*, 2004, 2006; Munoz-Jordan *et al.*, 2005, Lin *et al.*, 2008).

2.4 Life Cycle of West Nile Virus

The entrance of WNV into the cell is through receptor-mediated endocytosis and DC-SIGNR which is the main receptor for this form of endocytosis (Davis *et al.*, 2006). As the virus matures in the cell, the pH drops and becomes slightly acidic within the envelope and as the endosome become mature, the envelope protein changes conformation where the endocytic membrane fuse with the viral lipid membrane, which causes the release of the viral RNA genome into the cell cytoplasm (Modis *et al.*, 2004). This leads to the replication and translation of the single RNA into proteins in the cell. The viral RNA is replicated by cellular and viral proteins (Brinton, 2013). The expressions of the three structural proteins from the viral RNA assemble onto the membranes in the endoplasmic reticulum and bud into the cytoplasm by the Golgi apparatus. The virus leaves the cell in a lipid envelope via exocytosis, when the cellular enzymes cleave the prM (Lindenbach and Rice, 2001).

2.5 Epidemiology of West Nile Virus Infection

West Nile virus (WNV) is one of the most widely distributed of all *arboviruses* with an extensive distribution throughout Africa, the Middle East, parts of Europe, South Asia, and Australia (Gubler, 2007). Since the first discovery of WNV, infrequent human outbreaks were mostly reported in groups of soldiers, children, and healthy adults in Israel and Africa (Petersen and Reohrig, 2001; Jupp, 2001). These outbreaks were associated with minor illness among many patients, and some fatal cases were associated with increase in age. In

one of the largest outbreaks reported, thousands of self-limited and relatively mild clinical cases, consisting of fever, rash, and polyarthralgias occurred in South Africa, resulting in an epidemic attack rate of 55 percent (McIntosh *et al.*, 1970). WNV infection has been reported from human in India (Balakrishnan *et al.*, 2013; Chowdhury *et al.*, 2014), the outbreak of the virus in Europe and the Middle East in 1995 appeared to have caused infection in less than 5% of affected population (Campbell *et al.*, 2001; Dauphin *et al.*, 2004).

In recent years human WNV disease in the Eastern Hemisphere has been reported mostly from areas in the Mediterranean Basin, such as in Algeria in 1994, Morocco and Romania in 1996, through 2000, the Czech Republic in 1997, Tunisia in 1997 and 2003, Israel in 1999 and 2000, Russia in 1999 through 2001, and France in 2003 (Dauphin *et al.*, 2004; Zeller and Schuffenecker, 2005). The first domestically acquired human cases of WNV disease in the Western Hemisphere were detected in New York City in 1999 (Nash *et al.*, 2001). WNV rapidly spread during the following years, and by 2005 the virus had established sustained transmission foci in the hemisphere with an overall distribution that extended from central Canada to Southern Argentina (Gubler, 2007). The transmission of WNV persists across this large, ecologically diverse expanse and as a result this virus is recognized as the most widely distributed *arbovirus* in the world (Kramer *et al.*, 2008). Since 1999, WNV has spread across almost all of North America, resulting in the deaths of more than 450 people and tens of thousands of birds, horses, and other animals (CDC, 2013).

In Northern Africa, WN outbreak was described in horses of Kenitra and Larache provinces in Morocco. The infection affected 94 animals and 42 of them died (Tber, 1996).

In 2010, the World Organisation for Animal Health confirmed 200 equine cases, and it was noticed that equine morbidity started three weeks earlier than humans in Europe (Paz *et al.*, 2013a). Between September and December 1997 in Tunisia, 173 patients were hospitalized with symptoms of meningitis and meningo-encephalitis in the districts of Sfax and Mahdia (Murgue *et al.*, 2001a; Triki *et al.*, 2001). In 2003 in Morocco, in the same area where the 1996 outbreak occurred, 9 cases of encephalitis, with 5 deaths, were reported in horses (Schuffenecker *et al.*, 2005). In 2004, seropositive equines were reported in northern Columbia, which was the first evidence of WNV activity in South America (Mattar *et al.*, 2005). Also in Ukraine during 2010 and 2011, 310 serum samples from apparently healthy horses were examined for WNV and 13.5% were seropositive (Ute *et al.*, 2013). The first outbreak of West Nile virus (WNV) infection in Italy was reported in 1998 among horses residing in Tuscany region (Autorino *et al.*, 2002). There was a re-emergence of the virus in Italy in 2008, when equine and human cases of West Nile neuroinvasive disease (WNND) were confirmed in Veneto and Emilia Romagna Regions (Rossini *et al.*, 2008; Barzon *et al.*, 2009).

The virus was recently reported and indicated on-going transmission in sub-Saharan Africa where seroprevalence was reportedly high in Chad (97%) and Senegal (92%) (Cabre *et al.*, 2006). Eleven cases of acute febrile illness were caused by WNV in Guinea in 2006 (Jentes *et al.*, 2010). In 2009 a seroprevalence study in humans in Ghana indicated that WNV is endemic, with most humans WNV cases occurring in childhood (Wang *et al.*, 2009). Sero-conversion of sentinel chickens was observed in Senegal in 2009 (Fall *et al.*, 2013). In Eastern Africa, human infections and mosquitoes positive for WNV lineage 2 were reported in Djibouti from 2010-2011 (Faulde *et al.*, 2012). Recent positivity for WNV

in Kenya has also been reported in ticks, collected from 2010-2012 and mosquitoes from 2007–2011 (Lwande *et al.*, 2013; Ochieng *et al.*, 2013).

A study in Nigeria demonstrated that 25% of tested febrile patients, many of whom were infected with *Plasmodium falciparum* or *Salmonella typhi*, were seropositive for WNV, suggesting that WNV infection in this region may be mistaken for these diagnoses or for other co-circulating *arboviruses* (Baba *et al.*, 2006). Another study in Nigeria also demonstrated the prevalence of WNV antibodies in apparently healthy humans and polo horses in some States in Nigeria (Ezeifeke *et al.*, 1986). However seropositivity to the virus was also reported in humans and different animals such as, camels, sheep, goats and cattle in Ibadan and Maiduguri (Olaleye *et al.*, 1990). West Nile virus antibody was also reported in domesticated birds in Kaduna State metropolis in Nigeria (Mshelia, 2014). High seroprevalence of WNV antibodies in horses was observed in South-western region of Nigeria (Sule *et al.*, 2015).

2.6 Transmission of West Nile Virus

West Nile Virus (WNV) is transmitted mainly through an enzootic cycle, involving birds as main amplifying hosts and ornithophilic mosquitoes of the genus *Culex* as main vectors (Komar *et al.*, 2003). The virus is transmitted through female mosquitoes, which are the primary vectors of WNV (Hayes *et al.*, 2005a). The species of mosquito that are most frequently infected with WNV feed primarily on birds (Kilpatrick, 2011). Some species of birds develop sufficient viraemia levels to WNV (Kilpatrick *et al.*, 2007). Thereafter, the infected birds transmit the virus to biting mosquitoes which in turn infect other birds (Komar *et al.*, 2003).

In mammals and some species of birds, the virus does not multiply to develop high viral load during infection. Mosquitoes that bite these infected mammals do not ingest sufficient virus to become infected; thus, they are called dead-end hosts (Kilpatrick *et al.*, 2007). Direct human-to-human transmission may be caused by occupational exposure, such as in laboratory setting (CDC, 2002b) and also conjunctiva exposure to infected blood (Fonseca *et al.*, 2005). Outbreaks in USA identified other route of transmission of WNV, which includes blood transfusion, organ transplantation, intrauterine exposure and breast feeding (CDC, 2002c). From 2003, blood banks in the United States have been undergoing routine screening for the virus among blood donors (CDC, 2003).

Mosquito saliva was recently demonstrated to affect the course of WNV disease (Styer *et al.*, 2006; Schneider *et al.*, 2006, 2007). Mosquitoes deposit their saliva into the skin during blood meal and secrete molecules which contain principal proteins that modulate vascular constriction, blood coagulation, platelet aggregation, inflammation, and immunity. The molecules clearly alter the immune response in such a way that is advantageous to the virus (Zeidner *et al.*, 1999; Limesand *et al.*, 2003; Wasserman *et al.*, 2004; Wanasen *et al.*, 2004). It has been reported that the virus can specifically modulate the immune response during early viral infection (Schneider *et al.*, 2004); and mosquito feeding can exacerbate WNV infection, leading to higher viraemia and more severe forms of the disease (Styer *et al.*, 2006; Schneider *et al.*, 2006, 2007).

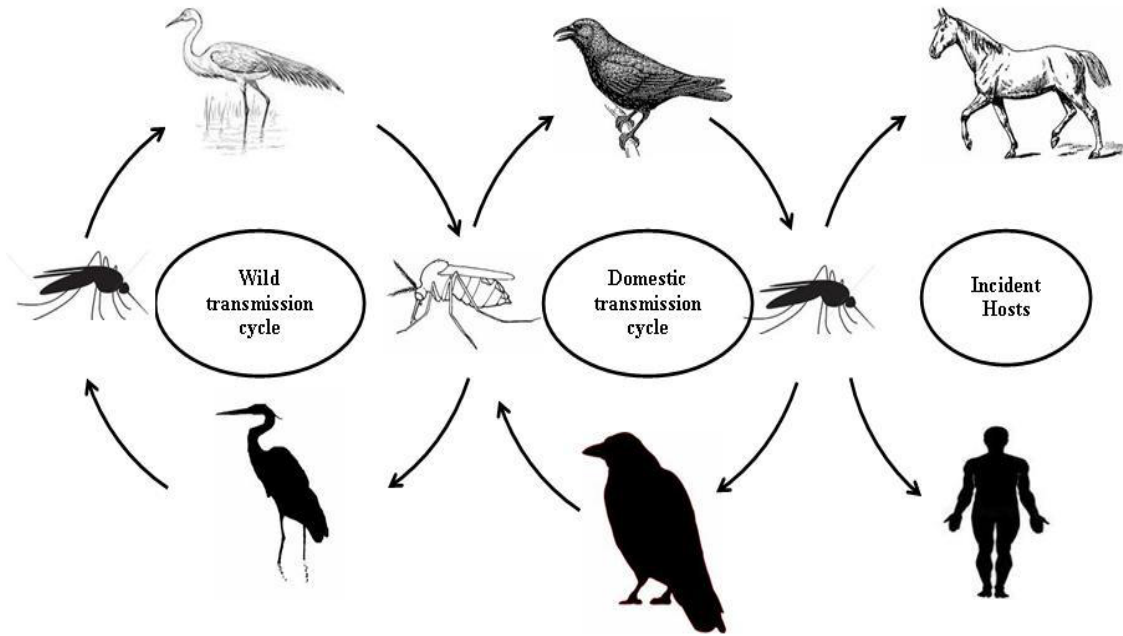


Figure 2.1. Schematic Diagram of West Nile Virus Transmission Cycle (Gulati *et al.*, 2014).

2.7 The Vector of West Nile Virus

Mosquitoes are the major vector responsible for natural transmission of WNV. After a mosquito feeds on a blood meal from an infected competent host, the virus multiplies within the mosquito, which it then transmits to a susceptible host through salivary gland secretions; WNV can be transmitted by a variety of mosquitoes with different host-feeding preferences (CDC, 2009). Mosquitoes that feed on both birds and mammals are referred to as bridge vectors for WNV because they act as a “bridge” between an infected reservoir (birds) and mammalian incidental hosts, which do not develop sufficient high level of viraemia capable of transmitting the Arbovirus to Mosquitoes (Turell *et al.*, 2002; Kilpatrick *et al.*, 2005). Ornithophilic mosquitoes play an important role in maintaining and amplifying transmission among birds, but typically, they do not play a role in transmission to humans (Turell *et al.*, 2002, 2005).

Experimental transmission was first documented in *Aedes albopictus* in 1942 (Hayes, 1989). Also Experimental trials in conjunction with field studies from 1952-1954 in the Sindbis Sanitary District of Egypt also demonstrated that mosquitoes were the vectors of WNV. Isolates were obtained during this study from *Culex antennatus*, *Culex univittatus*, and *Culex pipiens*. In Egypt, Israel, and South Africa, *Culex univittatus* has been implicated as the primary WNV vector, reported based on field isolation rates (Hayes, 1989). WNV was isolated in Africa from *Culex poicilipes* in Senegal, and *Aedes albocephalus* in Madagascar (Hubalek and Halouzka, 1999). In south-western Asia, the members of the *Culex vishnui* complex, including *Culex vishnui*, *Culex triaeniorhynchus*, and *Culex pseudovishnui* are the main vectors for WNV based on studies in Pakistan and India (Hayes, 1989). *Culex modestus* and *Culex pipiens* were confirmed as a WNV vector in

Europe during the 1996 WNV epidemic in Romania (Savage *et al.*, 1999; Campbell *et al.*, 2001).

Mosquitoes of the genus *Culex* have been reported as the most important bridge vectors in the United States, with *Culex pipiens* as the dominant bridge vector in the North-Eastern, North Central, and Mid-Atlantic United States, *Culex Quinquefasciatus* are seen in the South and South-West, and *Culex tarsalis* are found in the West (Kilpatrick *et al.*, 2005; Andreadis., 2012). *Culex spp* of mosquitoes have also been implicated in transmission of the virus in Europe, Australia, and South Africa (Balenghien *et al.*, 2008; Muñoz *et al.*, 2012). Mosquitoes of the genus *Aedes*, are the transmission vector for related *flaviviruses*, and the genus also serve as an important bridge vectors (Turell *et al.*, 2005; CDC, 2009).

Experimental transmission of WNV by ticks has been demonstrated, but a role for ticks in natural transmission and maintenance of WNV has not been determined (Abbassy *et al.*, 1993; Lwande *et al.*, 2013).

2.8 Clinical Signs of West Nile Virus Disease

2.8.1 Clinical signs of West Nile virus disease in man

About 80% of WNV infections in humans are asymptomatic (Petersen and Marfin, 2002a). West Nile fever presents as a minor influenza-like illness characterised by sudden onset of moderate-to-high fever that lasts for about 3 to 5 days with a maculo-papular or roseolar rash (Ferguson *et al.*, 2005). The rash appears in approximately 50% of WNV cases, spreading from the trunk to the extremities and head. Lymphadenopathy, anorexia, nausea, abdominal pain, diarrhoea, myositis, orchitis and respiratory symptoms are also observed (Smith *et al.*, 2004). Hepato-splenomegaly, hepatitis, pancreatitis, myocarditis (Pergam *et al.*, 2006) and haemorrhagic fever have been reported to be infrequent (Paddock *et al.*,

2006). Occasionally (< 15% of cases), acute aseptic meningitis or encephalitis occurs (Roos, 2004). Flaccid paralysis can accompany other symptoms generally without sensory loss (Sejvar *et al.*, 2005). However, there have been reported cases of axonal polyneuropathy, in which both sensory and motor neurones are apparently affected (Holman *et al.*, 2004). One of the major features of WNV encephalitis is uncoordinated movement; some of these abnormalities are characterized as Parkinsonism (Robinson *et al.*, 2003). Myalgia, confusion and light-headedness may persist beyond a year, and prolonged depression persists in about 31% of patients with the virus (Murray *et al.*, 2007).

2.8.2 Clinical signs of West Nile virus disease in animals

The disease in Equine present approximately 10% of neurological disorders following infection apart from fever of (>38.5°C), clinical signs of WNV in horses almost exclusively reflect lesions of the central nervous system (CNS) which include, ataxia in hind-limbs or fore-limbs, abnormal behaviour, paresis, paralysis, muscular tremors, myoclonia, lethargy and cranial nerve deficits (Murgue *et al.*, 2001b). Mortality rates among clinically affected horses have been reported to be approximately 40% to 50% (Murgue *et al.*, 2001b). The major clinical signs are those of infectious meningo-encephalomyelitis, but they appear in only 40% - 48% of infected animals respectively (Joubert *et al.*, 1971; Bunning *et al.*, 2002). Viraemia levels in infected horses are too low to infect vectors efficiently; this shows that horses are unlikely to serve as amplifying hosts for WNV (Bunning *et al.*, 2002).

In dogs encephalitis and myocarditis have also been reported (Lichtensteiger *et al.*, 2003). Mice and other laboratory rodents are the most thoroughly studied and are well characterised models for experimental studies. Infected animal models with the virus

develop encephalitis, showing many similarities to the human disease. However, the histological events that occur during infection, especially in peripheral tissues, have not been fully demonstrated (Kimura *et al.*, 2010). Apart from seropositivity, there is limited information on the occurrence of WNV in sheep (Juricová *et al.*, 1986). A case of a 4-year-old ewe with WNV infection and neurological symptoms has been described in Hungary (Kecskeméti *et al.*, 2007).

The disease in birds shows no symptoms in some species of birds; but the general signs of West Nile infection include lethargy, recumbency and in some cases, haemorrhage (Komar *et al.*, 2003).

Reptiles are also susceptible to the infection and clinical signs observed in alligators are anorexia, lethargy, intention tremors, swimming on their sides, spinning in the water, and opisthotonus (Jacobson *et al.*, 2005). Death occurs 24 - 48 hours after the onset of clinical signs (Bakonyi *et al.*, 2006).

2.9 Pathogenesis of West Nile Virus

The WNV replicates at the site of mosquito inoculation and then spread to lymph nodes and the bloodstream (Diamond *et al.*, 2003). Viral penetration of the central nervous system follow stimulation of toll-like receptors and produce high levels of tumour necrosis factor- α , which increases permeability of the blood-brain barrier (Wang *et al.*, 2004b). The WNV directly infects neurones, particularly in deep nuclei and gray matter of the brain, brainstem, and spinal cord (Kleinschmidt-DeMasters *et al.*, 2004; Ceccaldi *et al.*, 2004). Collateral destruction of by-stander nerve cells could contribute to paralysis (Darman *et al.*, 2004). Immune-mediated tissue damage could also contribute to pathologic changes in some cases (Leis and stokic, 2005). Genetic susceptibility for severe disease in mice has

been postulated to involve inadequacy in production of 2'-5'-oligoadenylate synthetase, but its genetic susceptibility has not been explicated in humans (Ceccaldi *et al.*, 2004). Most non-fatal WNV infections appear to be cleared by the host immune response, but the virus may persist in some vertebrate hosts (Kuno., *et al* 2001; Ceccaldi *et al.*, 2004).0

2.9.1 Pathologic changes in West Nile virus infection

Histological findings of WNV encephalitis include perivascular inflammation, microglial nodules, variable necrosis, and loss of neurones (Kleinschmidt-DeMasters *et al.*, 2004; Guarner *et al.*, 2004). The deep gray nuclei, brainstem, and spinal cord are the most affected organs (Kleinschmidt-DeMasters *et al.*, 2004; Guarner *et al.*, 2004). Patients with flaccid paralysis have perivascular lymphocytic infiltration in the spinal cord and also microglial nodules, and loss of anterior horn cells (Guarner *et al.*, 2004). Inflammation of the spinal cord was reported in 17 of 23 people who died with WNV neuroinvasive disease, inflammation was more pronounced in the anterior horns than in the posterior horns of 9 patients (Guarner *et al.*, 2004). Endoneural mononuclear inflammation of cranial nerve roots and spinal nerves may occur in a small percentage of individuals, infected with the virus. Foci of demyelination, gliosis, and occasional perivascular infiltrates may be found in persons with prolonged clinical cases. Viral antigens are usually found within neurones and neuronal processes, predominantly in the brain-stem and anterior horns. In general, antigens are focal and sparse, except in immunosuppressed patients of which, they can be seen extensively throughout the central nervous system (Guarner *et al.*, 2004).

2.10 Diagnosis of West Nile Virus Infection

Diagnosis of WNV infection can be carried out by viral isolation methods, viral antigen detection methods and molecular detection of the virus.

2.10.1 Serological detection of West Nile virus

Antibody can be identified in equine serum by using IgM capture enzyme-linked immunosorbent assay (IgM capture ELISA), Haemagglutination inhibition (HI), IgG ELISA, Plaque reduction neutralisation (PRN), and Microtitre virus Neutralization test (VN) (Beaty *et al.*, 1989; Hayes., 1989). Serological testing still remains the primary method of identifying WNV infection which is mainly based on anti-E antibody detection. However, cross reactivity of the antibody response with related Flaviviruses limits the specificity of serological tests. Plaque reduction neutralisation test (PRNT) is the gold standard serological test for WNV detection (Dauphin and Zientara, 2007). It is used as a confirmatory method to detect WNV specific neutralising antibodies from serum or cerebrospinal fluid.

The HI test is used for seroprevalence study of WNV antibodies in a population. Demonstration of four fold rise or drop of antibody titre in paired serum samples confirm the infection; but HI cannot differentiate between closely-related *Flaviviruses* and thereafter, cannot be used as a confirmatory diagnostic method. Various enzyme-linked immunosorbent assays (ELISA) has been developed for WNV (Blitvich *et al.*, 2003a; Johnson *et al.*, 2003; Long *et al.*, 2006; Choi *et al.*, 2007; Kitai *et al.*, 2007).

ELISA tests are rapid, reproducible and less expensive (De Filette *et al.*, 2012). MAC-ELISA enables the detection of acute infections, since it detects early IgM antibodies in

serum or cerebrospinal fluid. The IgM antibodies can identify WNV within 4 to 7 days after initial exposure, and may persist for more than one year (Roehrig *et al.*, 2003; Rossi *et al.*, 2010).

2.10.2 Molecular detection of West Nile virus

Reverse transcription polymerase chain reaction (RT-PCR), Real-time PCR and Multiplex reverse transcription PCR ligase detection reaction can be used for the detection of WNV (Johnson *et al.*, 2001; Shi and Kramer, 2003; Linke *et al.*, 2007; Rondini *et al.*, 2008). SYBR Green-based assay has been developed to detect 100% of different WNV target region variants (Papin *et al.*, 2004), but this SYBR-based assay has lower sensitivity as compared to WNV specific TaqMan RT-PCR assays (Johnson *et al.*, 2010). Other alternatives to SYBR Green-based assay are TaqMan and molecular beacons, both of which use hybridisation probes and rely on fluorescence resonance energy transfer (FRET) for quantification (Lanciotti *et al.*, 2000; Jimenez-Clavero *et al.*, 2006).

A loop-mediated isothermal amplification (LAMP) assay was developed by Parida *et al.*, in 2004 for WNV, which is relatively inexpensive. During the year 2010 to 2011, in Indian, conventional RT-PCR, real-time RT-PCR and RT-LAMP assays were used to detect WNV, associated with multifocal retinitis in patients from southern India (Shukla *et al.*, 2012).

2.10.3 Antigen detection and West Nile virus isolation

Antigen detection is the method of choice for detection of WNV in vertebrates, mosquito pools and avian samples. The virus can be isolated from cerebrospinal fluid, blood or brain tissues in cell cultures Vero/BHK 21/RK-13/mosquito (AP61) cell lines (Castillo-Olivares

and Wood, 2004). Antigen capture ELISA has been developed for the detection of WNV using NS-1 (Macdonald *et al.*, 2005; Chung and Diamond, 2008). A membrane-based electrochemical nanobiosensor has been developed for the detection of WNV that recognises viral particles or virus E protein during the early stage of infection (Nguyen *et al.*, 2009). In India, WNV has been isolated by intra-cerebral inoculation in 3 days old infant mice and inoculation in BHK-21 and Vero cell lines (Bondre *et al.*, 2007; Balakrishnan *et al.*, 2013; Chowdhury *et al.*, 2014).

The VecTest[®] is an antigen panel assay designed by Medical Analysis Systems to detect WNV, SLE and Eastern equine encephalitis. The test uses a detection dipstick, coated with specific antibodies, and the procedure is fast, but less sensitive as compared to plaque assay in Vero cells or RT-PCR (Ryan *et al.*, 2003).

2.11 Treatment and Vaccination against West Nile Virus Infection

Supportive treatments are the only available treatments for WNV related infections. Ribavirin, interferon- α or WNV specific antibodies have been considered as possible treatments, but none of them has been assessed in controlled clinical trials (Hayes *et al.*, 2005b; Oliphant *et al.*, 2005). Vaccination is one of the control methods of reducing the risk of WNV infection. Presently, there are no commercially available vaccines for WNV in humans (Iyer and Kousoulas, 2013). There are good and effective licensed WNV vaccines for horses (De Filette *et al.*, 2012). A vaccine under the trade name West Nile-Innovator[®], which is formalin-inactivated whole West Nile virus, was developed by Fort Dodge Animal Health (subsidiary of Pfizer) (Ng *et al.*, 2003). This vaccine is currently commercialised in the USA, and is quite effective. Another killed virus vaccine (Vetera[®] WNV vaccine), made by Boehringer Ingelheim Vetmedica is also licensed by the United

States Department of Agriculture (USDA). A third commercialised WNV vaccine in the United States for horses is Recombitek® Equine West Nile Virus Vaccine by (Merial, now Sanofi Aventis), which is a chimeric recombinant canarypoxvirus vaccine (El Garch *et al.*, 2008).

2.12 Prevention and Control of West Nile Virus Disease

Protection against WNV infected mosquitoes remains the fundamental means for preventing WNV disease; application of larvicides and targeted spraying of pesticides can reduce greater numbers of adult mosquitoes (Ruiz *et al.*, 2004). People in WNV endemic areas should wear insect repellent on skin and clothes, when exposed to mosquitoes, and avoid being outdoors during dusk to dawn when mosquitoes are in abundant. The insect repellents recommended for use on skin are those containing *N,N*-diethyl-m-toluamide (DEET), picaridin (KBR-3023) or oil of lemon eucalyptus (*p*-menthane-3,8 diol). They provide long-lasting protection (Barnard and Xue, 2004). DEET and permethrin are effective protection against mosquitoes, when applied to clothing (Herrington, 2003).

In other to prevention WNV infection through blood transmission by transfusion, blood donations in WNV endemic areas, should be screened by using nucleic acid amplification tests. Screening of organ donors for WNV infection has not been universally implemented because of concern about rejecting essential organs after false-positive screening results (Kiberd and Forward, 2004). Pregnant women should avoid exposure to mosquito bites to reduce the risk for intrauterine WNV transmission (O'Leary *et al.*, 2006).

2.12.1 Vector control

West Nile virus vector management and control includes:-

2.12.1.1 Cultural control

Personal protection, reduction of larval habitats, and chemical control are the best ways to reduce mosquito bites and therefore preventing the transmission of mosquito-borne pathogens, such as WNV, dengue fever and JEV. Most *Culex spp* feed at night, so long-sleeve shirts and insect repellents are recommended for use during night-time activity (Mosquito Information Website, 2009). It is essential to reduce or eliminate man-made aquatic environment around homes. This can be done by reducing watering plants, frequent changing of water in pet water dishes, removal of unnecessary water-holding containers, and keeping ponds stocked with mosquito fish (Kern, 2007). Water-holding containers that cannot be removed can be covered or turned upside down, old tires need to be removed, and drainage ditches need to be kept clear of debris that will obstruct water flow (Mosquito Information Website, 2009).

Large man-made aquatic habitats such as storm-water catch basins and waste water containers should be eliminated or reduced (O'Meara, 2014).

2.12.1.2 Chemical control

Insecticides can be used to control larvae and adults mosquitoes; Larvicides are applied to water bodies near where mosquito larvae are concentrated. This method can reduce large numbers of immature mosquitoes with small amount of pesticide; Adulticides are used to quickly reduce the population of adult mosquitoes in an infested environment, but mosquito resistance to specific insecticides may reduce the effectiveness of chemical control. Some chemicals require a licensed pesticide applicator to perform the application. (AMCA, 2005).

2.13 Risk Factors of West Nile Virus

The most important risk factor for acquiring WNV infection is through exposure to infected mosquitoes (Hayes *et al.*, 2005a). Climatic conditions, specifically ambient temperature and rainfall, are responsible for the abundance of mosquitoes and amplification of WNV (Kunkel *et al.*, 2006; Manore *et al.*, 2014). Also warmer temperatures could allow expansion of mosquitoes distribution and accelerate the mosquito life cycle, biting rates, and rate of WN viral replication; warming temperatures associated with climate change could increase the risk of WNV disease in humans (USGCRP, 2016).

Studies on *Culex pipiens* have found a direct correlation between increases in ambient temperature and increases in vector populations (Paz *et al.*, 2003). Increase in temperatures also affects the rate of virus replication, leading to rapid infectivity of the virus by mosquitoes (Kilpatrick *et al.*, 2008). Precipitation such as stagnant water is another key component to promoting vector abundance by providing ample breeding environments for mosquitoes (Takeda *et al.*, 2003). Although heavy rain and flood can reduce mosquito breeding site by flushing out their preferred stagnant water sources (Koenraadt and Harrington, 2008) but drought conditions can promote WNV transmission by forcing birds and mosquitoes to share the same habitat by competition for scarcity of water source (Roehr, 2012; Murray *et al.*, 2013).

People at old age from 60–89 years of age are at highest risk for developing meningo-encephalitis (Petersen *et al.*, 2002b; O'Leary *et al.*, 2004; Hayes *et al.*, 2005b). Immunosuppressed patients of transplanted organs have an increased risk of developing neuroinvasive diseases (DeSalvo *et al.*, 2004; Kumar *et al.*, 2004). And when

disease develops it is often more severe than immune-competent individuals (Kleinschmidt-DeMasters *et al.*, 2004). Diabetes, hypertension and cerebro-vascular disease have also been considered as possible risk factors (Tyler, 2004).

Abiotic factors, such as temperature and precipitation, with biotic factor, such as type of habitat are important factors that determine the distribution of vectors and vector-borne diseases in the environment (Reisen, 1995; Reisen *et al.*, 2006a; Reisen *et al.*, 2008; Chuang *et al.*, 2011). Changes in climatic conditions affect vector-borne diseases indirectly through its effects on habitat, vegetation and host community composition (Lafferty, 2009; Ostfeld, 2009).

Ecological factors also affect seasonal and spatial overlap among the primary hosts in the sylvatic amplification cycles. For example drought-induced concentration of mosquitoes and birds on shrinking wetland habitats which may enhance the transmission of WNV (Wang *et al.*, 2010). Also the abundance of primary competent vectors is usually an indicator for the risks of vector-borne diseases (Reeves, 1965; Saugstad *et al.*, 1972; Murray, 1995). However vector abundance of *Cx. tarsalis*, *Cx. quinquefasciatus*, *Cx. pipiens* and *Cx. restuans* has been reported as an indicator used to predict the risk of humans infection to WNV in different regions (Kilpatrick *et al.*, 2005; Reisen *et al.*, 2009; Kwan *et al.*, 2010).

2.14 West Nile Virus Disease Surveillance

The aim of disease surveillance for safety of public health purposes is to monitor existing epidemiological situation and predicting the likelihood of human disease outbreaks and, thus permitting interventions for such outbreaks (Eldridge, 1987).

2.14.1 Mosquitoes surveillance

Mosquito vector testing for human pathogenic viruses such as West Nile virus is a major feature of successful surveillance approaches and an early predictor of human epidemics (Wheeler *et al.*, 2016). Vector-borne disease surveillance programs primarily rely on techniques such as virus isolation, standard RT-PCR, and RT-qPCR to detect the presence of vector-borne viruses in targeted mosquito vectors and they are the gold standard for Arbovirus disease surveillance. RT-qPCR is the most sensitive assay for detecting vector-borne viruses, but the technique generally requires expensive equipment, and high quality RNA purified from the mosquito samples. Loop-mediated isothermal amplification (LAMP) is an isothermal nucleic acid amplification technique that is a useful alternative to PCR for pathogen detection and diagnostics (Nagamine *et al.*, 2002; Polley *et al.*; 2013).

Entomological surveillance for WNV was carried out in some region in Italy, from May through November placing CDC-CO₂ traps in five provinces of the region and mosquitoes were collected fortnightly, identified and pooled in up to 50 specimens and tested using a One-Step SYBR Green-based Reverse Transcriptase-Real-Time PCR (Ravagnan *et al.*, 2011). Pools of *Culex pipiens* infected with WNV were identified in 4 of the 7 provinces of the region surveyed (Federico *et al.*, 2014)

2.14.2 Human surveillance

Epidemiological surveillance measures of WNV in human quantify disease burden and identify seasonal, geographic, and demographic patterns in human morbidity and mortality (Lindsey *et al.*, 2008; CDC, 2010). This has also detected and quantified alternative routes

of WNV transmission to humans through contaminated blood donations and organ transplantation (Pealer *et al.*, 2003; Nett *et al.*, 2012). The blood supply in U.S. since 2003 has been routinely screened for WNV RNA, which has made blood transfusion-associated with WNV infection rarely, seen (CDC, 2003). The Food and Drug Administration recommends and ensure that blood collection agencies to perform WNV nucleic acid amplification test (NAAT) throughout the year on all blood donations, Organ and tissue donors are usually not screened for WNV infection (Nett *et al.*, 2012). WNV specific antibody may be absent in suspected WNV disease cases in immune-compromised individuals due to prolong viremia level observed in this patients. In this case testing serum and CSF samples could be useful for detection of the viral RNA (CDC, 2003).

The types of surveillance system used for monitoring WNV infection in human includes; Enhanced passive surveillance which involved collection of information from hospitalized cases of encephalitis and individuals with IgM antibody to WNV in test carried out in diagnostic or reference laboratory (CDC, 2003).

Active surveillance is strongly considered in WNV endemic areas. The active surveillance system approach should base on contacting physicians in different areas of specializations such as infectious disease specialist, neurologist, and critical care specialist, Also implementation of laboratory based surveillance for CSF specimen identification and making enquiry about patients with *arboviral* infection from hospital infection control personnel. Other human surveillance system for monitoring WNV infections includes; Special surveillance project, surveillance case definition and Minimal components of human surveillance system (CDC, 2003).

2.14.3 Equine surveillance

WNV surveillance in horse population involves both human and animal public health purpose and horses are good sentinels for WNV circulation in a geographical area and also an indicator of possible transmission to human. In monitoring WNV infection in equine in a geographical area, passive and active surveillance should be put in place (Ecdc, 2013).

Active surveillance for monitoring equine WNV infection should target clinical surveillance on horses in various farms and stables and also serological surveillance can be carried out using sentinel horses in selected geographical locations in country that is endemic to WNV disease in horses and humans. Clinical examinations of horses can be conducted and available information can be gathered from the farmers, horse grooms and attending veterinarians with relevant laboratory results. Serological surveillance can be used to detect specific-WNV IgM antibodies in equines to confirm recent WNV circulation in a geographical area (Ecdc, 2013).

Passive surveillance for WNV in equine should include investigation of horse cases with fever and neurological signs in an endemic area. Laboratory test should be conducted for equine samples using RT-PCR, cELISA, Mac-ELISA for confirmation of the virus in an area with circulating WNV infection (Andriani *et al.*, 2013).

2.14.4 Birds surveillance

The surveillance of the disease in dead birds has served as a key method for tracking WNV activity in the U.S. (Eidson *et al.*, 2001a; Watson *et al.*, 2004). Different types of laboratory diagnostic procedures may be used for serologic and virologic diagnosis of WNV (Stone *et*

al., 2004; Trevej and Eidson, 2008). Sightings of dead crow are very useful for surveillance when immediately reported, could provide a more immediate indicator of WNV activity than WNV positive birds. However, more rapid laboratory methods are in use in many areas, such as VecTest and RAMP, which can be used to provide a laboratory result from swabs taken where the bird is found (Stone *et al.*, 2005). Reports have indicated that WNV is sometimes first detected in an area in a bird species other than a corvid species (Eidson *et al.*, 2001b)

2.14.5 Environmental surveillance

Epidemiological and environmental surveillance for WNV was facilitated by development and implementation of ArboNET, the national *Arbovirus* surveillance system (Lindsey *et al.*, 2012). The main objective of WNV environmental surveillance is to quantify the intensity of transmission of the virus in a region in order to provide a predictive index of human infection risk. Risk prediction on information about local conditions and habitats that aid WNV vector can be used to inform an integrated vector management program and the associated decisions about implementing prevention and control interventions (Nasci, 2013).

Outbreak of WNV infection can quickly develop with lots of human cases occurring over a few weeks during the peak of transmission (CDC, 2010). Conditions associated with increasing human risk can be detected 2-4 weeks in advance of human disease onset (Kwan *et al.*, 2012). Enhanced surveillance for human disease cases should be considered, particularly when environmental or human surveillance suggests that an outbreak is suspected or anticipated. Educating healthcare providers and infection control nurses about

the need for *arbovirus* testing, and reporting of all suspected cases could increase the sensitivity of the surveillance system (Nasci, 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

This study was conducted in Kaduna State, which is located in North West Zone of Nigeria, between Longitude E006.5⁰ – E008.6⁰ and Latitude N09.2⁰ – N11.3⁰. The State shares boundaries with Niger State to the West, Zamfara, Katsina and Kano States to the North, Bauchi and Plateau States to the East, FCT and Nasarawa State to the South. The state occupies an area of about 46,063 square kilometers, (Jallo, 2000). Kaduna State has an estimated human population of 5,200,000, (KDSG, 2007).

Kaduna State has a total amount of annual rainfall varying between 1,000mm and 1,500mm and a rainy season, which is between 120 and 150 days long. The Northern part of the State is semi-arid, further south as the rain level increase, the climate becomes sub-humid. The extreme South of the State is marked by a series of rocky hills, which are responsible for the island of rainfall, (National Livestock Report, 1992). The State is essentially an agrarian society with about 75% engaging in farming and it also has potentials for livestock industry.

The State has a strong traditional institution with Emirs in Zaria and other major towns that keep horses. Also there are presence of military, police and polo horses in Kaduna State.

This study was carried out in some selected Local Government area of Kaduna State, namely Sabon Gari, Zaria, Igabi and Kaduna North Local Government Areas.

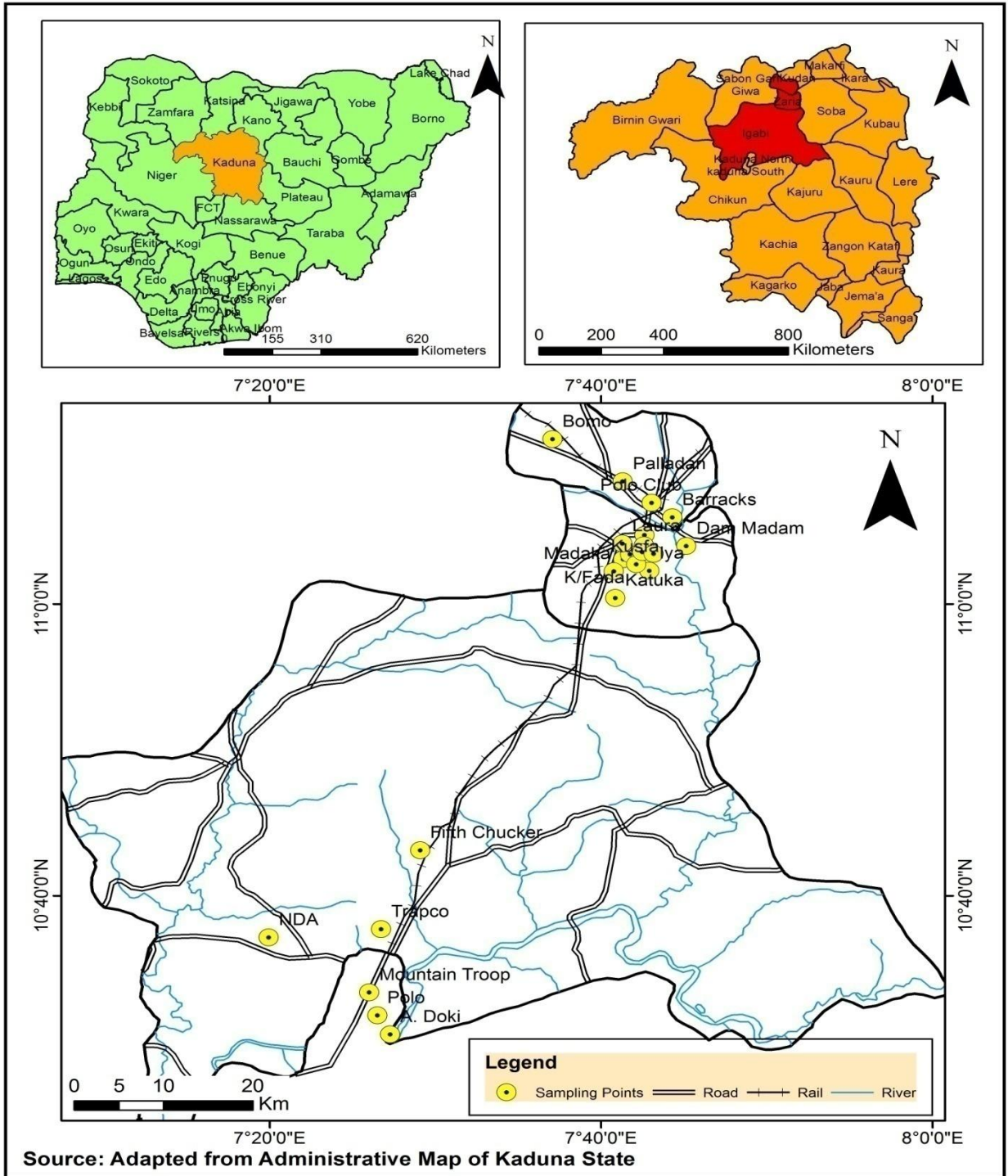


Figure 3.1: Map of Kaduna State, Nigeria.

3.2 Study Design and Sample Size

This study was conducted as a cross-sectional study of horses and mosquitoes associated with horses in stables.

The sample size was determined according to the formula described by Thrustfield (2005).

$$\text{Where, } N = \frac{Z^2 pq}{d^2}$$

N = Sample size

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

P = Prevalence (90.3% Sule *et al.*, 2015)

D = Desired precision (0.05)

$$\text{Where } q = 1 - P = (1 - 0.903) = 0.097$$

$$\frac{(1.96)^2 \times 0.903 \times 0.097}{(0.05)^2}$$

$$3.8416 \times 0.903 \times 0.097$$

$$\frac{3.8416 \times 0.903 \times 0.097}{0.0025}$$

$$0.0025$$

$$= 134.6$$

Eventually 368 Samples from horses were collected from the selected LGAs of Kaduna State, in order to maximally utilize the test kit. Also mosquito's pools were collected from the stables where the horse blood samples were collected. The mosquitoes were aggregated in pools containing 25 female *Culex* mosquitoes per pool

3.3 Sampling Technique for Horses

A non probability sampling technique was used and animals were sampled based on willingness of the horse owner to include their horses in the study. The sample collection was carried out from February to May 2016.

3.3.1 Sample collection and transport

3.3.1.1 Collection of blood from Horses

Five (5mls) of blood was collected aseptically from the jugular vein of apparently healthy horses using 18G needles. The samples were labelled and transported in a cold box to the Viral Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University Zaria. Clotted blood was centrifuged at 3000rpm for 5mins. The Serum was harvested labelled and preserved at -20°C until it was used for analysis.

3.3.1.2 Collection of mosquitoes from Horse stables

The CDC miniature light trap (Model 512 by John W. Hock Company U.S.A) acquired from Biological Sciences, Ahmadu Bello University, Zaria; was used to trap adult mosquitoes from June to September 2016 in the stables where the horses were sampled. The trap makes use of light and carbon dioxide (CO₂) to attract mosquitoes. It has a fan generated air current that draws mosquitoes into a collection jar or bag, (Sudia and Chamberlain, 1988). The CDC light trap was hung in a dark corner of the horse stables and left to operate from dusk to dawn in order to attract mosquitoes. The trapped mosquitoes were emptied into a well labelled sterile sample bottle and transported preserved with a silica gel as described by (Mayagaya *et al.*, 2015). The trapped mosquitoes were taken to

the Entomology Laboratory of the Department of Veterinary Parasitology, Ahmadu Bello University, Zaria, they were sorted into species. Adult female Culex species were selected for the determination of West Nile virus antigen.

3.4 Laboratory Analysis

3.4.1 Detection of WNV antibody in horse sera

All serum samples were screened for the presence of antibodies to WNV using Competitive enzyme linked immunosorbent assay (cELISA) from (ID screen^(R) West Nile Competitive Multi- Species, ID-VET).

3.4.1.1 Description and principles of the cELISA

The micro-wells are coated with a purified extract of the West Nile virus. Samples to be tested and controls are added to the wells. The anti-pr-E antibodies, if present, form an antigen-antibody complex. An anti-pr-E antibody peroxidase (HRP) conjugate is added to the wells. It fixes to the remaining free pr-E epitopes, forming an antigen-conjugate-HRP complex, after washing in order to eliminate the excess conjugate, the substrate solution (TMB) is added. The resulting coloration depends on the quantity of specific antibodies present in the sample to be tested: - in the absence of antibodies, a blue solution appears which becomes yellow after addition of the stop solution. In the presence of antibodies, no coloration appears. The micro-plate is read at 450 nm.

3.4.1.2 Laboratory procedure

The test was carried out using 96 wells micro-titre plate, a competitive enzyme linked immunosorbent assay plate (ID-VET, France). All the reagents were allowed to come to room temperature at 21⁰C before use, and they were homogenized by inversion.

Fifty micro-liters (50µl) of dilution Buffer 2, Mm NaCl, (A complex protein solution) were added to each micro-well. Fifty micro-litres (50µl) of the positive control, (dilution of a positive horse serum in a stabilizing buffer) were added to wells A1 and B1 which contains a purified WNV anti-Pr-E antibody (Horseradish peroxidase), (standard).

Fifty micro-litre (50µl) of the negative control, (Negative horse serum diluted in a stabilizing buffer) was added to wells C1 and D1. Then 50µl of each horse serum (horse serum sampled), were added in the remaining wells and incubated for 90 mins at 21°C. After which the well was emptied and washed 3 times with approximately 300µl of the wash solution containing, (0.3M NaCL, 0.5M KCL, 0.13M Na₂HPO₄, 0.03M NaH₂PO₄, Tween 20 0.005%). Drying of the wells in between washings was avoided.

The Conjugate 1× anti-Pr- antibody (Horseradish peroxidase) was prepared by diluting the concentrated conjugate 10× to 1/10 in dilution buffer 2 and 100µl of the conjugate 1× was added to each of the micro wells. The micro-titre plates were then incubated for 30mins at 21⁰C after which the micro-wells were emptied and each well was washed 3 times with approximately 300µl of the wash solution. One hundred micro-litre (100µl) of Substrate solution, containing (Tetramethylbenzidine (TMB) and Oxygenated water in a sterilizing buffer), which is a revealing solution was also added to each well and incubated again for 15 mins at 21⁰C in the dark. And 100µl of the stop solution, containing (Sulphuric acid solution 0.5M) was added to each well in order to stop the reaction. The results were read and recorded using ELISA reader at an optical density of 450nm (ID-VET Innovative Diagnostics).

3.4.1.3 Result validation

The test result was validated by:

The mean value of the positive control (OD_{PC}) is less than 30% of the OD_{NC} ,

That is: $OD_{PC} / OD_{NC} < 0.3$

The mean value of the negative control O.D. (OD_{NC}) is greater than 0.700,

That is: $OD_{NC} > 0.700$

3.4.1.4 Interpretation of the result

Samples that tested positive to WNV antibodies were colourless, and samples that tested negative to the viral antibodies appeared bluish and thereafter turned yellowish in colour when the stop solution was added. All the samples tested were interpreted as percentage of the sample value divided by the negative control (S/N).

$$S/N\% = \frac{OD_{\text{sample}} \times 100}{OD_{NC}}$$

Samples are presented as S/N%

- Tested samples that were less than 40% were considered positive.
- Tested samples that were less than or equal to 50% and greater than 40% were considered doubtful
- Tested samples that were greater than 50% were considered negative.

Table 3.1. Interpretation of the cELISA test result.

RESULT	STATUS
$S/N\% \leq 40\%$	POSITIVE
$40\% < S/N\% \leq 50\%$	DOUTFUL
$S/N\% > 50\%$	NEGATIVE

3.5 Detection of West Nile Virus Antigen in Mosquitoes

3.5.1 Mosquitoes identification

All the mosquitoes that were collected were sorted into species in Entomology Laboratory of the Department of Veterinary Parasitology, Ahmadu Bello University Zaria by visual observation using simple stereomicroscope to separate different species of the adult mosquitoes using their morphological characteristics of the head and thorax, in addition to the wing anatomy and markings on the hind legs, abdomen and nature of the palps as described by (Sirivanakarn and White, 1987; Darsie and Ward, 2005).

Thirty one pools of adult female *Culex* mosquitoes were used for the detection of WNV antigen.

3.5.2: Vector test[®] for West Nile virus antigen assay.

VecTest[®] for West Nile Virus (WNV) Antigen Assay (VECTOR Test[®] System Inc, USA) was used to determine WNV antigen in the mosquitoes identified as *Culex* species. VecTest[®] is a rapid immunochromatographic assay intended for the qualitative determination of WNV antigen in infected mosquitoes.

3.5.2.1 Principles of VecTest[®] for West Nile virus antigen detection

The VecTest[®] for WNV Antigen Assay is based on the dual monoclonal antibody “sandwich” principle. The test is initiated by placing one VecTest[®] WNV dipstick into 250 ml (0.25 ml) of ground mosquito extract. Antigen present in the solution binds to the specific antibody with a gold sol particle label. As the antigen-antibody-gold complexes migrate through the test zone containing immobilized WNV antibody, they bind to the immobilized antibody forming a “sandwich”. The unbound dye complexes migrate out of the test zone and can be captured later in the control zone. A reddish-purple line develops on the specific area of the test zone when antigen is present. The control line, farthest from the sample, should always develop provided the test has been carried out correctly.

3.5.2.2 Test procedure for VecTest[®] West Nile virus antigen assay

A pool containing 25 adult female *Culex* mosquitoes was placed into a conical grinding tube (Eppendorf tube) and was well positioned in a tube stand. Then, 250µl of the grinding solution was dispensed into then tube containing the mosquitoes. A pestle was placed into the grinding tube and was vigorously rotated to homogenise the mosquitoes, and the pestle was washed in between uses. The vector test strip from the canister was inserted into the mosquito suspension in the grinding tube with the arrows pointing downward for 20 – 30 minutes to complete the test procedure, after which the test result was read, and determined by removing the test strip and comparing it to the pictorial sample provided.

3.5.2.3 Interpretation of the VecTest[®] result

The Vector test[®] kit contain an antigen assay dip sticks with a control line and positive line which appear as reddish-purplish colour when there is a reaction.

The presence of two lines indicates the presence of positive WNV antigen, and the presence of a single line, which is the control line on the dip-stick, indicates a negative test result.

The presence of a faint line below the control line indicates a doubtful result.

3.6 Questionnaire Based Assessment on Risk Factors of West Nile Virus

A structured questionnaire was administered to the horse owners or horse grooms in all the sampled areas in order to determine the demographic, management and environmental factors associated with the occurrence of West Nile virus infection. (Appendix I). Questions such as breed, sex, age, presence of wild birds, grasses, stagnant water, mosquitoes were asked.

3.7 Data Analysis

The data generated were analyzed using statistical package for social sciences (SPSS) version 20.0 to carry out descriptive analysis of distribution of disease and variables.

Prevalence was calculated with the formula,

$$\text{Prevalence} = \frac{\text{Total no. of positive samples}}{\text{Total no. of samples}} \times 100$$

Chi square test was used to determine associations between demographic and management variables and occurrence of WNV infection. Values of $P \leq 0.05$ were considered significant.

Also OR and (95% confidence interval) was used to assess risk factors of WNV infection.

CHAPTER FOUR

RESULTS

4.1 Over All Prevalence of West Nile Virus Antibody in Horses from Selected L.G.As of Kaduna State, Nigeria.

A total of 368 horse sera were tested using cELISA and 89.9% (331/368) were positive for WNV antibody (Table 4.1).

4.1.1 Seroprevalence of West Nile virus infection according to sex of horses.

Based on sex, a total of 268 samples were tested for male and 100 for female of which 90.3% (242/268) tested positive among the male horses and 89.0% (89/100) tested positive for the female horses. There was no statistical association between WNV infection and sex of the horses (OR= 1.150; CI on OR: 0.546 < OR: 2.425) (Table 4.2).

4.1.2. Seroprevalence of West Nile virus infection based on age of horses

Based on age of the horses 89.3% (50/56) within the age range of 1-5 years were positive for the virus those between the ages of 6-10 years had 86.9% (93/107) sero-positive to WNV infection and had the higher risk of the disease occurrence with OR= 1.254; 95% CI on OR: 0.454 < OR: 2.509 (Table 4.3) as compare to the other age categories. However, 90.2% (120/133) for 16-20 years of age were also positive for the virus. Horses that are within the age of 20 years and above were 100% (19/19) sero-positive to WNV antibodies. There was no statistical significant difference based on age as reported in (Table 4.3.).

Table 4.1: Over all Prevalence of West Nile Virus antibody in horses in selected L.G.A of Kaduna State.

Variables	Frequency	Percentages (%)
Positives	331	89.9
Negatives	37	10.1
Total	368	100.0

Fr- (frequency), (%) - Percentages

Table 4.2: Seroprevalence of West Nile virus infection according to sex of Horses in selected Local Government Areas of Kaduna State.

Sex	Number Tested	No. Positive (%)	Odds ratio	95% CI	
				Lower	upper
Male	268	242(90.3)	1.150	(0.546–2.425)	
Female	100	89(89.0)	1(REF)		
Total	368	331(89.9)			

$\chi^2 = 0.136, df = 1, p = 0.713$

Table 4.3. Seroprevalence of WNV infection based on age of horses in selected LGAs of Kaduna State.

Age(years)	Number Tested	No. of WNV Positive (%)	OR (95% C.I.)
1-5	56	50 (89.3)	1(REF)
6-10	107	93 (86.9)	1.254 (0.454-3.466)
11-15	133	120 (90.2)	0.903 (0.325-2.509)
16-20	53	49 (92.5)	0.680 (0.181-2.560)
> 20	19	19 (100.0)	0
Total	368	331 (89.9)	

$\chi^2 = 3.617$; $df = 4$; $p = 0.460$

4.1.3 Prevalence of West Nile virus antibody according to location.

In Zaria LGAs a total of 150 horses were sampled and 88.7% (133/150) were seropositive to WNV infection, while in Igabi LGAs, 86.0% (86/100) were also positive to WNV antibody. Moreover, 98.9% (94/95) and 78.3% (18/23) were seroprevalence for the virus in Kaduna North and Sabon Gari LGAs respectively. There was a statistical significant difference based on locations ($p \leq 0.05$). Sabon Gari showed a higher likelihood of having the infection as compared to the other LGAs OR 2.173; 95% CI on OR: 0.715 < OR: 6.608 as shown in (Table 4.4).

4.1.4 Prevalence of West Nile virus antibody among breed of horses

The prevalence of WNV infection among breed of horses revealed that the Sudanese breed of horses had the highest seroprevalence for WNV infection 94.7% (18/19) as at the time they were sampled. A Total of 189 Arewa horses were tested and 91.0% (172/189) were positive for WNV antibody. Argentine and Talon breed of horses had a seroprevalence of 85.9% (79/92) and 91.2% (62/68) respectively for WNV infection. There was no statistically significant association between seroprevalence of WNV and breed of horses ($p \geq 0.05$). (Table 4.5).

Table 4.4: Prevalence of West Nile virus antibody according to location of horses sampled in selected Local Government Area of Kaduna State.

Local Govt Area	Number Tested	No. of WNV Positive (%)	OR (95% C.I.)
Zaria	150	133 (88.7)	1(REF)
Igabi	100	86 (86.0)	1.274 (0.597-2.717)
Kaduna	95	94 (98.9)	0.083 (0.011-0.636)
SabonGari	23	18 (78.9)	2.173 (0.715-6.608)
Total	368	331 (89.9)	

$\chi^2 = 13.977$; $df = 4$; $p = 0.003$

Table 4.5: Prevalence of West Nile virus antibody among breed of horses sampled in selected Local Government Area of Kaduna State.

Breed	Number Tested	No. of WNV Positive (%)	OR (95% C.I.)
Arewa	189	172 (91.0)	1(REF)
Argentine	92	79 (85.9)	1.665 (0.771-3.595)
Talon	68	62 (91.2)	0.979 (0.369-2.596)
Sudanese	19	18 (94.7)	0.562 (0.071-4.474)
Total	368	331 (89.9)	

$\chi^2 = 2.521$; df = 4; P = 0.471

4.1.5 Serological evidence of West Nile virus infection based on colour of horses

Serological evidence of the virus based on colours of horses showed that, horses with chestnut colour had a seroprevalence of 89.9% (208/187) for WNV infection, the black coloured horses had 88.1% (59/67), the white horses had 92.1% (35/38) while, the gray and bay horses had prevalence of 92.9% (26/28) and 85.7% (12/14) respectively. The albino horses had 92.3% (12/13) seroprevalence for WNV infection. There was no statistical association between colours of horses and seroprevalence for WNV infection ($P \geq 0.05$). The bay coloured horses had a higher likelihood of having the infection as compared to the other categories (OR= 1.229; 95% CI on OR: 0.232 < OR: 6.524) (Table 4.6).

4.1.6 Serological evidence of West Nile virus infection in horses based on activities of horses

Seroprevalence of WNV infection in horses based on their activities at the time they were sampled revealed that horses used for both parade and race has the highest prevalence of 100% (49/49) and 100% (12/12) respectively. Those that were engaged in polo activities had a seroprevalence rate of 85.0% (164/193). While those used for entertainment purposes had prevalence rate of 93.0% (106/114). There was statistical association of WNV seroprevalence and horse activities ($p \leq 0.05$). (Table 4.7).

Table 4.6: Serological evidence of West Nile virus infection based on Colour of horses sampled in selected Local Government Area of Kaduna State.

Colour	Number Tested	No. of WNV Positive (%)	OR (95% C.I.)
Black	67	59 (88.1)	1 (REF)
White	38	35 (92.1)	0.632 (0.157-2.541)
Chestnut	208	187 (89.9)	0.828 (0.349-1.968)
Gray	28	26 (92.9)	0.567 (0.113-2.857)
Bay	14	12 (85.7)	1.229 (0.232-6.524)
Albino	13	12 (92.3)	0.615 (0.07-5.380)
Total	368	331 (89.9)	

$\chi^2 = 1.080$; $df = 5$; $P = 0.956$

Table 4.7: Serological evidence of West Nile virus infection among horses based on Activities of horses in selected Local Government Area of Kaduna State.

Activities	Number Tested	No. of WNV Positive (%)	OR(95% CI)
Celebration	114	106 (93.0)	1 (REF)
Polo	193	164 (85.0)	2.343 (1.032-5.319)
Race	12	12 (100.0)	0
Parade	49	49 (100.0)	0
Total	368	331 (89.9)	

$\chi^2 = 13.256; df = 4; p = 0.004$

4.1.7 Prevalence of West Nile virus antibody based on source of horses

Seroprevalence of the virus based on source of the horses showed that the locally sourced horses had a highest prevalence of 92.9% (250/269). The imported ones had a prevalence of 81.8% (81/99). There was statistical significant difference of WNV infection based on source of the horses ($P \leq 0.05$) with odds ratio showing OR = 2.924; 95% CI on OR: 1.464 < OR: 5.839). (Table 4.8.).

Table 4.8: Prevalence of West Nile virus antibody based on source of horses sampled in selected Local Government Area of Kaduna State.

Source	Total Tested	Number	No. of positive (%)	Odds ratio	95% CI
Local	269		250 (92.9)	2.924	(1.464 - 5.839)
Imported	99		81 (81.8)	1(REF)	
Total	368		331 (89.9)		

$\chi^2 = 9.893, df = 1, P = 0.002$

4.2. Detection of West Nile Virus Antigen in Mosquitoes.

A total of 775 mosquitoes contained in 31 pools of 25 adult female *Culex* mosquitoes per pool were trapped in stables from the four selected LGAs having high population of horses in Kaduna State, Nigeria.

Out of the 31 mosquito pools tested, only one mosquito pool 3.2% (1/31) tested positive to WNV antigen in Kaduna State. The positive pool 7.1% (1/14) was obtained from Zaria LGA (Table 4.8). Doubtful results were obtained from four mosquito pools 23.1% (3/13) from Sabon Gari and 7.1% (1/14) from Zaria LGAs respectively. There were no WNV antigen reactions in the pools sampled from Igabi and Kaduna North L.G.As. As shown in table 4.8.

Table 4.9: Detection of West Nile virus antigen in *Culex* mosquito sampled in the stables from the selected Local Government Area of Kaduna State.

Local Govt Areas	No. of Mosquito pools	No. of positive pools (%)	No. of doubtful pools (%)
Sabon Gari	13	0 (0.0)	3 (23.1)
Zaria	14	1 (7.1)	1 (7.1)
Igabi	2	0(0.0)	0 (0.0)
Kaduna North	2	0(0.0)	0 (0.0)
Total	31	1 (3.2)	4 (12.9)

4.3: Evaluation of the Respondents on the Factors that

Could Facilitate The Transmission of West Nile Virus.

A total of 42 questionnaires were administered to the horse grooms. All the 42 questionnaires were evaluated and completed for all the local governments areas visited in Kaduna state, as shown in Table 4.10 and Table 4.11.

4.3.1: Demographic features of the respondents

The demographic information on the respondents are revealed that, Zaria LGA had the highest numbers of respondents with a total of 47.6% (20/42) followed by Sabon Gari 31% (13/42), Kaduna North 11.9% (5/42) and Igabi L.G.A., had the lowest numbers of respondents of 9.5% (4/42). Based on the age range of the grooms, 9.5% were above the age of 40 years. Grooms with the age range of 20 to 30 years are the majority 27/42 (64.3). Most of the grooms had a formal education of which those with tertiary and secondary educational status had a close percentage of 19/42 (45.2%) and 18/42 (42.9%) respectively. A total of 100% (42/42) of the respondents were all male. According to the numbers of persons in household living in close proximity to the horse stable; number of persons ranging from 1 to 5 had the highest percentage of 18/42 (42.9%). (Table 4.10).

Table 4.10. Demographic features of the respondents interviewed in Kaduna State

Variables	Frequency	Percentages (%)
Location		
Zaria	20	47.6
Sabon Gari	13	31.0
Kaduna North	5	11.9
Igabi	4	9.5
Total	42	100
Age		
< 19 years	1	2.4
20-30 years	27	64.3
31-40 years	10	23.8
> 40 years	4	9.5
Total	42	100
Sex		
Male	42	100
Female	0	0.0
Total	42	100
Educational status		
Secondary	19	45.2
Tertiary	18	42.9
Primary	4	9.5
No formal education	1	2.4
Total	42	100
Nos. of person in household		
1-5 persons	18	42.9
6-10 persons	11	26.2
11-15 persons	1	2.4
16-20 persons	6	14.3
Above 20 persons	6	14.3
Total	42	100

4.3.2. Management and environmental risk factors of West Nile virus infection.

Fifty percent of the respondents know that mosquitoes can transmit diseases to horses while 21/42 (50.0%) were not aware of the fact that mosquitoes could transmit diseases to horses. Majority 27/42 (64.3%) of the grooms feed their animals in the stable as compared to those that feed their horses in the open 12/42 (28.6%) and under the tree 3/42 (7.1%). About 26/42 (61.9%) of the respondents said that wild birds were usually present around the stables and also 37/42 (88.1%) indicated that they do not control mosquitoes and other vector in their stables. Most of the grooms said they have witness a horse having a symptom of febrile illness and sudden death but few of them have seen a horse showing nervous signs as reported in (Table 4.11). Of the 42 respondents 33/42 (78.6%) responded they have vet doctors attending to their horses. About 19/42 (45.2%) of the Grooms had worked in the stables between 1-10 year. Others that had also worked in the stables for about 11-20 years had 12/42 (28.6%), 21-30 years had 10/42 (23.8%) and 31- 40 years had 1/42 (2.4%) respectively. Respondents who use personal protective equipment when attending to horses were few 19/42 (42.9%) as shown in (Table 4.11).

Table 4.11: Responses on management and Factors that could aid the transmission of west Nile virus.

Variables	Frequency (%)
Where do you feed your horses	
In the stable	27(64.3)
In the open	12(28.6)
Under the tree	3(7.10)
Total	42(100)
Are there presence of wild birds around your stable	
Yes	26(61.9)
No	16(38.1)
Total	42(100)
Do you control mosquitoes and other vectors in your stable	
Yes	5(11.20)
No	37(88.1)
Total	42(100)
Do you often clear grasses around your stables	
Yes	12(28.6)
No	30(71.4)
Total	42(100)
Is there waste bins near your stables	
Yes	17(40.5)
No	25(59.5)
Total	42(100)
Is there stagnant water body near your stable	
Yes	16(38.1)
No	26(61.9)
Total	42(100)
Do you know mosquitoes can transmit disease to horses	
Yes	21(50.0)
No	21(50.0)
Total	42(100)

Responses on management and Factors that could aid the transmission of west Nile virus.

Variables	Frequency (%)
Have you seen a horse with signs of anorexia, weakness, sudden death	
Yes	26(61.9)
No	16(38.1)
Total	42(100)
Have you seen a horse with nervous signs	
Yes	17(40.5)
No	25(59.5)
Total	42(100)
How do you dispose your dead animal	
Deep burial	28(66.7)
Sell off	8(19.0)
Others	6(14.3)
Total	42(100)
Do you have a vet doctor attached to your farm	
Yes	33(78.6)
No	9(21.4)
Total	42(100)
For how long have you been with horses	
1-10 years	19(45.2)
11-20 years	12(28.6)
21-30 years	10(23.8)
31-40 years	1(2.40)
Total	42(100)
Do you use personal protective equipment when attending to horses	
Yes	19(42.9)
No	23(57.1)
Total	42(100)
If yes what type of personal protective equipment do you use	
Face mask	4(9.50)
Lab coat	2(4.80)
Cover all,	6(14.3)
Complete personal protective equipment	1(2.40)

CHAPTER FIVE

5.0: DISCUSSION

West Nile virus (WNV) is a Flavivirus and causes a disease of public health importance (WHO, 2011). This study was carried out to determine the seroprevalence of WNV infection in horses and also to determine the presence of West Nile virus antigen in mosquitoes in some parts of Kaduna State Nigeria.

The overall seroprevalence of WNV antibodies in horses was 89.9% from the selected Local Government Areas (LGAs) in Kaduna State Nigeria. This suggests that the horses have been exposed to the virus and this could be attributed to the poor environmental management system adopted by some of the grooms in and outside the horse stables and as seen during sampling and also the wet and warm season during which the horses were sampled. Mosquitoes tend to be more in abundance during the rainy and hot season and transmission of the virus could be possible at this time.

Majority of the horses were sourced locally while a few were imported. It could be possible that most of the horses sampled might have acquired the infection from where they were sourced. Also most of the horses travel out for polo tournament; enzootic infection might have also been possible for acquiring WNV infection.

Sero-prevalence of WNV infection in this study is comparable to the levels reported by previous researchers in Nigeria and in other countries Sule *et al.* (2015) reported a prevalence of 90.3% in horses in South-western Nigeria. The works of Ezeifeke *et al.*, (1986) and Baba *et al.* (2014) reported a lower WNV prevalence of 25.0% and 11.5% in horses respectively. Similar low prevalence reports were described from Djibouti (9.0%),

Cote d' Ivoire (28.0%), Democratic Republic of Congo (30.0%) and Gabon (3.0%) (Cabre *et al.*, 2006). In other parts of the world, two provinces of Pakistan had a prevalence of 65.0% as reported by Zohaib *et al.* (2015). Also Ute *et al.* (2013), Sadegh *et al.* (2013) and Strahinja *et al.* (2014) demonstrated a low WNV prevalence of 13.5%, in Ukraine, 2.8% in Iran and 28.6% in Northern Serbia respectively. Perhaps these low prevalence levels may be due to the differences in types and sensitivities of tests used such as Complement Fixation Test and Micro neutralization test. In addition, Bradley *et al.* (2003) reported prevalence of 62.5% among horses tested for WNV antibodies in Coahuila State, Mexico, using Epitope-blocking ELISA and Plague reduction neutralization test (PRNT). It is known that use of less sensitive serological tests will increase the number of false negative results, resulting in lower prevalence estimates (Thrusfield, 2005). While other reports described similar levels of high prevalence as found in this study including Cabre *et al.* (2006), who reported a high seroprevalence of West Nile virus in Chad 97% and in Senegal 92% using ELISA techniques. Differences in this prevalence could also be in part, due to environmental or ecological differences. For example some reports indicated surveillance in regions that are characterized by semiarid climate and grassland vegetation which are frequently reported areas for WNV isolations in birds and mosquitoes (Traore-Lamizana *et al.*, 1994; Centre Collaborateur, 2006). Kaduna State lies between the Sudan and Guinea Savannah ecological zones of Nigeria, it is expected to be environmentally favourable for WNV transmission.

There was no statistically significant difference in seroprevalence based on sex in this study, even though male horses had a higher percentage of infection. Previous works such as Baba *et al.* (2014), have described higher prevalence (100%) in male horses. It has been

reported that, development of neuroinvasive disease in man due to WNV infection occurs more frequently among males, on the other hand, the risk for initial infection with WNV has not been found to be significantly high among males according to sero-surveys and studies among human blood donors (Tsai *et al.*, 1998; CDC, 2000; Brown *et al.*, 2007).

There is no statistical association between age group and WNV infection in this study. Age has been attributed as one of the risk factors of WNV infection due to suppressed immunity. Pradier *et al.* (2012) reported that the incidence of neuroinvasive WNV disease and death is associated with increase in age. The lack of correlation between age and seroprevalence may suggest equal opportunity of exposure to mosquitoes by all age categories of horses, there is no preferential housing system for young or old horses in stables in this study. Since the ELISA kit used in detecting infection in this study is an IgG based ELISA, it is difficult to partition infection into recent and old infections thus newly infected horses and those with repeated exposure will react similarly to the test. An IgM-based ELISA kit would have detected recent infections which could partition by age.

Furthermore, based on location, Kaduna north had the highest seropositivity of 98.9% while Sabon Gari had the lowest. There was a statistical significant association of the infection among the locations. This could be due to the bad state of the environment precipitated with bushes, trees, waste bins, and old tires which are some of the environmental risk factors of WNV infection, because WNV vector and reservoir host activities is favourable in an unkempt environment. Stagnant water body is another key component that promotes vector abundance by ample breeding environments for mosquitoes (Takeda *et al.*, 2003). It's also a source of drinking water for wild birds.

Based on the breed of horses, there was no statistical significant difference of the disease. The Sudanese breed had a high serological evidence of 94.7%, than the Arewa, Talon and the Argentine. Sule *et al.* (2015) had a similar report that Dongola horses (Arewa horses) had a high sero-positivity of 97% but Ezeifeke *et al.* (1986) reported 30.1% for the Arabian cross (Talon breed) higher than the other breeds as shown in their work. These differences could be due to a long time exposure to indigenous mosquito vector as described by (Sule *et al.*, 2015). Dark colour is also a contributing factor to mosquito attraction, because mosquitoes seem to be more attracted to dark colours. But in this study the white, albino and gray colours are more sero-prevalence for the virus with a prevalence level of 92.1%, 92.3% and 92.9% respectively; although there was no statistical significant difference based on skin colour.

The purpose, for which the horses are used, revealed that horses used for parade and race activities had 100% prevalence as compared to those that indulge in polo and entertainment. And there is statistical significant association of the infection based on activities of the horses. Multiple-infection might have occurred in the stables where the horses were kept among other horses as at when they travelled out for a competition in other places. The locally sourced horses had a prevalence rate of 92.9% than the ones that were imported. This could be attributed to the climatic condition, poor environmental management condition that is concentrated with mosquitoes and wild birds around the stable surroundings. Transmission cycle between the vector and birds could pose a great risk in transmission of the virus to animals and humans especially when they are precipitated in the environment. There is statistical significant association based on the source of the animal and WNV infection.

Mosquitoes, which are the main vector of WNV, have played a major role in the transmission of the virus in birds, humans, animals and reptiles. This study detected WNV antigen in a pool of mosquitoes trapped in horse stables in Kaduna State. This evidence suggests that there has been an on-going circulation of WNV infection in Kaduna State. Other work done such as, in New York State around the year 2000 White *et al.* (2001) detected WNV in 363 mosquito's pools during a surveillance season using Polymerase Chain Reaction (PCR) which is more sensitive than the vector test used in this study. A pool of mosquitoes was positive for WNV in mosquitoes trapped from zoo in two States in Tabasco as reported by Hidalgo-Martinez *et al.* (2008); this result is in concordance with the report of this study.

To the best of my knowledge this is the first reported positive result of West Nile virus antigen in mosquitoes in Nigeria. Sule and Oluwayelu. (2016) reported no WNV activity in mosquitoes in south-western Nigeria. Also Baba *et al.* (2006) reported no WNV positivity from the 52pools of *Culex* mosquitoes tested in semi arid zones of Nigeria. Mshelia, (2014) also could not detect the virus antigen in mosquitoes trapped in live bird markets in Kaduna State. Even though they used RT-PCR which is a more sensitive test than the Vector Test used in this work, they could not detect the virus. This could be due to poor handling and poor storage facility which might have altered the quality of the virus particles in the mosquitoes they trapped and used for their research work.

The questionnaire surveyed revealed the views of the respondents about West Nile virus infection. About 50% of them were ignorant about mosquitoes transmitting diseases to horses. This shows that they have little or no knowledge about the risk of the disease infection and mosquito-borne diseases.

Respondents that are more into horse grooming were (64.3%) and they were between the ages of 20 to 30 years followed by those that are between the ages of 31 to 40 years old (23.8%) and majority of them had formal education and some are still undergoing tertiary education. All of the respondents were males. According to previous studies, age and sex, especially the old age and male genders have shown to be one of independent risk factors of WNV infection. Jean *et al.* (2007), documented risk factors that are independently associated with developing neuroinvasive diseases rather than West Nile fever included older age, male sex, hypertension, and diabetes mellitus.

Almost all the respondents (88.1%) indicated that they do not control mosquitoes and other vectors in their stables and surrounding environment, because they thought that fumigation is detrimental to the health of the horses. Water logs, grasses, waste bins, trees, also old tires and old buckets around the stables as seen and responded to during the course of questionnaire survey are factors that favour mosquitoes breeding which could enhanced the risk of transmission of WNV.

Some of the grooms with the percentage of (61.9%) and (40.5%) responded that they have seen horses showing nervous signs and febrile illness such as anorexia, weakness, severe emaciation and sudden death, which are some of the signs of WNV infection. It could be possible that these signs and deaths observed may have been as a result of WNV infection as the seroprevalence was high in this study. About 28.6% of the respondents feed their horses in the open while majority (64.3%) of them feed in the stable and few (7.1%) feed their horses under the tree, this showed that the respondents are ignorant of the disease transmission and so preventive measures were not been taken in environmental management practices, this could expose the horses to risk of getting infected. About 78.6% have veterinary doctors attached to their stables while 21.4% had none. Some of the

respondents have been working as a groom for over 30 years and about 57.1% do not make use of personal protective equipments. This showed that they are ignorant of possible disease transmission and they might have been exposed to *Arbovirus* zoonotic infections like WNV.

The transmission cycle of WNV exists in rural ecosystems and also in the urban areas where the virus infects birds, humans, horses and other mammals (Turell *et al.*, 2005; Paz and Semenza, 2013b). West Nile fever is a serious illness for humans and approximately 1 in 150 infected persons develop a serious illness with symptoms that might last for several weeks. Up to 20% of patients have milder symptoms and approximately 80% show no symptoms at all (CDC, 2015b). West Nile virus is widely distributed as encephalitic *flaviviruses*, and it is a vector-borne pathogen of global importance (May *et al.*, 2011).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study has demonstrated high seroprevalence of WNV infection in horses in Kaduna North, Igabi, Sabon-Gari and Zaria Local Government Areas of Kaduna State, with an overall seroprevalence of 89.9%. This infection was detected in apparently healthy horses. The seroprevalence was higher in male (90.3%) than in female horses (89.0%) and was more common (100%) in older horses aged 20 years and above than in younger horses. The prevalence was more in Sudanese breed (94.7%) than in other breeds of horses. Seroprevalence was statistically higher in Kaduna North LGA (98.9%) than in other LGAs of Kaduna State studied. Locally sourced horses had higher seroprevalence (92.9%) than imported horses (81.8%). Evidence of contemporary WNV circulation was found in 1 pool (3.2%) out of the 31 pools of *Culex* mosquitoes trapped in horse stables in the 4 LGAs, indicating an on-going circulation of WNV in the state. There is ignorance (50%) about the disease and its transmission amongst horse handlers in the State with preponderance of management practices and environmental conditions favourable for WNV infection occurrence, including presence of wild birds, stagnant bodies of water, unkempt vegetation and other conditions suitable for mosquito breeding around the stables in the 4 LGAs studied. There is evidence of widespread WNV infection in horses and on-going virus carriage in mosquitoes in Kaduna State suggesting high risk for humans and animal infection.

6.2 Recommendations

Based on the findings of this study, the following are recommended:

- Adequate protective measures should be adopted against mosquitoes-borne diseases including WNV infection.
- Public awareness and campaign should be carried out on mosquitoes-borne zoonotic diseases including WNV infection.
- Eliminate mosquito breeding sites around horse stables and homes to reduce or eliminate the presence of the mosquito vector in the environment.
- Health authorities should include WNV infection as differential in the diagnosis of fevers of unknown origin and include WNV in the list of viral agents being screened for blood transfusion.
- Vector test should be adopted for entomological and ornithological surveillance for WNV in the field because, it's handy, easy and fast assay.
- Other species of mosquitoes should also be tested for WNV antigen.
- Further studies should be carried out in all other local government areas not covered in Kaduna State.
- Further studies should be carried out on WNV in other animals to increase understanding of the epidemiology of WNV infection in Nigeria.

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APPENDIX

1.0 DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE FACULTY OF VETERINARY MEDICINE, AHMADU BELLO UNIVERSITY, ZARIA.

RESEARCH QUESTIONNAIRE

Instructions: Please tick Yes / No in the following boxes and specify where necessary. The information obtained will be used strictly for academic purpose only and absolute confidentiality will be ensured.

1. DEMOGRAPHIC VARIABLES

1. Date _____

2. Location: _____

3. Age:

a. <19 [] b. 20 – 30 [] c. 31 – 40 [] d. > 40 []

4. Sex of Respondents:

a. Male [] b. Female []

5. Educational qualification:

a. Primary [] b. Secondary [] c. Tertiary [] d. None [] e.

Others: _____

6. No. of persons in household/ location: _____

7.

2. ANIMAL INFORMATION

8. Name and Identification Number:

9. Age:_____

10. Sex:_____

11. Color:_____

12. Breed of horse(s):

a. Arewa [] b. Argentine [] c. Talon [] d. Sudanese []

13. What is the source of your horse(s)

a. Imported [] b. Locally sourced [] c. both []

3. ENVIRONMENTAL MANAGEMENT AND PRACTICE OF THE RESPONDENTS

14. Where do you feed your animals?

a. In the open [] b. Under the tree [] c. In the Stable []

15. Are there presences of wild birds in the environment? Yes [] No []

16. Do you control mosquitoes and other vectors in your horse stable or farms? Yes []

No []

17. Do you often clear grasses around your stables? Yes [] No []

18. Are there waste bins near your horse stable or farms? Yes [] No []

19. Is there stagnant water body near your horse stable or in the farm? Yes [] No []

20. Do you know mosquitoes can transmit disease to your horse(s)? Yes [] No []

21. Have you seen any horse diseases with nervous signs? Yes [] No []

22. Have you seen any horse diseases with weakness, uncoordinated movement, eating while sleeping, lack of appetite, severe emaciation and sudden death? Yes No

23. How do you dispose off your dead horse? a. Deep burial b. Sell off c. Used to feed other animals Others_____

24.. Do you have a Veterinary doctor attach to your stable or farm? Yes No

25. For how long have you been attending to horses: _____ [a]1-10yrs [b] 11-20yrs [c] 21-30yrs [d] 31-40yrs

26. Do you wear personal protective clothen, when attending to your horse(s)? Yes No .

27. Which of the personal protective clothens do you wear?

a. Cover all b. Lab coat c. Boot d. Face mask

APPENDIX 2. PLATES

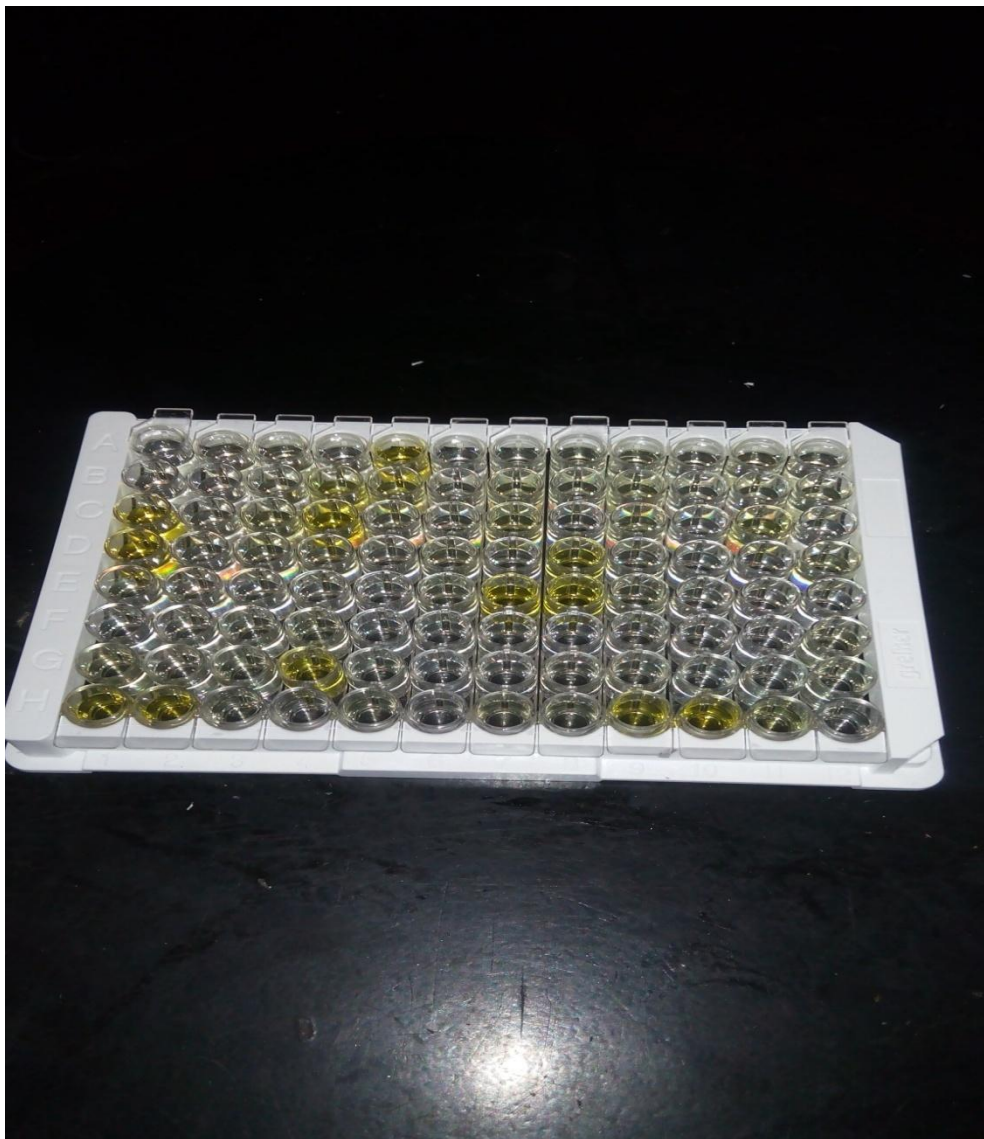


Plate I: Screening of test samples using Competitive Enzyme Link Immunosorbent Assay. The colourless wells indicate positive samples, while the yellow wells indicate the negative samples.



Plate II: One of the horses tested for WNV antibody.



Plate III: A VectorTest^(R) dip stick for a positive pool of mosquitoes. The second band below the control band is an indication of positive result for presence of WNV antigen in mosquito pool.



Plate IV: Center for Disease Prevention and Control (CDC) miniature light trap for mosquitoes collection.



Plate V: *Culex spp* of mosquitoes trapped in horse stables.

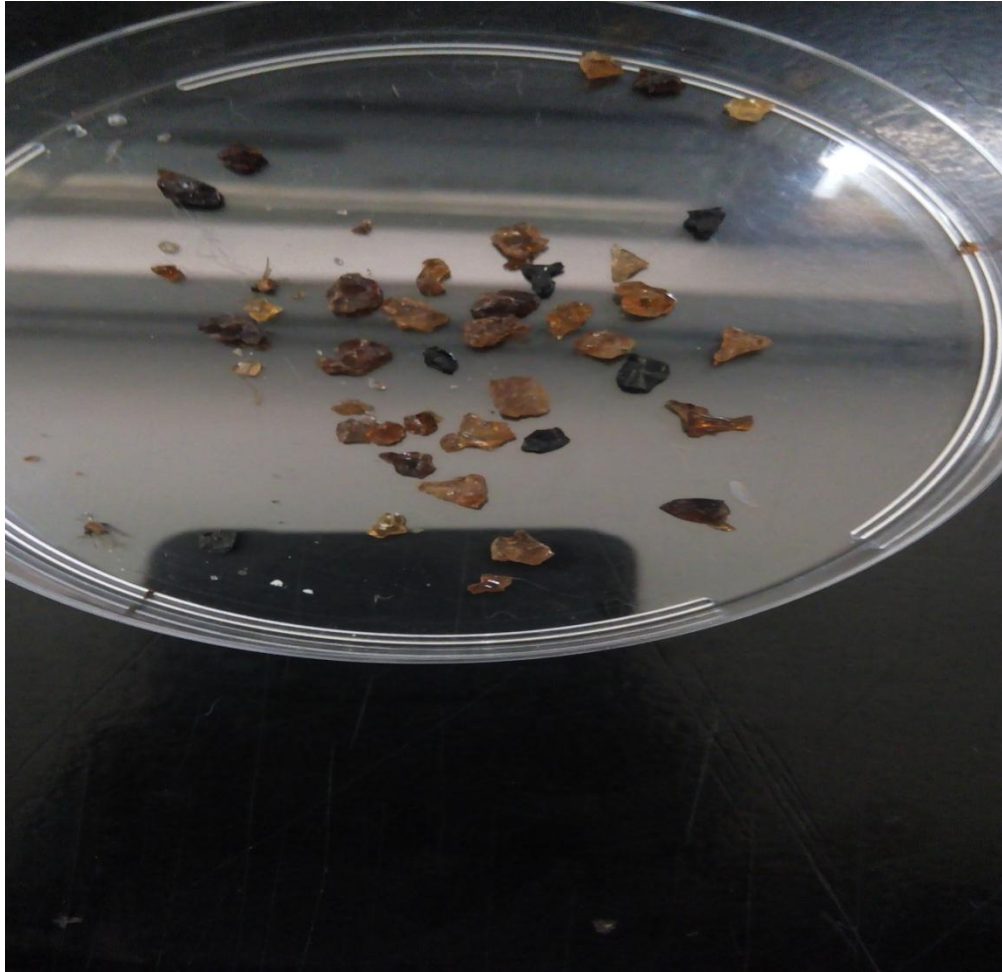


Plate VI: Silica gel used for mosquitoes preservation.



Plate VII: The vector test[®] kit for WNV Antigen Assay