SEROPREVALENCE AND MOLECULAR DETECTION OF WEST NILE VIRUS IN FEBRILE PATIENTS ATTENDING SOME HOSPITALS IN KADUNA STATE, NIGERIA

BY

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JULY, 2017

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JOSEPHINE ASHULEE MA'AJI, B.Sc (JOS) 2009

(P13SCMC8057)

A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES,

AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD

OF A MASTER OF SCIENCE DEGREE IN MICROBIOLOGY,

DEPARTMENT OF MICROBIOLOGY,

FACULTY OF LIFE SCIENCES,

AHMADU BELLO UNIVERSITY,

ZARIA, KADUNA STATE,

NIGERIA.

JULY, 2017

DECLARATION

I declare that the work in this Dissertation entitled ‘’**Seroprevalence and Molecular Detection of West Nile Virus in Febrile Patients attending some Hospitals in Kaduna State, Nigeria**.’’ has been carried out by me in the Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this Dissertation was previously presented for another degree or diploma in this or other Institution.

_____ _____

Ma’aji, Josephine Ashulee Date

CERTIFICATION

This Dissertation entitled '**Seroprevalence And Molecular Detection Of West Nile Virus In Febrile Patients Attending Some Hospitals In Kaduna State, Nigeria.**' by MA'AJI, JOSEPHINE ASHULEE (P13SCMC8057) meets the regulations governing the award of degree of Master of Science of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This Dissertation is dedicated to God Almighty the giver of all that is good, wisdom and knowledge and to my Parents Late Mr Apilimi Ma'aji and Late Mrs Christiana Ma'aji.

ACKNOWLEDGEMENTS

All thanks to my creator in heaven who has made this achievement possible, the wisdom, direction and resources all came from You, I live all my days for You.

My profound gratitude goes to my supervisors Prof. O. S. Olonitola and Dr E. E. Ella for their relentless effort in guiding and encouraging me through the period of this work. Thank you for all your contributions and support, may you be blessed and favoured in all that you do.

I am most indebted to my parents Late Mr and Mrs Apilimi Ma'aji for teaching and helping me in the pursuit of my dreams. If my wishes were to be granted, you will both be here to share in this achievement. May God rest your souls.

My appreciation goes to the University of Ahmadu Bello University especially the Department of Microbiology for this opportunity to fulfill my dreams, together we can make the world a better place. My head of Department, lecturers and other staff of the Department you have all helped made history. God bless you all.

Special thanks to my wonderful siblings Susan (Mummy Ella), Aye, Onumushi, Grace (Mama) who stood by me through every difficulties. Your show of affection is appreciated. To Peter, thanks for always being there for me and to kind hearted friends; Salamat, Judy, Azumi, Bumni, Deborah, Bety and my Uncle Mr Idris Dahiru your words of encouragement and friendship is well appreciated. I cannot forget Emmanuel Atai who has helped in the statistical analysis of this work, thank you and God bless.

Thanks to Mr Y. Y. Pala from the Department of Public Health, Faculty of Veterinary Medicine who assisted me with some parts of the work, sir your effort is appreciated. To all the Laboratory technicians in virology laboratory, Department of Microbiology that helped in the course of this work, your efforts have contributed to the success of this work. I am most grateful.

To all my course mates the 2013 postgraduate set, you have all made my stay in school enjoyable and fun filled, may all our dreams in life come true.

Finally to all who have contributed to the success of this work but have not been mentioned here, your effort will not go unrewarded, God will richly bless you all.

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ABBREVIATIONS

ADE – Antibody Dependent Enhancement

bp – Base pair

CCL – Chemokine Ligand

CCR – Chemokine Receptors

CDC – Centre for Disease Control and Prevention

cDNA – complimentary Deoxyribonucleic acid

CNS – Central Nervous System

CSF – Cerebro Spinal Fluid

Cx – *Culex*

DC-SIGN – Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

ELISA – Enzyme-linked Immunosorbent Assay

GTPase – Guanosine Triphosphatase

IFN – Interferones

IgG – Immunoglobulin G

IgM – Immunuglobulin M

ISR –Immune Status Ratio

JEV – Japanese Encephalitis virus

LBP – Laminin Binding Protein

MHC – Major Histocompatibility Complex

M-MuLV – Moloney Murine Leukemia virus

MTase – Methyl Transferase

NCA – Normal Cell antigen

NK – Natural Killer cells

NS – Non Structural-Protein

NTPase – Nucleoside Triphosphatase

PUO – Pyrexia of Unknown Origin

RTPase – RNA Triphosphatase

RT-PCR – Reverse Transcription-Polymerase chain reaction

SLEV – Saint Louis Encephalitis virus

TLR – Toll-like Receptors

WNND – West Nile Neuroinvasive Disease

WNRA – West Nile antigen

WNV - West Nile Virus

ABSTRACT

West Nile Virus (WNV) infection has become a major public health problem throughout the world. It is a mosquito-borne virus that is frequently included in the routine diagnosis of febrile illness in some countries excluding Nigeria. This research was aimed at determining the seroprevalence of WNV in patients with febrile illness attending some hospitals in Kaduna state, Nigeria. Three hospitals were selected for this study, one from each senatorial district of Kaduna state. Serological screening for WNV antibodies was done using Enzyme Linked Immunosorbent Assay (ELISA) to screen for IgM antibodies against the virus, using West Nile IgM ELISA test kit (Diagnostic Automation/Cortez Diagnostics, Inc. USA) at the Virology Laboratory, Department of Microbiology, ABU Zaria. A total of one hundred and thirty five (135) samples were collected from febrile patients out of which 7(5.2%) were seropositive for IgM antibodies. From the three senatorial district of Kaduna state, Kaduna Central had the highest prevalence of 3.0%, Kaduna South had a prevalence of 2.2% and there was no positive sample recorded in Kaduna North. This study showed that the male population had a higher prevalence of 3.7% and the result also showed that patients in the age group 31-40years had a higher prevalence of 2.2% as compared to the other age groups. In this study, there was a statistically significant association (P=0.04) between WNV infection and occupation, thereby revealing that farming may pre dispose one to WNV infection. The result from this study shows that the use of mosquito nets is protective against WNV infection. Certain risk factors such as Blood transfusion, Organ transplant, chemotherapy, cancer, hypertension and diabetes were not statistically significant in this study. All the seven serum samples that were seropositive for WNV IgM were subjected to Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) to detect the viral RNA. The RNA was first extracted, amplified then the products were resolved using gel electrophoresis. The nucleotide sequence of primers used for the reaction included WNENV-both reverse and forward, WN233 and 640, WN3'NC-both reverse and forward, where RNA to WNV; WNENV (431bp) targeting the envelop gene and WN (408bp) targeting the C terminal portion of the C gene and the N terminal part of the PrM gene were detected in the study area. PCR detected the presence of WNV in Kaduna metropolis and so the prevaence of WNV in the area was established through the findings in this study. Infection by WNV should be a consideration in the diagnosis and treatment PUOs.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

West Nile virus (WNV) is a member of the genus *Flavivirus* in the family *Flaviviridae* (Heinz *et al*., 2000). It is a member of the Japanese encephalitis virus serocomplex, which contains several medically important viruses associated with human encephalitis. It is found in both tropical and temperate regions (Fonseca *et al*., 2005).

West Nile Virus is a genetically and geographically diverse virus requiring birds as an important host. This is because the virus replicates in birds; attaining viremia that is sufficiently high to infect mosquitoes which then serve as vector to other hosts such as humans, horses (Fonseca *et al*., 2005). Humans and horses may suffer serious disease or death from WNV infection but are considered incidental hosts which do not participate in the WNV life cycle because they do not develop sufficient viremia to infect mosquito vectors (Van Der Meulen *et al*., 2005). The main route of human infection is through the bite of infected mosquitoes, which are the prime vector, with birds being the most commonly infected animal and serving as the prime reservoir host (Fonseca *et al*., 2005). Mosquitoes of the genus *Culex* have been reported as the most important bridge vectors, though the Genus *Aedes* may also serve as important bridge vectors.WNV has also been identified in some reptilian species, including alligators and crocodiles and also in amphibians (Steinman *et al*., 2003)

West Nile virus is recognized as being widespread among flaviviruses (Asnis *et al*., 2001). It was first isolated from a human experiencing a febrile syndrome in the West Nile district of Uganda in 1937 (Smithburn *et al*., 1940). West Nile Virus disease occurred only sporadically and was considered a minor risk for humans, until an outbreak in Algeria in 1994, with cases of WNV-

associated encephalitis, and the first large outbreak in Romania in 1996, with a high number of cases with neuroinvasive disease (Nash *et al.,* 2001). Today, the West Nile Virus is widely distributed throughout Africa, the Middle East, Europe, Asia, Central America, Mexico, Caribbean and the Unites States of America (Komar, 2003).

Researchers believe West Nile virus is spread when a mosquito bites an infected bird and then bites a person. Few people develop severe disease or even notice any symptoms at all. West Nile Virus has three different presentations in humans. The first is an asymptomatic infection; the second is a mild febrile syndrome termed West Nile Fever; the third is a neuroinvasive disease which can be life threatening leading to West Nile meningitis or encephalitis, depending on the part of the body that is affected (Chowers *et al*., 2001).

The second, febrile stage has an incubation period of 2 to 8 days followed by fever, headache, chills, diaphoresis (excessive sweating), weakness, lymphadenopathy (swollen lymph nodes), drowsiness, pain in the joints and symptoms like those of influenza or the flu (Chowers *et al*., 2001). Occasionally there is a short-lived truncal rash and some patients experience gastrointestinal symptoms including nausea, vomiting, loss of appetite, or diarrhoea. All symptoms resolve within 7 to 10 days, although fatigue can last for some weeks and lymphadenopathy can take up to two months to resolve (Smithburn *et al*., 1940)

West Nile virus can also be spread through blood transfusions and organ transplants (CDC, 2002). It is possible for an infected mother to spread the virus to her child through breast milk (Chowers *et al*., 2001). Risk factors independently associated with developing a clinical infection with WNV include a suppressed immune system, history of organ transplantation, recent chemotherapy, aging or advance in age, and pregnancy (Kumar *et al*., 2004). For neuroinvasive

disease the additional risk factors include aging, sex, hypertension, and diabetes mellitus (Jean *et al*., 2007).

The most accurate way to diagnose WNV infection employs a serological test, which analyse blood or cerebrospinal fluid (CSF) sample for antibodies against the virus (Naides, 2007). Definitive diagnosis of WNV is obtained through detection of virus-specific antibody IgM and neutralizing antibodies. West Nile virus meningitis and encephalitis produce similar degrees of CSF pleocytosis and are often associated with substantial CSF neutrophilia (Tyler *et al.*, 2006). A positive test for West Nile IgG in the absence of a positive West Nile IgM is indicative of a previous flavivirus infection and is not by itself evidence of an acute West Nile virus infection. Consideration of a differential diagnosis is required when a patient presents with unexplained febrile illness, severe headache, encephalitis or meningitis. Diagnostic and serologic laboratory analysis using polymerase chain reaction and viral culture of CSF to identify the specific pathogen causing the symptoms, is the only currently available means of differentiating between aetiology of encephalitis and meningitis (Bleck, 2007)

Complications from severe West Nile virus infection include: Permanent brain damage, permanent muscle weakness (mimicking polio) and death (Naides, 2007). Approximately 10% of patients with brain inflammation do not survive (Klee *et al.*, 2004).

1.2 Statement of Research Problem

WNV is recognized as being widespread among flaviviruses (Asnis *et al*., 2000). Its geographical distribution spans from Africa, West Asia and Middle East as well as Eastern Europe and the United States (Cantile *et al*., 2001). Generally the information on the current status of infections in Nigeria is poor. In other countries several arboviruses are frequently

considered in the etiology of acute febrile illness. Most health institutions in Nigeria including the Kaduna metropolis lack appropriate diagnostic facilities for this group of viruses and also seem to attach less importance to viral fever.

West Nile Virus shares the same vector and transmission route as malaria fever and this vector is very common in the tropics especially in Nigeria. West Nile virus infection could be non-specific that it, often escapes medical attention (Monath *et al*., 2001). In such situation West Nile virus and other arbovirus infections are misdiagnosed and so inappropriately treated thereby resulting in high rate of morbidity, complications and mortality (Baba *et al*., 2006).

About 70-80% of individuals infected by WNV are asymptomatic and do not seek medical attention, serving as reservoir for transmission through direct blood contact, if not treated. While approximately 20-30% have "West Nile Fever" without evidence of neurologic involvement (Tsai *et al.,* 1998; Mostashari *et al*., 2001; Busch *et al.,* 2006; Sejvar, 2007), neuroinvasive disease accounts for less than 1% of WNV cases, but can result in severe neurological sequelae and death (Debiasi *et al.,* 2006). The mortality rate is reported to be between 10% and 30% for patients with neuroinvasive WNV disease, or <0.1% of all infected patients (Sejvar, 2014).

With the lack of data on the disease in the study area and the country at large, WNV remains a serious threat to the public health, especially to very young, elderly and immunocompromised individuals and there is currently no antiviral treatment to cure WNV infections and only supportive care can be administered (Loginova *et al.*, 2009).

1.3 Justification

West Nile Virus has not been widely studied in Nigeria due to lack of awareness of the disease as well as diagnostic tools. Most cases of fever in Nigeria are considered and treated in the light of the malaria and typhoid, overlooking WNV as a possible cause of some feverish presentation.

Many presentations in Nigeria are documented as pyrexia of unknown origin (PUO) especially if they fail to respond to anti malaria and anti microbial drugs (Olaleye *et al*., 1990).

Moreover, the early symptoms of West Nile virus mimic malaria, typhoid, measles and influenza thereby rendering the clinical diagnosis of this virus confusing.

To further achieve a success in the advocacy to combat the presence of this virus of medical importance, there is the need to create awareness on the importance of the screening for West Nile viruses in health facilities in the country. Therefore, results gotten from this study could provide information that will stimulate the relevant bodies to include West Nile virus and other arboviruses diagnosis in the routine diagnosis of febrile illnesses, and also identify risk factors responsible for the transmission of the West Nile virus.

1.4 Aim

The aim of this study is to determine the seroprevalence and detect West Nile virus among febrile patients attending selected hospitals in Kaduna metropolis, Nigeria.

1.5 Objectives

The objectives of this study were to:

1. Determine the presence of IgM antibody specific for West Nile virus among the study population using the enzyme linked immunosorbent assay (ELISA).
2. Detect the West Nile virus in the febrile patients that are seropositve for the virus using Reverse Transcriptase –Polymerase Chain Reaction (RT-PCR).
3. Determine the risk factors as well as socio-demographic factors associated with West Nile virus infection in the study population using structured questionnaire.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of West Nile Virus (WNV)

West Nile virus (WNV) was first isolated in Uganda (West Nile district) in 1937 from the blood of a native Ugandan woman (Smithburn *et al*., 1940) and until the end of the 20th century was considered a cause of viral encephalitis limited only to Africa and Asia. It became a global public health concern after the virus was introduced to North America and especially New York in 1999 (Lanciotti *et al*., 1999). Romania had recorded the first large outbreak of West Nile neuroinvasive disease (WNND) in Europe in 1996, with 393 confirmed cases (Tsai *et al*., 1998). Since then, major outbreaks of WNV fever and encephalitis have been reported in several regions throughout the world causing human and animal deaths (HCDCP, 2012).

While the introduction and progress of WNV through the New World could be studied as an isolated case, epidemics of WNV were believed to have occurred throughout most of Africa, the Middle East, and South Asia before clinical WNV was observed in humans in those areas (Smithburn *et al*., 1940). A 1939-1940 serosurvey found widespread human seropositivity for WNV, determined by comparison of neutralization titers for WNV, St. Louis encephalitis virus (SLEV), and Japanese encephalitis virus (JEV), in Uganda, Sudan, the current Democratic Republic of the Congo, and Kenya, with seropositivity over 50% in some localities (Smithburn *et al*., 1940). Seropositivity was also found in western Nigeria, in samples collected in 1951 and 1955 (Macnamara *et al*., 1959). In South Africa, seropositivity in humans who had not traveled, monkeys, domestic animals, and juvenile birds was also demonstrated (Zaayman and Venter,

2012). Therefore, WNV had been demonstrated over a wide geographic range in Africa before clinical infections were observed in most locations.

In the last decade, lineage 2 strains, considered of low virulence, was introduced in Central and South Eastern Europe and were incriminated as causative agents of major human and animal disease outbreaks. A great number of WNV infections in humans occurred in 2010 and 2011 in Greece, with 363 laboratory confirmed cases and 44 deaths (HCDCP, 2012). West Nile Virus lineage 2 strains were first detected from pools of *Culex* mosquitoes (Papa *et al*., 2011). The unexpected high virulence of lineage 2 strains created major concerns regarding the pathogenic potential of evolving and mutating WNV strains.

2.1.1 Classification of WNV

West Nile virus is a single-stranded RNA virus of the family *Flaviviridae*, genus *Flavivirus*. It is a member of the Japanese Encephalitis Virus serocomplex, which comprises of several medically important viruses that cause encephalitis in humans: Japanese encephalitis, St. Louis encephalitis, Murray Valley encephalitis, and Kunjin. More than 58 members belong to the *Flaviviridae* family, whose name is derived from the word "flavi", Latin for "yellow", because one of the most famous flaviviruses is the Yellow Fever Virus (ICTV, 2012).

Flaviviridae family is further divided into three genera: flaviviruses, pestiviruses and hepaciviruses. The Flavivirus genus is the largest with at least 53 species divided into 12 serologically related groups. Of these, the Japanese Encephalitis Virus (JEV) group is the one with the most human-associated disease viruses; Japanese Encephalitis Virus, St. Louis Encephalitis Virus, Murray Valley Encephalitis Virus and West Nile Virus are four members of the JEV group that have been associated with widespread human and animal disease outbreaks (Schweitzer *et al*., 2009).

West Nile Virus is a genetically and geographically diverse virus. Four or five distinct WNV genetic lineages have been proposed based on phylogenetic analyses of published isolates (Bakonyi *et al*., 2005). Their genomes differ from each other by more than 20–25% and correlate well with the geographical point of isolation. Lineages 1, 2, and 5 of WNV have been associated with significant outbreaks in humans (Bakonyi *et al*., 2005). Lineage 1 is distributed widely throughout the world and consists of two clades: 1a and 1b (Petersen and Roehrig, 2001).

2.1.2 Structure and Genome of WNV

The WNV genome is a positive single stranded RNA of approximately 11000 nucleotides surrounded by an icosahedral nucleocapsid which is contained in a lipid bi-layered envelope, of approximately 50nm in diameter (**Figure 2.1B**). The genome is transcribed as a single polyprotein that is cleaved by host and viral proteases into three structural (C, prM/M, and E)-Figure 2 and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins as seen in **Figure 2.2** (Chambers *et al*., 1990). Studies also reported that a larger NS1-like (NS1') viral protein, which is often detected during infection, is the possible resultant of ribosomal frame shifting (Chambers *et al*., 1990).

The viral capsid is approximately 30 nm in diameter and consists of C protein dimers, the basic component of nucleocapsids, with the RNA binding domains located at the C- and N-termini separated by a hydrophobic region (Diamond and Brinton, 2009). The hydrophobic regions of the C dimers form an apolar surface which binds to the inner side of the viral lipid membrane (Ma *et al*., 2004). In immature virions, the lipid bi-layered envelope that coats the nucleocapsid contains 180 molecules each of E and prM proteins (**Figure 2.1B**) organized into 60 asymmetric trimeric spikes consisting of prM-E heterodimers (Zhang *et al*., 2003). The

transition from immature to mature virions starts with the release of the N-terminal prepeptide from the prM protein after cleavage by a furin-like protease in the trans-Golgi compartment of the infected cell (Stadler *et al*., 1997).

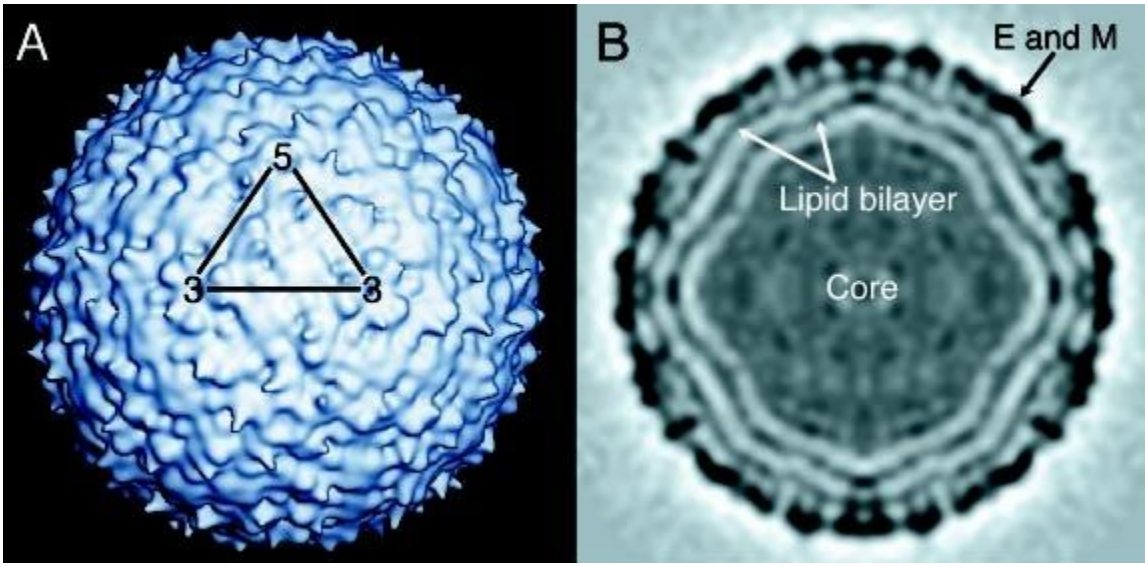


Figure 2.1: Structure of West Nile virus determined by Cryo-Envelope Membrane. (A) A surface shaded view of the virion, one asymmetric unit of the icosahedron is indicated by the triangle. The 5-fold and 3-fold icosahedral symmetry axes are labeled. (B) A central cross section showing the concentric layers of density. Virion core, lipid bilayer and proteins E and M are indicated.

Source: http://www.intechopen.com/books/viral-replication/west-nile-virus-basic-principles-replication-mechanism-immune-response-and-important-genetic-determi

2.1.3 Proteins in the Genome of WNV

The viral nonstructural proteins are responsible for regulating viral mechanisms of transcription, translation, replication and attenuation of host antiviral responses (**Table 2**).

NS1 protein functions as a cofactor for viral RNA replication and is the only nonstructural protein that is secreted in high levels (up to 50μg/ml) in the serum of WNV infected patients and has been connected with severe disease (Macdonald *et al*., 2005). Many theories have been proposed regarding the contribution of NS1 to the pathogenic mechanism of WNV: it has been proposed to elicit hazardous autoantibodies (Chang *et al*., 2002), to contribute to the formation of various immune complexes circulating in the host organism (Young *et al*., 2000), elicit antibodies against NS1 which cause endothelial cell damage (Lin *et al*., 2003), or to minimize immune response targeting of WNV decreasing recognition of infected cells by the complement system.

The NS2A is a hydrophobic, multifunctional membrane-associated protein which plays an important role in RNA replication and viral particles assembly (Kummerer and Rice, 2002). NS2A is also the major suppressor of beta interferon (IFN-β) transcription, thus inhibiting interferon response, one of the first lines of defense of the host (Liu *et al*., 2006). NS2B is a cofactor required for NS3 proteolytic activity. NS3 is a multifunctional protein, with two distinct functional domains. The protease comprises the N-terminal amino acid residues of NS3, while the carboxylated terminus contains a helicase, a nucleoside triphosphatase and a RNA triphosphatase (Chappell *et al*., 2008).

The NS3 trypsin-like serine protease is only active as a heterodimeric complex with its cofactor, NS2B. In the cytoplasm of infected host cells, this heterodimeric complex (NS2B-NS3pro) is responsible for post-translational cleavage of the viral polyprotein to release structural and non-

structural viral proteins that are essential in viral replication mechanism and virions assembly. Cleavage takes place at the C-terminal side of two basic residues (e.g., RR, KK, and RK), a sequence motif that occurs at the junctions of NS2A/B, NS2B/3, NS3/4A, and NS4B/5. It also cleaves the viral polyprotein within the C-terminal region of protein C and protein NS4A as a necessary precursor to cleavage of prM and NS4B, respectively, by cell signalase in the lumen of the endoplasmic reticulum (Gorbalenya *et al*., 1989 ; Robin *et al*., 2009). The C-terminal of NS3 is characterized by the presence of motifs with homology to supergroup II RNA helicases, to a RNA-stimulated nucleoside triphosphatase (NTPase) and to a RNA triphosphatase (RTPase). (Wengler, 1993). The NTPase activity provides the chemical energy which is necessary to unwind RNA replication intermediates into forms that can be amplified by the NS5 RNA-dependent RNA polymerase (Li *et al*., 1999). The RTPase dephosphorylates the 5' end of viral RNA, before cap addition by the N-terminal methyl transferase region of NS5 (Luo *et al*., 2008). RNA helicases travel along RNA in a 3' to 5' direction fueled by ATP hydrolysis; this movement opens secondary structures and displaces proteins bound to RNA (Frick *et al*., 2007). Thus, together with the NS5 polymerase, with which NS3 is in tight association and interaction, the NS3hel plays an important role in flavivirus replication. However, a complete picture of the mechanism by which NS3hel associates with RNA template is not yet completely known. NS4A, along with NS4B and NS2A, are the least known flavivirus proteins (Frick *et al*., 2007).

The NS4A precise functional role has not been sufficiently characterized, although evidence suggests a role of "organizer" of the replication complex in flaviviruses (**Table 2**). Its N-terminal is generated in the cytoplasm after cleavage by the NS2B-NS3 protease complex, whereas the C-terminal region (frequently designated 2K fragment) serves as a signal sequence for the translocation of the adjacent NS4B into the endoplasmic reticulum lumen. The 2K fragment is

removed from the N terminus of NS4B by the host signalase, however a prior NS2B-NS3 protease complex activity at the NS4A/2K site is required (Lin *et al*., 1993). Proteolytic removal of the 2K peptide also induces membrane alterations (Miller *et al*., 2007). NS4A was proven to act as a cofactor for NS3 helicase allowing the helicase to sustain the unwinding rate of the viral RNA under conditions of ATP deficiency (Shiryaev *et al*., 2009).

The NS4B colocalizes with viral replication complexes and dissociate NS3 from single-stranded RNA, thereby enabling it to bind to a new dsRNA duplex, consequently enhancing the helicase activity and modulating viral replication (George *et al*., 2013). In addition, NS4A, NS4B, along with NS2A and NS5 proteins appear to inhibit the interferons α/β response of the host (Muñoz-Jordán *et al*., 2003).

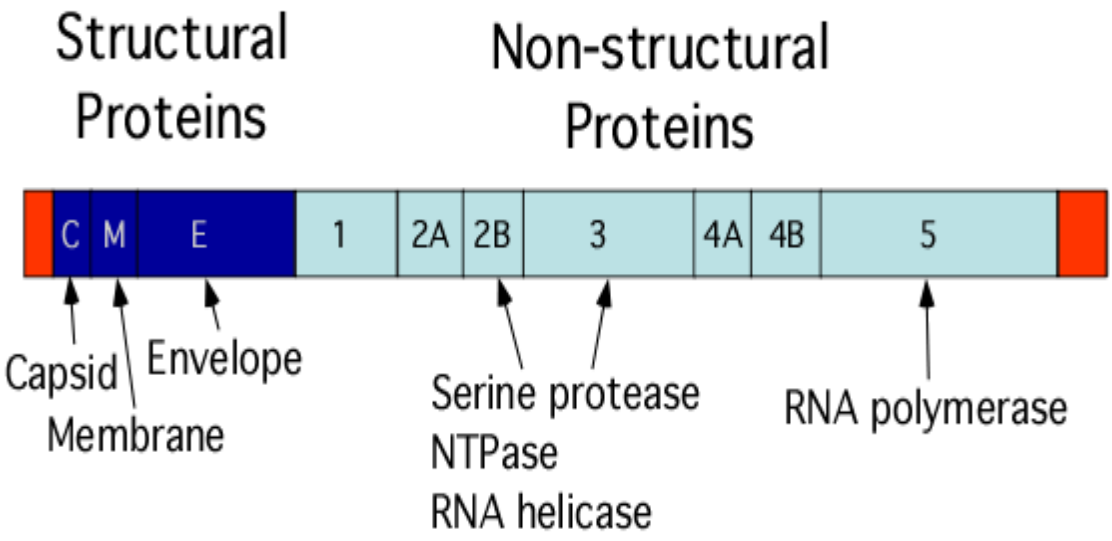


Figure 2.2: The Genome of West Nile Virus

Source: (http://www.microbiologytext.com/index.php)

Table 2.1 Functions of West Nile virus Nonstructural Proteins

Non structural Proteins	Functions
NS1	Co-factor for viral RNA replication, pathogenic mechanism in early infection (decrease complement recognition
NS2A	Viral RNA replication and virions assembly, major suppressor of IFN- β transcription
NS2B	Co-factor for NS3 pro activity, interferons antagonist
NS3	Serine protease, RNA helicase, RTPase, NTPase
NS4A	"Organizer" of replication complex, inhibitor of interferonsα/b B host response.
NS4B	Inhibitor of interferon α/ β host response,enhancer of NS3 activity.
NS5	Methyltransferase, RNA-dependent RNA polymerase, Interferon antagonist

(George *et al*., 2013)

NS5 is the C-terminal protein of the viral polyprotein and is the largest and most conserved of flaviviruses proteins. The N-terminal region of NS5 contains an S-adenosyl methionine methyltransferase (MTase) domain, part of the viral RNA capping machinery. The cap is a unique structure found at the 5' end of viral and cellular eukaryotic mRNA, critical for both mRNA stability and binding to the ribosome during translation (Egloff *et al*., 2002). The C-terminal region of NS5 contains a RNA-dependent RNA polymerase which is required for the synthesis of the viral RNA genome (Davidson, 2009). NS5 remains in close interaction with NS3, constituting the major enzymatic components of the viral replication complex, which promotes efficient viral replication in close association with cellular host factors.

2.1.4 Replication Cycle of West Nile Virus

2.1.4.1 WNV attachment, receptors employed and penetration

West Nile Virus has the ability to replicate in various types of cell cultures from a wide variety of species (mammal, avian, amphibian and insect) (George *et al*., 2013). The first step in the infectious cell entry involves the binding of E protein to a cellular molecule-receptor (Smit *et al*., 2011). Several cell molecules have been proven to function as co-receptors for *in-vitro* virion attachment: WNV interacts with DC-SIGN and DC-SIGN-R on dendritic cells (Davis *et al*., 2006). It has been reported to attach to the integrin αvb3, through DIII RGD/RGE sequence, which is an integrin recognition motif (Chu and Ng, 2004).

However a study showed that WNV entry does not require integrin αvb3 in certain cell types suggesting that receptor molecule usage is strain-specific and/or cell type-dependent (Medigeshi *et al*., 2008). Rab 5 GTPase was found to be a requirement for WNV and Dengue Virus cellular entrance (Krishnan *et al*., 2007). Laminin binding protein (LBP) is also a putative receptor for

the WNV, with proven high specificity and efficiency between LBP and DII of E protein (Bogachek *et al*., 2008).

Many other attachment factors have been identified for flaviviruses, including CD14 (Chen *et al*., 1999), GRP78/BiP, 37-kDa/67-kDa laminin binding protein (Jindadamrongwech *et al*., 2004), heat-shock proteins 90 and 70 (Reyes-del *et al*., 2005), and even negatively charged lycoaminoglycans, such as heparan sulfate, which are expressed in various cell types, though, for the latter, some studies did not reveal specific binding of WNV with heparan sulfate (Lee *et al*., 2006). After the viral attachment via the cellular receptors, WNV enters the cell through clathrin-mediated endocytosis. The treatment of cells with chemical inhibitors such as chloropromazine that prevent formation of clathrin-coated pits or expression of negative mutants of Eps15 in cells, a protein involved in clathrin-coated pit formation inhibited WNV infection (Chu *et al*., 2006). The endosome environment is characterized by acidic pH, which triggers conformational changes of the E glycoprotein. The first step involves the disruption of the E protein rafts and dissociation of the E homodimers to monomers. An outward projection of DII takes place, and the fusion loop of DII is exposed to the target membrane. The E proteins insert their fusion loops into the outer leaflet of the cell membrane. Three E monomers interact with one another via their fusion loops to form an unstable trimer which is stabilized through additional interactions between the DI domains of the three E proteins (Liao *et al*., 2010). Next, DIII is believed to fold back against the trimer to form a hairpin-like configuration. The energy released by these conformational changes induces the formation of a hemifusion intermediate, in which the monolayers of the interacting membranes are merged (Liao *et al*., 2010).

2.1.4.2 WNV viral uncoating and replication within host cells

Finally, a fusion pore is formed and after enlargement of the pore, the nucleocapsid is released into the host cell. The viral RNA is released by the nucleocapsid with a yet unknown mechanism and is translated (George *et al*., 2013). The early gene product; a polyprotein is cleaved at multiple sites by the NS3 serine protease and the host signal peptidase within the lumen of the endoplasmic reticulum. At the same time, the viral RNA-dependent RNA polymerase copies complementary negative polarity (–) strands from the positive polarity genomic (+) RNA template, and these negative strands serve as templates for the synthesis of new positive viral RNAs (Cleaves *et al*., 1981). Studies showed that RNA replication can continue without protein synthesis, and that from a (+) strand RNA only one (-) strand RNA can be synthesized at a time, while from a (-) strand RNA multiple (+)strand RNAs can be simultaneously copied as seen in **Figure 2.3**, however virion assembly cannot take place if sufficient protein synthesis has not been performed: Each virion contains 180 copies each of E and prM structure proteins and only one genomic copy (Chu and Westaway, 1987).

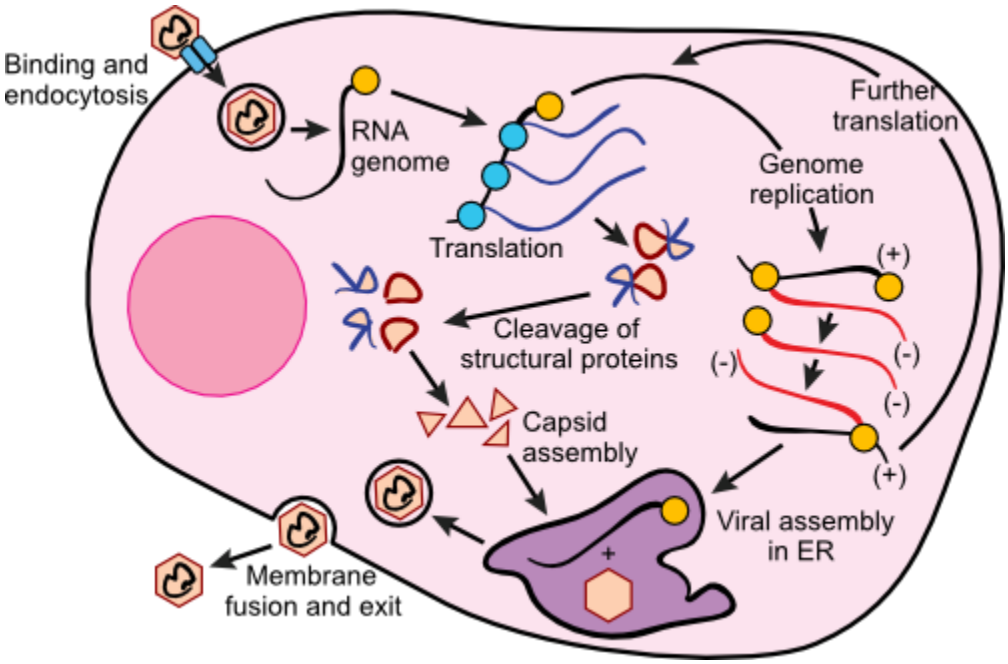


Figure 2.3: The life Cycle of West Nile Virus

Source: (http://www.microbiologytext.com/index.php)

2.1.4.3 Viral assembly and release

During West Nile virion assembly, C proteins bind to the newly replicated RNA and wrap around it to form an icosahedral shell. The nucleocapsid acquire envelope from cellular membrane of the endoplasmic reticulum and bud into the lumen as immature virions on which E and prM proteins form 60 heterotrimeric spikes. Immature virions are then transported to the mildly acidic compartments of the trans-golgi network triggering a rearrangement of E proteins on the immature virion; the lower pH induces a structural transition such that E proteins form 90 antiparallel homodimers on the surface of the virion (Konishi and Mason, 1993)**.** Under acidic conditions, prM remains associated with the virion and protrudes from the surface of an otherwise smooth virus particle. This pH-dependent conformational change increases the susceptibility of prM to a furin-like serine protease Wengler, 1989). The pr peptide dissociates from the particle upon release of the virion to the extracellular milieu by exocytosis, which starts 10-12 h after cell infection. However, this furin processing of prM is rather inefficient and many virions still contain prM proteins even after their release to the extracellular milieu, which will reorganize back to prM/E heterodimers. This inefficient and incomplete maturation leads to the secretion of a mixture of mature, immature and partially mature particles from flavivirus-infected cells. A high number of prM-containing particles have been described for WNV (George *et al*., 2013). The immature virions were considered to be unable to cause infection as they cannot undergo the structural rearrangements required for membrane fusion (Moesker *et al*., 2010). However, newer studies proved that even immature virions of flaviviruses can cause infections (Rodenhuis-Zybert *et al*., 2010). Regarding partially immature virions, a study have shown that they can also be infectious (Plevka *et al*., 2011). It seems that the matured part of these virions is responsible for cell binding and entry after which the further processing of remaining prM may

take place inside the cell. Further studies are needed to estimate the "cut-off" regarding the number of prM proteins on viral surface that allow the viral particle to be infectious (George *et al*., 2013).

2.1.5 Immunity in WNV

Immune response of animals and humans to WNV infection is divided into Innate and Adaptive Response.

2.1.5.1 The role of innate immunity

Interferons play an essential protective role limiting infection of many viruses. IFN-α/β are produced by most of the cells following viral infection and are capable of inducing an antiviral state in the cell by "activating" the relevant genes. They also create a linkage between innate and adaptive immune responses by various mechanisms such as activation of B and T cell lineages or dendritic-cell maturation (Marrack *et al*., 1999; Asselin-Paturel *et al*., 2005; Le Bon *et al*., 2006). IFN-γ is produced by γδTcells, CD8+T cells, and natural killer cells and limits early viral dissemination to the CNS through several mechanisms (Schroder *et al*., 2004). However, WNV has evolved various counter measures against interferons functions (Diamond, (2009). Hence, IFN administration cannot be considered to be of much significant therapeutic importance for WNV disease control (Chan-Tack and Forrest, 2005).

Several nucleic acid sensors e.g. TLR3, cytoplasmic dsRNA, RIG-I and MDA5 bind to viral RNA and activate transcription factors like IRF3 and IRF7 as well as IFN-stimulated genes (Yoneyama *et al*., 2004).

Complement is a system of proteins in serum and molecules on cell surface that recognize pathogens and induce pathogen clearance. Three pathways exist for complement activation the

classical, the lectin and alternative pathways, which are initiated by binding of C1q, mannan-binding lectins or hydrolysis of C3 respectively. All three pathways have been found to be important for controlling WNV lethal infections (Mehlhop *et al*., 2005; Roozendaa and Carroll, 2006).

There are data suggesting that macrophages and dendritic cells may directly inhibit WNV. Macrophages can control infection through cytokine and chemokine secretion, enhanced antigen presentation and direct viral clearance (Ben-Nathan *et al*., 1996). γδT cells also limit WNV infection in an early stage (Wang *et al*., 2006).

2.1.5.2 Adaptive response

Humoral immunity plays a vital role in protection from WNV infection. Experimental studies demonstrated complete lethality of B-cell-deficient and IgM/mice infected with WNV, whereas they were protected by transfer of immune sera (Diamond *et al*., 2003). IgM titers at day 4 p.i. could predict the disease outcome at prospective experiments. IgG can also protect from infection, however, in primary infection their role is less vital: as it is produced after days 6-8, the disease outcome would have been determined, since both viral shedding to CNS and clearance from tissues must have occurred (Shrestha and Diamond, 2004). The vast majority of neutralizing antibodies are directed against all three domains of E protein. However the most potent neutralizing antibodies are directed on DIII possibly inhibiting viral fusion at post-attachment stage (Gollins and Porterfield, 1986; Nybakken *et al*., 2005).

In humans, antibodies against prM have also been recognized but with limited neutralizing activity (Nybakken *et al*., 2005). Antibody neutralization is a procedure where multiple antibodies, above an estimated threshold “manage” to neutralize the activity of the virion and

render it non-infectious. This threshold was estimated to be 30 antibodies per virion for a highly accessible epitope of DIII of E protein (Pierson *et al*., 2008). The level of neutralizing antibodies does not always correlate with protection against WNV. Steric phenomena because of the dense icosahedral arrangements of these proteins do not allow the equivalent display of all the epitopes. Maturation of the virus thus reduces the accessibility of some of the epitopes on the virion to neutralize Abs (Nelson *et al*., 2008). Thus, these antibodies cannot efficiently neutralize the virus even at high levels of concentration. This can lead antibody dependent enhancement (ADE) of infection in cells bearing activating Fc-γ receptors (Klasse and Burton, 2007; Pierson *et al*., 2007) and thus a mild infection with sufficient levels of antibodies can become even life threatening due to the inability of the antibodies to neutralize the virus.

Antibodies against NS1, a protein secreted in the serum of patients during acute phase of disease and expressed on the surface of infected cells considered to be a cofactor in virus replication, have been found to be non-neutralizing but protective through both Fc-γ receptor-dependent and independent mechanisms (Chung *et al*., 2006).

The mechanism of T lymphocytes (part of cellular response mechanism) have been demonstrated to be vital for the protection against WNV infection. On recognizing an infected cell through the viral antigen fragments associated with MHC class I molecules on the infected cells' surface, cytotoxic (CD8+) T cells secrete cytokines and lyse the cells directly (perforin, granzymes A and B) or indirectly via Fas-Fas ligand interactions (Russell and Ley, 2002; Shrestha *et al*., 2006). Studies showed that for the protection against lineage I, perforin played the most important role and, in contrast, lineage II strain Sarafend was controlled more efficiently by granzymes (Wang *et al*., 2004). CD4+T cells contribute through multiple mechanisms, and preliminary data suggest that CD4+T cells restrict pathogenesis *in-vivo* (Sitati and Diamond, 2006). Except IFN–α/β, T-

cell immune response is extremely essential regarding the control of WNV in the CNS, their presence being correlated with virus clearance (Wang *et al*., 2003, Shrestha *et al*., 2006). West Nile Virus infection induces the secretion of the chemokine CXCL10 from neurons, recruiting effector CD8+T cells via the chemokine receptor CXCR3 (Klein *et al*., 2005). Expression of chemokine receptor CCR5 and its ligand CCL5 is up-regulated by WNV and is associated with CNS infiltration of CD4+and CD8+T cells, NK1.1+and macrophages expressing the receptor (Glass *et al*., 2005).

2.1.6 Epidemiology

Since its first isolation in the West Nile district in Uganda in 1937, West Nile Virus has become endemic throughout Africa and areas of the middle east (Hayes, 2001).

Avian species are considered the primary hosts of West Nile virus, and in an endemic region, the virus is maintained in an enzootic cycle between mosquitoes and birds (Work *et al*., 1955). Birds from more than 300 avian species have been reported killed from West Nile virus (CDC, 2009). Disease can also be caused in humans and other mammals, particularly horses, considered as alternative hosts of WNV; main route of infection is through the bite of infected mosquitoes. However, the virus can also spread between individuals by blood transfusion and organ transplantation and few reports have also proposed the transmission from mother to newborn via the intrauterine route or via breast-feeding (CDC, 2002; Charatan, 2002). Most human infections remain asymptomatic. West Nile fever (a mild flu like fever) develops in approximately 20 to 30% of infected persons and West Nile neuroinvasive disease in <1% (Mostashari *et al*., 2001), characterized by encephalitis, meningitis, acute flaccid paralysis and even long-term neurological sequeale (Lim *et al*., 2011).

Seroepidemiological studies suggested that one in four to one in five (20–25%) WNV-infected individuals develop mild illness (Zou *et al*., 2010) and one person in 150 (0.67%) develops West Nile neuro-invasive disease (WNND) (Fratkin *et al*., 2004). Subsequent epidemiological studies using asymptomatic infection data obtained from nucleic acid testing to screen blood donations combined with the reported cases to the CDC concluded that one in 244 to one in 353 infections will progress to WNND (Carson *et al*., 2012 ; Busch *et al*., 2006). These findings suggested that more asymptomatic WNV infections could be identified when prospective studies focused on healthy populations such as blood donors are conducted (Busch *et al*., 2006). A serosurvey following lineage 2 WNV infections in Greece in 2010 yielded estimates of one in 124 to one in 141 infections leading to WNND, with approximately 18% of infected individuals showing symptoms (Ladbury *et al*., 2013).

Nonetheless, horses and humans develop viremia levels of low magnitude ($<10^5$pfu/ml) and in short durations insufficient to infect mosquitoes and thus do not serve as amplifying hosts for WNV in nature (Bunning *et al*., 2002). On the contrary, various avian species, both migratory and sedentary, develop viremia levels sufficient to infect most feeding mosquitoes (Komar *et al*., 2003). Hence, WNV is maintained in an enzootic cycle with wild and domestic birds being the main amplifying hosts and ornithophilic mosquitoes, especially of the *Culex* species remains the main vectors. Moreover, both local movements of resident birds and long-range travel of migratory birds may contribute to the spread of WNV to distant communities (Komar *et al*., 2005). Various studies have provided indirect evidence that WNV is transported by migratory birds, especially via their migration routes from breeding areas of Europe to wintering areas in Africa (Calistri *et al*., 2010 : Valiakos *et al*., 2011).

West Nile Virus strains are grouped into at least 7 genetic lineages (Mackenzie and Williams, 2009). Lineage 1 is the most widespread, comprising of isolates found in Europe, North America, Asia, Africa and Australia. This lineage is further divided into at least two different clades: WNV-1a is found mainly in Africa, Europe, North America and Asia and is further divided in six evolution clusters (May *et al*., 2011). West Nile Virus 1-b comprise of the Australian Kunjin virus. While a third clade comprising of Indian isolates is now classified as Lineage 5 (Bondre *et al*., 2007). Lineage 2 strains are mainly distributed in Sub-Saharan Africa and Madagascar, but in the last decade they have been introduced in Europe. Lineage 3 contain a strain circulating in certain *Culex* and *Aedes* species mosquitoes in southern Moravia, Czech Republic, namely "Rabensburg virus", not known to be pathogenic to mammals (Bakonyi *et al*., 2005). Lineage 4 is represented by a strain isolated from *Dermacentor marginatus* ticks from the Caucasus (Lvov *et al*., 2004). A re-classification of Sarawak Kunjin virus as lineage 6 has been proposed as this strain is different to other Kunjin viruses. The African Koutango virus is closely related to WNV and a seventh lineage has been proposed for this strain. An eighth lineage has been proposed for WNV strains detected in *Culex pipiens* mosquitoes captured in Spain in 2006, which could not be assigned to previously described lineages of WNV (Vazquez *et al*., 2010).

Lineage 2 was considered to be endemic in Sub-Saharan Africa and Madagascar, however, since 2004 the strains have been observed in Hungary from birds of prey (Bakonyi *et al*., 2006) and in 2007 in Russia from mosquito pools during a disease outbreak with 67 human cases (Platonov *et al*., 2008). In 2010 it caused outbreaks in Romania (Sirbu *et al*., 2011) and Greece (HCDCP, 2012) and in 2011 it was detected for the first time in Italy (Savini *et al*., 2012). The Greek and Italian strains showed the highest homology to Hungarian and South African strains, differing from the Russian lineage 2 strains detected in 2007, however, in Italy no major human disease

outbreak occurred; only one human case was reported with mild clinical expression (Bagnarelli *et al*., 2011). Genetic analysis of the Italian strains revealed the presence of histidine at 249 amino acid position of NS3, just like the Hungarian strains, in contrast to the Greek strains that contained proline at that position, the presence of which has been already implicated with high pathogenicity of lineage 1 strains (Brault *et al*., 2007).

2.1.6.1 WNV in Africa, Middle East and Nigeria

West Nile virus was first observed in Africa, in the West Nile district of Uganda, 1937 (Smithburn *et al*., 1940), and thus had been known in the Old World for over 60 years before it crossed the Atlantic. Though it was first isolated from a febrile human case, WNV was observed to cause relatively mild disease in humans and no deaths were reported from the early epidemics studied. (Smithburn *et al*., 1940). A 1939-1940 serosurvey found widespread human seropositivity for WNV, determined by comparison of neutralization titers for WNV, SLEV, and JEV, in Uganda, Sudan, the current Democratic Republic of the Congo, and Kenya, with seropositivity over 50% in some localities.(Smithburn and Jacobs, 1942).

Following its first isolation in 1937, WNV was not isolated again until 1950. During a serosurvey conducted of 251 individuals, mostly children, living in Cairo, isolates were generated from the serum of three children, only one of whom had been diagnosed with a fever (Kokernot *et al*., 1956). The same serosurvey noted that more than 70% of the study participants aged 4 and above had neutralizing and complement-fixing antibodies to WNV (Muñoz-Jordán *et al*., 2005). WNV was also isolated from *Culex* spp. mosquitoes in Egypt in 1952 (Smithburn *et al*., 1954).

The first known isolation of West Nile virus in Israel was from a febrile child in 1951, as part of an outbreak that occurred on an agricultural settlement near Haifa (Bernkopf *et al*., 1953). Morbidity in children in this outbreak was substantially higher than in adults, and subsequent outbreaks in Israel in 1952 and 1953 occurring primarily in adolescents and adults were also identified as WNV, on the basis of isolation of the virus from human cases and serology from human cases and chickens (Marberger *et al*., 1956). However, WNV is believed to have been present in Israel prior to these isolations, because prior outbreaks between 1942 and 1950 were observed to have been similar clinically and epidemiologically to the ones in 1951 and 1952. Illnesses in these cases were generally self-limiting with recovery slower in adults than children (Goldlum *et al*., 1954). West Nile Virus fever was described as a "benign specific short-term fever occurring in epidemic form" and was believed to cause only mild neuroinvasive cases (Marberger *et al*., 1956).

The first fatalities due to WNND were reported in a cluster of elderly patients in 1957; however, overall, neurological involvement in WNV cases was considered unusual (Weinberger *et al*., 2001). In 2000, the first WNV outbreak in Israel since 1980 was reported, with 417 serologically confirmed cases and 35 deaths (Weinberger *et al*., 2001). Viral isolates from this outbreak were most closely related to isolates from the 1996 Romanian and 1999 Russian outbreaks (Smithburn *et al*., 1954). Since then, Israel has experienced regular annual summertime outbreaks of varying size, similar to those observed in the United States (ECDPC, 2013).

Human seropositivity for WNV in Turkey was documented in the 1970s, and again beginning in the mid-2000s (Ozkul *et al*., 2006). An outbreak of WNV occurred in Turkey in 2010-11, concurrent with other outbreaks in the Mediterranean region, causing 47 cases including 40 WNND cases and 10 fatalities (Radda, 1973). Seropositivity for WNV was also reported in Iran

in the 1970s (Kalaycioglu *et al*., 2012). A 2008-2009 survey of patients with fever and loss of consciousness in Isfahan, Iran identified 3 cases which were positive by RT-PCR and 6 more that were positive by IgG (Chinikar *et al*., 2012). Serologic evidence for infection has also been found in Jordan and Lebanon although no human cases have been reported from those countries (Ahmadnejad *et al*., 2011).

West Nile virus continued to circulate in Northern and sub-Saharan Africa throughout the late 20th and early 21st Century, causing outbreaks in Algeria, Morocco, Tunisia, the Democratic Republic of the Congo, and South Africa, along with sporadic cases and seropositivity in humans and/or horses distributed throughout the continent (Hindiyeh *et al*., 2010). Active transmission has continued in Northern Africa, with outbreaks reported in Morocco in 2010 and Tunisia in 2012 and ongoing sporadic transmissions in Egypt and Algeria (Kading *et al*., 2013).

The regular pattern of infection in South Africa prior to 1974 was sporadic, relatively mild human infections and epizootics, with epidemics in humans occurring in 1974 and 1984 (Jupp, 2001). The relative nonpathogenicity of human and equine infections in South Africa had been attributed to reduced pathogenicity of lineage 2 WNV strains; however, later reports of WNND caused by lineage 2 WNV infections in South Africa suggested that the full clinical extent of WNV infection in earlier epidemics may not have been recognized (Zaayman and Venter, 2012). In 2010, the first case of lineage 1 WNV occurring in South Africa caused the death of a pregnant mare (Soliman *et al*., 2010). Infections caused by lineage 2 in Madagascar have also generally been considered mild to inapparent; one fatal case of WNND originated in Madagascar in 2011, although it was speculated that the patient had a deficient antibody response (Venter *et al*., 2011)

Reports have indicated ongoing transmission in other regions of sub-Saharan Africa (Cabre *et al*., 2006). Eleven cases of acute febrile illness were caused by WNV in Guinea in 2006 (Jentes *et al*., 2010). A 2009 seroprevalence study in Ghana indicated that WNV is endemic, with most WNV cases occurring in childhood (Wang *et al*., 2009). A fatality due to WNND was reported in Gabon in 2009 (Lawson *et al*., 2009). 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 cocirculating arboviruses (Baba *et al*., 2013). In Eastern Africa, human infections and mosquitoes positive for WNV lineage 2 were reported in Djibouti (Faulde *et al*., 2012). Positivity for WNV in Kenya has also been reported in ticks collected between 2010 to 2012 and mosquitoes from 2007 to 2011 (Lwande *et al*., 2013).

Various researches have been carried out on WNV in Nigeria, report has it that WNV and other viral types including other arboviruses were isolated from livestock in the Northen part of Nigeria from 1966-1970 (Kemp *et al*., 1973). Since then, other studies on the status of WNV infection in Nigeria has been carried out.

In 1990, there was a study where Haemagglutination Inhibition antibodies (HI) were demonstrated in both animal and humans (Olaleye *et al*., 1990), that study covered the Rain Forest (Ibadan) and the Sahel Savanna (Maiduguri) ecological zones. Another study was carried out in semi – arid zone in Nigeria where WNV was isolated from both mosquitoes and febrile patients (Baba *et al*., 2006). A seroepidemiological study was also carried out in an urban and rural settlement of Ogbomosho to check for West Nile Virus in Humans (Elijah and Julius, 2015). Babatunde *et al.*, 2016 reported the evidence of WNV vectors in Abeokuta, Southwest, Nigeria after their study to determine seasonal abundance and molecular identification of WNV

vectors in *Culex pipiens* and *Culex quinquefasiatus*. Therefore the endemicity of the virus in Nigeria cannot be over emphasized.

2.1.7 Transmission

West Nile virus (WNV) is transmitted through female mosquitoes, which are the prime vectors of the virus. Only females feed on blood, and different species take a blood meal from different types of vertebrate hosts. The important mosquito vectors vary according to geographical area (Hayes *et al*., 2005).

The mosquito species that are most frequently infected with WNV feed primarily on birds (Kilpatrick, 2011). Mosquitoes show further selectivity, exhibiting preference for different species of birds. In the United States, WNV mosquito vectors feed on members of the Corvidae and thrush family more often than will have been expected in other members (George *et al*., 2013).

Some species of birds develop sufficient viral levels (>~$10^{4.2}$ log PFU/ml;) after being infected to transmit the infection to biting mosquitoes that in turn go on to infect other birds. In birds, death usually occurs after 4 to 6 days (Komar *et al*., 2003). In mammals, and several species of birds the virus does not multiply as readily (do not develop high viremia during infection), and mosquitoes biting these infected hosts are not believed to ingest sufficient virus to become infected, making them dead-end hosts (Kilpatrick *et al* 2007). As a result of the differential infectiousness of hosts, the feeding patterns of mosquitoes play an important role in WNV transmission (Kilpatrick *et al*., 2007), and they are partly genetically controlled, even within a species.

The potential for mosquito saliva to affect the course of WNV disease has been demonstrated (Schneider *et al*., 2007). Mosquitoes inoculate their saliva into the skin while obtaining blood. Mosquito saliva is a pharmacological cocktail of secreted molecules, principally proteins that can affect vascular constriction, blood coagulation, platelet aggregation, inflammation, and immunity, that alters the immune response in a manner that may be advantageous to a virus (Wasserman *et al*., 2004) Studies have shown it can specifically modulate the immune response during early virus infection, (Schneider *et al*., 2004) and mosquito feeding can exacerbate WNV infection, leading to higher viremia and more severe forms of disease (Styler *et al*., 2006).

Direct human-to-human transmission initially was believed to be caused only by occupational exposure, such as in a laboratory hazards (CDC, 2002[a]) or conjunctive exposure to infected blood (Bunning *et al*., 2002), through tansfused blood (CDC, 2002[b], Pupo *et al*., 2006), transplanted organs (CDC, 2002[d]; Hunsperger *et al*., 2009), transplacental transmission (CDC,2002[e]; Elizondo-Quiroga *et al*., 2005), occupational transmission via percutaneous exposure (Vieira *et al*., 2015) and in a dialysis center by unidentified means (Busch *et al*., 2005). Transmission via breast milk is also likely (Chowers *et al*., 2001; CDC, 2002[f]).

2.1.8 Host and Vector of WNV

The host and the vector both play important roles in the transmission of West Nile Virus

2.1.8.1 Host range

Maintained in nature in an enzootic transmission cycle between birds and mosquitoes, WNV can also infect humans and other vertebrates and cause serious disease and death (**Figure 2.4**). Birds are considered the most important hosts for the WNV life cycle because they can develop

viremia sufficiently high to infect mosquitoes (Van Der Meulen *et al*., 2005). Birds in the family *Corvidae* such as American crows (*Corvus brachyrhynchos*) and blue jays (*Cyanocitta cristata*) become ill or die from WNV, but other birds such as common grackles (*Quiscalus quiscula*) and house sparrows (*Passer domesticus*) develop high viremia with lower mortality rates (Komar *et al*., 2003). American robins (*Turdus migratorius)* and house finches (*Carpodacus mexicanus*) are considered important amplifying hosts in different regions of the U.S. (Kilpatrick *et al*., 2006). In addition to birds, at least 30 other vertebrate species, including reptiles, amphibians, and mammals, are susceptible to WNV infection. However, only a few nonavian vertebrates, including the brown lemurs (*Lemur fulvus*), lake frogs (*Rana rinibunda*), hamsters, fox squirrels (*Sciurus niger*), eastern gray squirrels (*Sciurus carolinensis*), eastern cottontail rabbits (*Sylvilagus floridanus*), and eastern chipmunks (*Tamias striatus*) have been reported to develop viremia levels expected to support vector transmission (Kostiukov *et al*., 1985).

Humans and horses may suffer serious disease or death from WNV infection but are considered incidental hosts which do not participate in the WNV lifecycle because they do not develop sufficient viremia to infect mosquito vectors. Although transmission between hosts by mosquitoes is by far the most common route of transmission, WNV can also be transmitted directly if infected animals or mosquitoes are consumed by susceptible hosts or if susceptible birds come in close contact with cloacal or oral fluids from other birds with high WNV viremia (Van Der Meulen *et al*., 2005). In spite of the routine screening of blood for WNV by nucleic acid technique in the United States, thirteen (13) transfusion-associated transmissions have occurred (Meny *et al*., 2011).

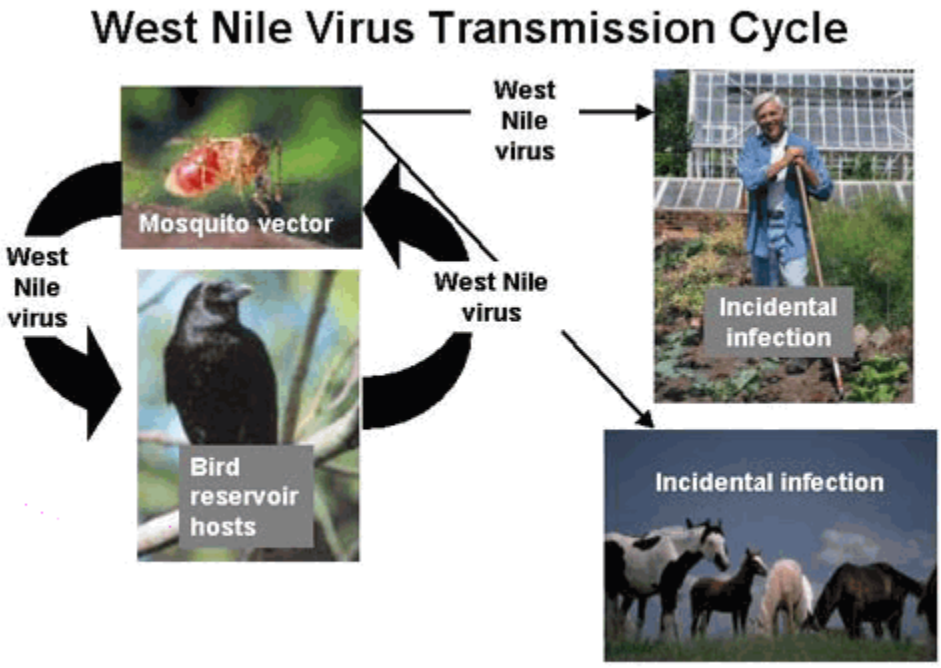


Figure 2.4: West Nile Virus transmission cycle
Source: (http://www.microbiologytext.com/index.php)

2.1.8.2 Vectors associated with WNV transmission

Mosquitoes are the vector for natural transmission of WNV. After a mosquito feeds on an infected competent host, the arbovirus replicates within the mosquito and can then be transmitted to a susceptible host through salivary gland secretions (**Figure 2.4)**. Compared to related arboviruses such as dengue virus and yellow fever virus, WNV can be transmitted by a variety of mosquitoes with different host-feeding preferences with up to 45 species and 8 genera reported in the U.S. between 2004 and 2008 (CDC, 2009). However, not all mosquito species reported as WNV-positive are competent vectors of WNV, and not all species that are transmission-competent in the laboratory play a role in natural transmission (Turell *et al*., 2005).

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 (Turell *et al*., 2002). Mosquitoes of the genus *Culex* have been reported as the most important bridge vectors in the United States, with *Cx. pipiens* as the dominant bridge vector in the Northeastern, north-central, and mid-Atlantic United States, *Cx. quinquefasciatus* in the south and southwest, and *Cx. tarsalis* in the west (Andreadis, 2012). *Culex* mosquitoes have also been implicated in transmission in Europe, Australia, and South Africa (Muñoz *et al*., 2012). Mosquitoes of the genus *Aedes*, the transmission vector for related flaviviruses, may also serve as important bridge vectors (Turell *et al*., 2005). While experimental transmission of WNV by ticks has been demonstrated, a role for ticks in natural transmission and maintenance of WNV has not been determined (Lwande *et al*., 2013).

2.1.9 Pathogenesis of WNV

Most of our knowledge regarding WNV dissemination and pathogenesis derives from the study in rodent models. After an infected mosquito bite, WNV infects keratinocytes and Langerhans cells (Byrne *et al*., 2001). They migrate to lymph nodes resulting in a primary viremia (Johnston *et al*., 2000). Then the virus spreads to peripheral visceral organs like kidney and spleen where a new replication stage occurs, as well as epithelial cells and macrophages (Rios *et al*., 2006). Depending on the level of viremia, the peak of which comes at day 3 p.i. in mice, the virus may cross the blood-brain barrier (BBB) and enter the central nervous system (CNS), causing meningo-encephalitis (Wang *et al*., 2004). Various ways have been proposed for WNV entry to CNS; TNF- mediated change in endothelial cell permeability have been proposed to facilitate CNS entry (Wang *et al*., 2004), as well as infection of olfactory neurons and spread to the olfactory bulb (Monath *et al*., 1983).

Other ways involve direct axonal retrograde transport from infected peripheral neurons or transport of the virus by infected immune cells trafficking to the CNS (Garcia-Tapia *et al*., 2006). WNV infects neurons in various parts of the CNS causing loss of architecture, degeneration and cell death. In latter stage mononuclear cells infiltrate the infected regions although it is not really clear if they help stop infection or contribute to pathogenesis destroying infected cells and releasing cytokines (Lazear *et al*., 2011). Infection and injury of brain stem, hippocampal and spinal cord was observed in both humans and rodents that succumb to the disease (George *et al*., 2013).

Persistence of WNV in mice was found to be tissue dependent. Infectious virus could persist as long as 4 months especially in mice that did not exhibit disease during acute infection and especially in the skin and spinal cord (Appler *et al*., 2010). This persistence may also occur in humans after mild febrile illness or subclinical infections; 3% of WNV-positive blood donors

were found to have detectable WNV RNA in blood between 40 and 104 days after their index donation (Busch *et al*., 2008).

In wild birds, less is known regarding pathogenesis of WNV. The virus has been detected by histology and RT-PCR in various tissues e.g. brain, liver, lungs, heart, spleen and kidneys of various avian species e.g. crows, blue jays, goshawks, magpies (Panella *et al*., 2001 ; Bakonyi *et al*., 2006 ; Valiakos *et al*., 2011). Various avian species were found to be viremic for 6 days post inoculation and viremic titers high enough to transmit the virus to mosquitoes via their bites (Owen *et al*., 2006). In wild birds, infectious WNV was detected for as long as six weeks in tissues, however, it is important to clarify that immune response, virulence and viral persistence is to a great degree species-dependent, with great variations among various avian species in different geographical areas, as well as strain dependent, implicating various genetic determinants of virulence (Reisen *et al*., 2006).

2.1.10 Clinical Manifestation of WNV

Most human infections with WNV (~80%) are asymptomatic, and symptomatic infections may vary from flu-like malaise to serious neuroinvasive diseases, for which there is no specific treatment (Pealer *et al*., 2003).

West Nile virus disease cases are categorized into two primary groups: the non-neuroinvasive disease and neuroinvasive disease.

The non-neuroinvasive disease is an asymptomatic infection, followed by an acute systemic febrile illness that may include headache, myalyias, arthralgias, rash or gastrointestinal symptoms.

The neuroinvasive disease may include aseptic meningitis, encephalitis or acute flaccid paralysis (AFP). These illnesses are usually characterized by the acute onset of fever with stiff neck,

altered mental status, seizures, limb weakness or cerebrospinal fluid (CSF) pleocytosis (WSDH, 2014).

Fewer than 1% of human infections progress to severe disease, for which the most frequently reported risk factors include advanced age, immune suppression, and chronic medical conditions including, but are not limited to, hypertension, diabetes, and chronic renal failure (Bode *et al*., 2006 ; Patnaik *et al*., 2006)

West Nile virus infection can present as encephalitis, meningitis, or flaccid paralysis. Encephalitis or meningoencephalitis are more common than meningitis in contemporary outbreaks (Petersen and Marfin, 2002). Older age, alcohol abuse, and diabetes was associated with West Nile encephalitis in a retrospective study of 221 patients with West Nile virus infection, 65 of whom presented with encephalitis (Bode *et al*., 2006). Encephalitis that is associated with muscle weakness and flaccid paralysis is particularly suggestive of WN virus infection (Petterson and Marfin, 2002).

Among WNND patients, 50–71% develop WN encephalitis, 15–35% develop meningitis, and 3–19% develop acute flaccid paralysis (Asnis *et al*., 2001). Severe cases have fatality rates ranging from 3% to 19% in encephalitis cases (Klein *et al*., 2002; Emig and Apple, 2004; Kopel *et al*., 2011).

Loeb *et al*., 2008 reported that physical and mental impairment resolve in about a year, but patients with preexisting morbid conditions take longer to recover. A study of patients infected with WNV have noted physical symptoms and/or cognitive defects persisting over a year after infection in more than half of WNND cases (Sadek *et al*., 2010). Persistence of WNV symptoms >6 months was reported most often in patients with WNND, hypertension, and diabetes (Cook *et al*., 2010). WNND has also been reported as a risk factor for development of chronic kidney

disease in a long-term follow-up study of WNV patients (Nolan *et al*., 2012). Severe disease is commonly associated with the elderly (Watson *et al*., 2004).

At one year, less than 40 percent of patients with severe disease achieve a full recovery; full recovery is most likely to occur in people who are less than 65 years of age (Klee *et al*., 2004).

2.1.11 Diagnosis of WNV

There are several laboratory methods, which can be used to diagnose WNV. The most efficient diagnostic method is detection of virus using the IgM antibody capture enzyme immunoassay (MAC-ELISA) or microsphere immunoassay (MIA) to check for IgM antibody to WNV in serum or cerebrospinal fluid (CSF); more than 90% of those infected have detectible serum IgM (WSDH, 2014). WNV-specific IgM antibodies are usually detectable 3 to 8 days after onset of illness and persist for 30 to 90 days, but longer persistence has been documented *(*Papa *et al*., 2011).

In addition, most WNV infections are asymptomatic and IgM can persist in the serum for up to 500days (CDC, 2003). Immunoassays for WNV-specific IgM are available commercially. The presence of WNV-specific IgM in blood or CSF provides good evidence of recent infection but may also result from cross-reactive antibodies after infection with other flaviviruses or from non-specific reactivity (CDC, 2013). IgM-capture ELISA is a valuable tool for the detection of recent infection in all species. Therefore, the presence of IgG antibodies alone is only evidence of previous infection with other flaviviruses and clinically compatible cases with the presence of IgG, but not IgM, should be evaluated for other etiologic agents (Tyler *et al*., 2006).

Other methods that can be used for the diagnosis of WNV include;

2.1.11.1 Plaque-reduction neutralization tests (PRNTs)

This can help determine the specific infecting flavivirus. PRNTs can also confirm acute infection by demonstrating a fourfold or greater change in WNV-specific neutralizing antibody titer between acute and convalescent-phase serum samples collected 2 to 3 weeks apart. Serological evidence of recent WNV infection is confirmed by a 4-fold or greater rise in plaque-reduction neutralizing (PRNT) antibody in paired sera. The first serum should be drawn as soon as possible after the onset of clinical signs and the second between 14 and 21 after the first. Neutralizing antibody may not be present until 2 weeks or more after exposure to WNV; so it is possible that clinical signs will be observed before a serum is PRNT positive. According to product inserts for commercially available WNV IgM assays, all positive results obtained with these assays should be confirmed by neutralizing antibody testing of acute and convalescent-phase serum specimens (CDC, 2013).

2.1.11.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

This method can be performed on serum, CSF, and tissue specimens that are collected early in the course of illness and, if results are positive, can confirm an infection. An RT-nested PCR (RT-nPCR) has proved to be a reliable and rapid method for detecting WNV (Johnson *et al*., 2001).

2.1.12 Treatment of WNV

Treatment is supportive, often involving hospitalization, intravenous fluids, respiratory support and prevention of secondary infections for patients with severe disease (Kightlinger, 2003). Ribavirin has demonstrated *in vitro* activity against WN virus, but therapeutic efficacy has not yet been demonstrated in animal models, it increased mortality in Syrian golden hamsters when administered two days after inoculation (Morrey *et al*., 2004). During an outbreak in Israel,

ribavirin was used in an uncontrolled, non-blinded fashion in some patients with WN virus neuroinvasive disease, it appeared to be ineffective and possibly detrimental (Chowers *et al*., 2001).

Interferon — The rationale for the use of alfa interferon is based upon evidence of efficacy against WN virus *in-vitro* and in animal models (Sayao *et al*., 2004). Two patients with serologic confirmation of WN virus infection presented with deteriorating mental status and progression to coma, were treated with standard interferon within 72 hours of presentation (Kalil *et al*., 2005), there was rapid neurologic improvement within 48 hours. It remains unclear if the change in clinical status was due to interferon or to spontaneous improvement, which has been documented in untreated WN virus infection (Sejvar *et al*., 2003).

2.1.13 Prevention and Control Measures

Prevention of infection includes personal protective measures, mosquito control programmes, and blood donor screening (Han *et al*., 1999).

2.1.13.1 Personal protection measures

Persons 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 mosquito vectors of WNV are abundant. Insect repellents recommended for use on skin include those containing *N*,*N*-diethyl-m-toluamide (DEET), picaridin (KBR-3023), or oil of lemon eucalyptus (*p*-menthane-3,8 diol) which provides long-lasting protection (Barnard and Xue, 2004). A personal protection measure to avoid mosquito exposure is a mainstay of prevention. Pregnant women should avoid exposure to mosquito bites to reduce the risk for intrauterine WNV transmission. Reducing the risk of animal -to-human transmission, gloves and other protective clothing should be worn

while handling sick animals or their tissues and during slaughtering and culling procedures (WSDH, 2014).

2.1.13.2 Mosquito control programmes

Application of larvicides and targeted spraying of pesticides to kill adult mosquitoes can reduce the abundance of mosquitoes. It is important to drain standing water where mosquitoes are likely to breed. In a case control study examining risk factors, only spending increased amounts of time outdoors and the presence of flooded basements correlated with infection (Han *et al*., 1999).

2.1.13.3 Blood donor screening programmes

To prevent transmission of WNV through blood 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). Blood donor screening for WN virus has greatly reduced, but not eliminated, the risk of transfusion transmission (Busch *et al*., 2005). WN virus infection should be considered in recent transfusion recipients with unexplained, compatible illness.

Care is required when handling virus because of the zoonotic potential of WNV. Laboratories working with known WNV isolates should adhere to established containment requirements. Caution should be exercised when collecting material from live or dead specimens, particularly when central nervous tissue is examined. Precautions should include wearing two layers of waterproof gloves and a facemask.

2.1.14 Vaccine Development in WNV Control

There are currently various WNV vaccines licensed for horses, mice and other non-human primates which include West nile-innovator (Pfizer), Recombitek-Equine West Nile virus vaccine but no human vaccine is available yet (Shashikant *et al*., 2009).

At present, there are no FDA-approved vaccines for human use but several clinical trials are ongoing. In 2005, Acambis (Sanofi-Pasteur) successfully completed a Phase I clinical trial with its live-attenuated ChimeriVax-WN. ChimeriVax-West Nile (Acambis, Sanofi-Pasteur) utilizes the attenuated Yellow Fever Virus (YFV) vaccine strain (17D) to build a live chimeric virus that consists of the prM and E proteins of WNV in the context of the Yellow fever virus (YFV) capsid and non-structural proteins (Biedenbender *et al*., 2011).

In the first part of a Phase II trial in healthy adults 18-40 years of age, a single dose of ChimeriVax-West Nile raised neutralizing antibodies 28 days after vaccination (Biedenbender *et al*., 2011). The second part of the Phase II trial determined safety and tolerance in healthy individuals over 41 years of age. Seroconversion was achieved at day 28 in more than 96% of the healthy adults in both age groups. Another chimeric vaccine (WN-DEN4) that uses attenuated dengue virus as a backbone for prM-E genes of WNV (Pletnev *et al*., 2006) is being evaluated in a Phase II human trial at the John Hopkins School of Public Health in adults 18-50 years of age. The Vaccine Research Center (VRC) at the National Institute of Allergy and Infectious Diseases (NIAID) has developed a DNA plasmid-based vaccine. In 2005, the VRC initiated a successful Phase I clinical trial demonstrating its safety, tolerability and ability to induce neutralizing antibodies, a second-generation DNA vaccine using an improved vector was evaluated in a Phase I clinical trial (Ledgerwood *et al*., 2011). Naked plasmid DNA was administered via needle-free intramuscular injection on days 0, 28 and 56 with at least 21 days between injections. The plasmid in this vaccine is incapable of replicating in animal cells and does generate infectious

virions. The vaccine was well tolerated without serious adverse events. All individuals that completed the 3-dose vaccination schedule developed neutralizing antibodies (Ledgerwood *et al*., 2011). The majority of the subjects developed a $CD4^+$ response rather than a $CD8^+$response as assessed by intracellular cytokine staining. Vaccine-induced T cell responses were mainly directed against WNV E protein. Human vaccines are unlikely to be available for at least several years.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was carried out in Kaduna State, Nigeria **(Figure 3.1)**. Kaduna State is located in the North West zone of the country, it is located on Latitude 11˚12` N and Longitude 07˚37`E (NPC, 2006). The State was created out of the old Northern Region of Nigeria, which is now a state capital to about 6.3 million people (NPC, 2006). It is characterized by a tropical climate with two seasons; a rainy season of approximately 210 days (May to October), with a mean annual rainfall of 1092.8mm, and the dry season which spans between November and April. The monthly mean temperature shows a maximum of 39.7^0C in June and 13.8^0C in December (Ukegbu, 2005).

The State has twenty-three (23) Local Government Areas (LGAs) and three Senatorial Zones. These include; Kaduna North, Kaduna Central and Kaduna South Senatorial Zones.

The State has 23 hospitals and 3 dental centres, in 21 LGAs except Sabon Gari and Kubau. The Federal Government of Nigeria also has specialized tertiary health institutions in the State. In addition to these, there are 608 Local Government health facilities which include Primary Health Centres (PHCs) and Health Clinics (HCs) and 656 private health facilities which include private and faith based hospitals, clinics, laboratories and pharmacies, which are spread across the 23 LGAs of the State (KDG, 2013).

For the purpose of this study, three hospitals were selected; one from each senatorial district, the rationale behind the selection of the sampled population was to ensure that moderate representation is achieved in the three senatorial districts of the state. viz-

1. Hajiya Gambo Sawaba memorial Hospital Zaria City, Zaria, representing Kaduna North
2. Gwamna Awan General Hospital, Kakuri Kaduna, representing Kaduna Central
3. General Hospital (GH) Kafanchan, representing Kaduna South

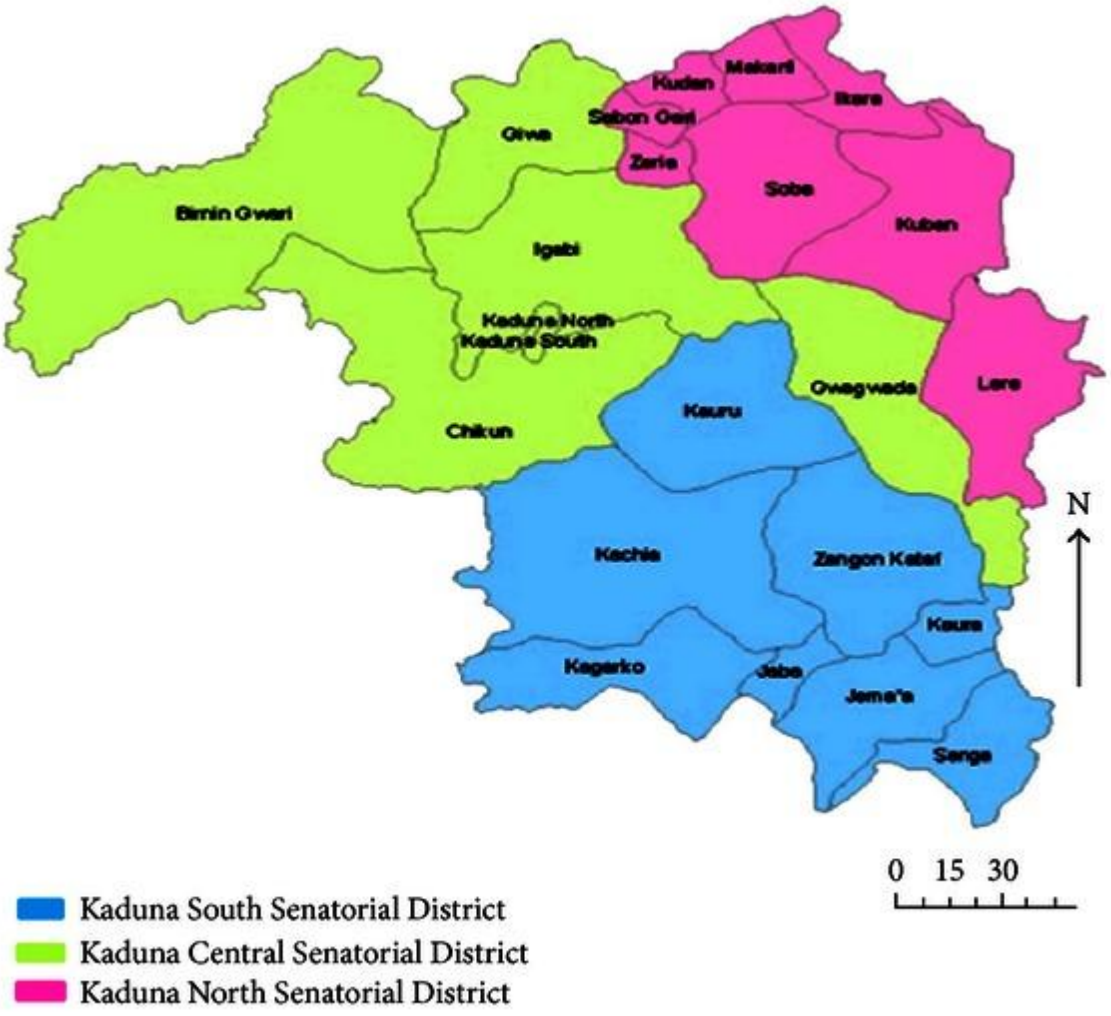


Figure 3.1: The map of Kaduna State showing the study areas (National Population Census, 2006)

3.2 Study Design

This study is a hospital-based cross-sectional study involving the use of self-designed, structured questionnaires, it was carried out between the months of January to July 2016. A non probability sampling method was used and equal numbers of samples were collected from each of the three senatorial districts. Subjects who consented were screened for WNV IgM antibody using West Nile IgM ELISA test kit (Diagnostic Automation/Cortez Diagnostics, Inc. USA) at the Virology Laboratory, Department of microbiology, ABU Zaria and Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) assay was carried out on IgM positive samples at the Public health Department, Faculty of Veterinary Medicine, ABU Zaria to confirm the presence of WNV.

3.3 Study Population

This study population included in-and-out patients of all ages irrespective of sex who presented with fever as diagnosed by the Clinicians and other symptoms which were headache, nausea, vomiting, loss of appetite, diarrhoea, diaphoresis, weakness, flu. These were those sent to the laboratory for malaria, widal, HIV and Hepatitis investigations.

3.4 Ethical Approval

Ethical approval (**Appendix 1**) was obtained from the Medical and Ethical Committee of the Kaduna State Ministry of Health. The purpose of the study was explained to patients in other to obtain their consent for the collection of samples and filling the questionnaire (**Appendix 2**).

3.5 Selection Criteria

This study included febrile patients as diagnosed by attending clinician of all age range attending the selected hospitals who gave their consent.

Febrile patients attending the selected hospitals who did not give their consent were excluded from the study, as well as non febrile patients.

3.6 Sample size Determination

For the purpose of this study, a prevalence rate of 2.19% as reported from a study conducted in Ogbomosho in Nigeria (Elijah and Julius, 2015), was used to determine the sample size, using the equation below, as derived by Naing *et al*. (2006) at 95% confidence interval.

$$n = \frac{Z^2pq}{L^2}$$

Where,

n = Sample size

Z = Standard normal distribution at 95% confidence interval = 1.96

P= known prevalence of WNV in patients in Ogbomosho = 2.19

q = 1- p ; 1- 0.0219 = 0.9781

L = allowable error, which is taken as 5% = 0.05

Substituting…

$$n = \frac{(1.96)^2 \times 0.0219 \times 0.9781}{0.05^2}$$

= 33 samples

However, in order to have more representation of the study population, a total of 135 samples were collected for this study. Equal numbers of samples were collected from the three selected hospitals.

3.7 Data Collection

A self-designed questionnaire (**Appendix 2**) was administered to each patient aimed at obtaining socio-demographic data, risk factors as well as clinical information related to WNV. Translation of the questions was done where necessary at the point of filling the questionnaire.

3.8 Sample Collection and Processing

Following the assemblage of all materials necessary for blood collection and the labeling of vacutainers appropriately, 2ml of venous blood was drawn by venipuncture aseptically from febrile patients who gave consent by medically qualified personnel. Each of the blood sample collected was allowed to clot at room temperature (25^{o}C) and serum was carefully collected after centrifugation for 5mins at 1000rpm and stored at -20˚C at the Department of Microbiology, ABU Zaria until it was time for analysis.

3.9 Sample Analysis

The samples were subjected to ELISA and all the positive cases were then screened for the WNV using RT-PCR as indicated below:

3.9.1 Screening for WNV IgM using Enzyme-Linked Immunosorbent Assay (ELISA)

A West Nile IgM capture ELISA kit for the qualitative detection of IgM antibodies to West Nile recombinant antigens (WNRA) in human serum was used, all analysis was done following the manufacturer's instructions

3.9.1.1 Test principle

The WNV IgM capture ELISA consist of one enzymatically amplified "two step" sandwich-type immunoassay. In this assay, controls and unknown serum samples are incubated in microtitre wells which have been coated with anti-human IgM antibodies. This was followed by incubation with the west Nile virus derived recombinant West Nile antigen (WRNA) protein and a control preparation NCA (normal cell antigen) separately. The serum samples were mixed with sample dilution buffer for WN IgM, and then added to the wells. After one hour incubation and washing, the wells were treated with a WNRA-specific antibody labeled with the enzyme horseradish peroxidase (HRP) to help determine the presence of a molecular target. After a second incubation and washing step, the wells were incubated with the tetramethylbenzidine (TMB) substrate. An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate was determined by absorbance measurement at 450 nanometers. Above a certain threshold, the ratio of the absorbance of the WNRA and the control wells presumptively determines whether antibodies to WNV are present or not.

3.9.1.2 Assay for WN IgM in the Patient Sample

This work was carried out in the Department of Microbiology, ABU, Zaria. All samples and reagents were brought to room temperature (20-25^{o}C) from -20^{o}C and mixed thoroughly by gentle swirling before use. The procedures followed were according to the manufacture's instruction. The Positive, negative controls and patients serum were assayed in duplicate. Test

sera and controls were diluted using 1:100 dilution ratio buffer for WN IgM (SD); 50µl of test sera and controls was applied to the plate by appropriate pipette. The plate was covered and incubated at 37^{o}C for 1hour in an incubator (Model: IH – 150).

After incubation, the plate was washed manually 6 times using 1X wash buffer provided in the kit, then 50µl of West Nile Antigen (WNRA) also provided in the kit was applied to row A-D and 50µl of Normal Cell Antigen (NCA) was applied to row E-H by multi-pipette on the plate respectively. The plate was then covered and incubated at 37˚C for another 1hour in an incubator, after which it was washed 6 times. Thereafter 50µl of ready to use Enzyme-HRP conjugate was added into all wells by multi-pipette and the plate was covered and incubated at 37˚C for 1hour in an incubator, after which it was washed as before. After washing, 150µl of wash solution was added into all the wells again and the plate was incubated at room temperature for 5 minutes. After incubation, the plate was washed 6 times. Seventy five micro litre (75µl) of liquid TMB substrate was then added into all the wells and incubated at room temperature in a dark place for 10minutes without covering the plate. After the incubation, 50µl of stop solution was added to all the wells and incubated at room temperature for 1minute, after this last incubation, the optical density (OD) was read with a microplate reader (Model: GF-M3000) at 450nm.

3.9.1.3 Interpretation of results

For the calculation of the immune status ratio (ISR): the average of the sample replicates with the WNRA and sample replicates with the NCA were computed, and the WNRA/NCA ratio (ISR) for all unknown sample were then calculated. The same calculation was performed for the positive and negative controls.

Results were therefore interpreted as follows according to the manufacturer's instructions:

Negative: WNRA/NCA ratio (ISR) <4.47 are seronegative for WNV IgM

Equivocal: WNRA/NCA ratio (ISR) of 4.47-5.66 are equivocal and should be retested

Positive: WNRA/NCA ratio (ISR) >5.66 are seropositive for WNV IgM.

3.9.2 Detection of WNV RNA using RT-PCR

All the seven serum samples that were seropositive for WNV IgM were subjected to Reverse Transcription - Polymerase Chain Reaction (RT-PCR) to detect the viral RNA. The RNA was first extracted, amplified then the products were resolved using gel electrophoresis. The RT-PCR was carried out following the manufacturer's instructions in the Department of Public Health, Faculty of Veterinary Medicine, ABU, Zaria.

3.9.2.1 Assay Procedure for RT-PCR

Table 3.1: Oligonucleotide Primers for RT-PCR

Primers	Genome position	Sequence (5′-3′)	Product size (bp)	References
WNENV-forward	212-236	TTGTGTTGGCTCTCTTGGCGTTCTT	431	Shi *et al*., 2001
WNENV-reverse	619-643	CAGCCGACAGCACTGGACATTCATA		
WN233	233-257	TTGTGTTGGCTCTCTTGGCGTTCTT	408	Lanciotti *et al*., 2000
WN640	640-616	CAGCCGACAGCACTGGACATTCATA		
WN3'NC-forward	10,668-10,684	CAGACCACGCTACGGCG	103	Lanciotti *et al*., 2000
WN3'NC-reverse	10,770-10,756	CTAGGGCCGCGTGGG		

3.9.2.2 RNA Extraction Protocol

Viral RNA was extracted using QIAamp® viral RNA mini extraction kit.

The procedures carried out were as follows; Serum samples were allowed to thaw at room temperature (28^{o}C), properly labeled and samples were inserted into the rotor and centrifuged at 21,000 to 25,000 × g for 60 minutes. The supernatant was carefully removed from the tube after centrigugation using a fine tip pipette leaving about 140µl of the sediment. Five hundred and sixty microliters (560µl) of prepared buffer AVL containing carrier RNA was added to the tubes and incubated at room temperature (28^{o}C) for 10 minutes. After the incubation, the tubes were spinned to remove drops from the inside of the lid. Five hundred and sixty microliters (560µl) of ethanol was then added to the samples, and mixed by vortexing for 15 seconds, after that the tubes were briefly centrifuged to remove drops from inside the lid. Six hundred and thirty microliters (630µl) of the reaction mixture from the previous step was transferred into the QIAamp mini column and the caps were closed and centrifuged at 6000×g (8000rpm) for 1 minute. The QIAamp mini column was placed into a 2 mls collection tube and the tubes containing the filtrate was discarded. The QIAamp mini column was carefully opened and the previous step was repeated. The QIAamp mini column was carefully opened and five hundred microlitres (500µl) of buffer AW1 was added, the caps closed and centrifuged at 6000×g (8000rpm) for 1 minute, the QIAamp mini column was then placed into a clean 2 mls collection tube and the tube containing the filtrate was discarded. The QIAamp mini column was opened again and five hundred microliters (500µl) of buffer AW2 was added and centrifuged at full speed for 3minutes, after that the QIAamp mini column was placed in a new 2 mls collection

tube and centrifuged for 1minute. The QIAamp mini column tube was placed again in a sterile 1.5 mls microcentrifuge tubes and sixty five microliters of elution buffer AVE was carefully added, allowed to stand at room temperature for 1minute, after that it was centrifuged at 6000×g (8000rpm) for 1 minute to elute the viral RNA. The viral RNA was then stored at -20^{o}C until it was needed.

3.9.2.3 Complementary Deoxyribonucleic Acid (cDNA) Synthesis Protocol

The cDNA synthesis protocol was carried out following one *taq*® RT-PCR kit procedures;

Five microlitres (5µl) of total RNA, two microliter (2µl) of $d(T)_{23}$ and one microliter (1µ) of nuclease free water making a total of eight microliters (8µl) sample volume was mixed in two sterile RNase free micro tubes. The RNA was denatured for 5 minutes at 70^{o}C, spinned briefly and an ice was promptly put to improve the yield of cDNA. Ten microliters (10µl) of Molony Murine Leukaemia virus (M-MuLV) reaction mix and two microliters (2µl) of M-MuLV enzyme mix making it a total volume of twenty microliters (20µl) was added, but to the negative control, two micro liters (2µl) of distilled water was added instead of enzymes. The 20µl of cDNA reaction mixture was incubated at 42^{o}C for 1 hour, and thereafter the enzyme was inactivated at 80^{o}C for 5 minutes. The reaction was diluted with 30µl of nuclease free water for polymerase chain reaction (PCR).

3.9.2.4 PCR Amplification

The reaction included 12.5µl of master mix, 3.1µl of both forward and reverse primers and 1.5µl of nuclease free water. From the 200µl cocktail mix, 20µl of the reaction mixture was dispensed into each of the PCR eppendorf tubes, 5µl of cDNA sample volume was added to each tube and to the negative control 5µl of nuclease free water was added. The preparation was brieftly

centrifuged for 30 seconds and afterward arranged in a thermocycler for PCR. The PCR conditions were as follows:

Initial denaturation	95^0C	5minutes	1cycle
Denaturation	94^0C	30seconds	40 cycles
Annealing	59^0C	30seconds	
Extension	72^0C	30seconds	
Final extension	72^0C	7minutes	

Fifteen micro litres (15µl) of the PCR product was electrophoresed using 2% agarose gel as prepared below and was visualized by staining with Ethidium bromide under the ultra violet light, for the separation of PCR product.

3.9.2.5 Determination of RT-PCR Amplicon of Gene with 100bp

Two grams of agarose powder was weighed and poured into a 500ml conical flask, one hundred miles (100ml) of 1×Tris Borate Ethyl Dietholtriacetic Acid (TBE) buffer was added, mixed and dissolved in a microwave oven for 2 minutes, after which it was allowed to cool to about 45^0C. The casting tray was assembled and combs put in position, 5µl of Ethidium bromide was added to the agarose and mixed, the mixture was then carefully poured into the casting tray and allowed to solidify. The combs were removed from the gel, the tray was transferred into the electrophoretic tank and the tank was filled to the maximum level with 1×TBE buffer. The first well was loaded with 100bp DNA ladder, the second well was loaded with 10µl nuclease free water mixed with 5µl of 6×loading dye and the other wells loaded with multiple 10µl of the amplified PCR products mixed with 5µl of 6×loading dye. The voltage was set at 80V for

45minutes, the gel was observed in the gel documentation unit, the image was saved in the computer for further analysis.

3.9.2.6 Data Analysis

Results and data obtained from questionnaires were reduced to percentages and presented on tables and graphs. All statistical analysis was done using the Statistical Package for Social Sciences (SPSS) version 20.0 software programme at 0.05 level of significance and at 95% confidence interval. Pearson's Chi square (χ^2) was used to determine the relationship between variables and the prevalence rate of the infection was calculated in percentage.

CHAPTER FOUR

4.0 RESULTS

West Nile Virus IgM antibodies were detected in 7(5.2%) patients (Figure 4.1). From Kaduna Central Senatorial district (Gwamna Awan General hospital, Kakuri) we had the prevalence of 3.0%, Kaduna South (General hospital Kafanchan) had the prevalence of 2.2%, and no positive sample was recorded in Kaduna North (Hajiya Gambo Sawaba General hospital). Table 4.1.

In relation to sex (Figure 4.2), males had higher prevalence of 3.7% and females had a lower prevalence of 1.5%.

Result analysed according to age showed that patients in the age group 31-40 had the highest prevalence of 2.2% and the least prevalence of 0.0% was recorded in age group 21-30.

Analysis according to marital status, showed that seropositive cases were high among married patients with prevalence of 3(2.2%) and least in the separated and single patients with both having prevalence of 1.5%.

Result analysed in relation to educational status showed those with secondary school education having the highest prevalence of 3.0% and those with primary education status had the least prevalence of 0.0%. However, the above data showed that infection rate with WNV did not differ significantly based on either sex, age, marital status or educational level.

In the analysis according to occupation of patients as shown in (Table 4.3), farmers had the highest prevalence of 3.0% and those with occupation of fishing and housewife all had the least

prevalence of 0.0%. Here the result was statistically significant (P<0.05) showing that farming is a socio-demographic factor that pre disposes to WNV infection.

Table 4.1: Seroprevalence of West Nile Virus among febrile Patients in some Hospitals in Kaduna State

Senatorial District	No. Examined	No. positive for WNV (%)	Prevalence (%)	Chi-square value	p-value.
Kaduna Central (GAGHK)	45	4	3.0	3.917	0.141
Kaduna South (GHK)	45	3	2.2		
Kaduna North (HGSGH)	45	0	0.0		
Total	**135**	**7**	**5.2**		

Keys:

HGSGH- Hajiya Gambo Sawaba Genaral Hospital, Zaria.

GAGHK- Gwamna Awan General Hospital, Kakuri.

GHK- General Hospital Kafanchan.

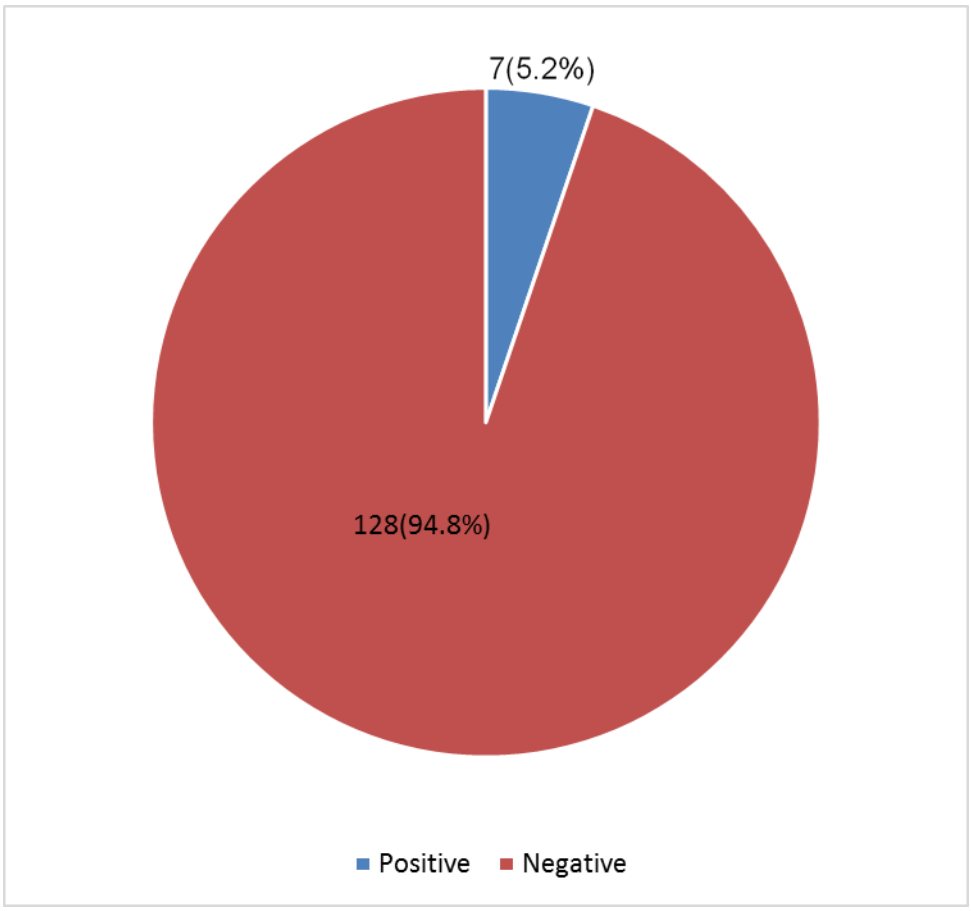


Figure 4.1: Seroprevalence of West Nile Virus among Febrile Patients attending some Hospitals in Kaduna State.

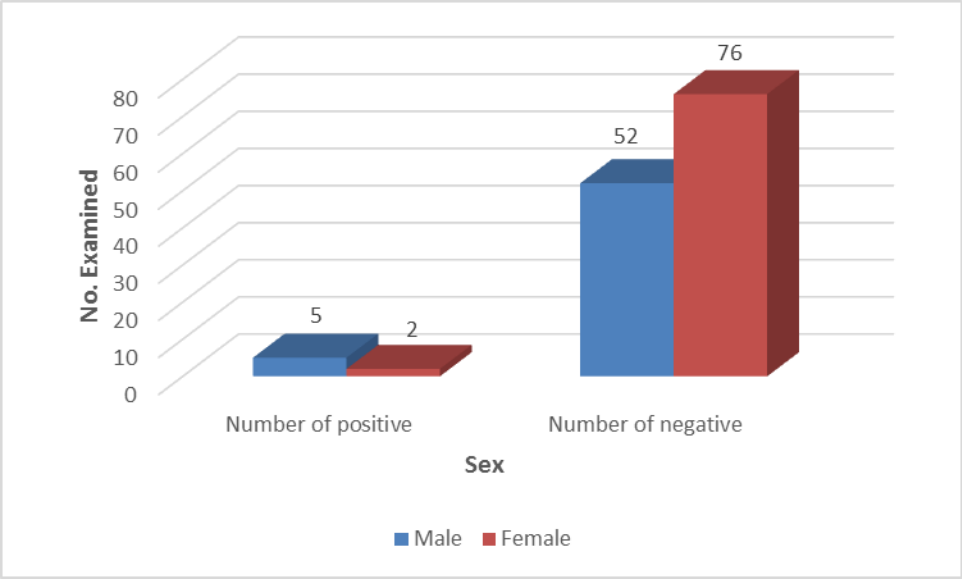


Figure 4.2: Seroprevalence of West Nile Virus among febrile Patients in some Hospitals in Kaduna State in Relation to Sex

Table 4.2: Age Distribution of West Nile Virus IgM among febrile Patients in some Hospitals in Kaduna State

Age group(years)	No. Examined	Number positive for WNV (%)	Prevalence (%)	Chi-square value	p-value
<20	22	1	0.7	3.373	0.497
21-30	36	0	0.0		
31-40	45	3	2.2		
41-50	20	2	1.5		
>50	12	1	0.7		
Total	**135**	**7**	**5.2**		

N.B : Not statistically significant (P>0.05)

Table 4.3: Socio-demographic factors and Seroprevalence of WNV among febrile patients attending some Hospitals in Kaduna State.

Demographic Factor	No Examined	No. positive for WNV (%)	Prevalence (%)	Chi-square value	p-value.
Marital Status					
Single	52	2	1.5	1.009	0.604**
Married	62	3	2.2		
Separated	21	2	1.5		
Total	**135**	**7**	**5.2**		
Educational Status					
Primary	21	0	0.0	7.982	0.053**
Secondary	22	4	3.0		
Tertiary	36	2	1.5		
Non-Formal	49	1	0.7		
Total	**135**	**7**	**5.2**		
Occupation					
Civil Servant	27	2	1.5	10.083	0.039*
House wife	28	0	0.0		
Fishing	2	0	0.0		
Butcher	2	1	0.7		
Farming	76	4	3.0		
Total	**135**	**7**	**5.2**		

KEYS:

**- Not statistically significant (P>0.05)

*- Statistically significant (P<0.05)

The seroprevalence of WNV infection in relation to some epidemiological risk factors, Table 4.4 showed no significant difference between WNV infection and the history of blood transfusion (P>0.05).

The findings showed that those not cancer chemotherapy had a higher prevalence of 4.4% as compared with those undergoing cancer chemotherapy (0.7%) and this difference was not statistically significant (P>0.05). In relation to the history of cancer, the seroprevalence of WNV in the study population revealed that those without cancer had a higher prevalence of 4.4% compared to those with cancer 0.7%. The patient that were not hypertensive and didn't have diabetes both had the highest prevalence of 3.7% and those with hypertension and diabetes had the least prevalence of 1.5%. Data collected showed that patients that do not use mosquito net had a higher prevalence of 5.2% while those that use mosquito net had the least prevalence of 0%. Those patients with net screen on doors and windows had the highest prevalence of 3.0% and those without net screen on doors and windows had the least prevalence of 2.2%. The use of mosquito net was statistically significant in this study which reveals that the use of mosquito net is protective against WNV infection.

The result analyzed according to some clinical data is as shown in Table 4.5. Participants presenting with headache had the highest prevalence of 5.2% and the lowest was shown in stomach ache, drowsiness and diarrhoea with the prevalence of 2.2%.

Plate 1 shows the result of the PCR, where RNA to WNV; WNENV (431bp) targeting the envelop gene and WN (408bp) targeting the C terminal portion of the C gene and the N terminal part of the PrM gene were detected in the study area.

Table 4.4: Seroprevalence of WNV in Relation to some Risk Factors among Febrile Patients attending some Hospital in Kaduna State

Risk Factor	No. Examined	No. positive for WNV (%)	Prevalence (%)	p-value (Fisher)/OR	CI
Blood Transfusion					
Yes	38	4	3.0	0.098/0.271	0.058-1.275
No	97	3	2.2		
Transplant					
Yes	-	-	-	-	-
No	135	7	5.2		
Cancer					
Yes	5	1	0.7	0.237/0.194	0.019-2.008
No	130	6	4.4		
Cancer chemotherapy					
Yes	3	1	0.7	0.149/0.095	0.008-1.203
No	132	5			
Hypertensive					
Yes	28	2	1.5	0.634/0.637	0.117-3.472
No	107	5	3.7		
Diabetes					
Yes	21	2	1.5	0.298/0.436	0.079-2.411
No	114	5	3.7		
Use of Mosquito net					
Yes	36	0	0.0	0.189/0.719	0.645-0.801*
No	99	7			
Net on doors and Windows					
Yes	71	4	3.0	1.000/0.824	0.177-3.830
No	64	3	2.2		

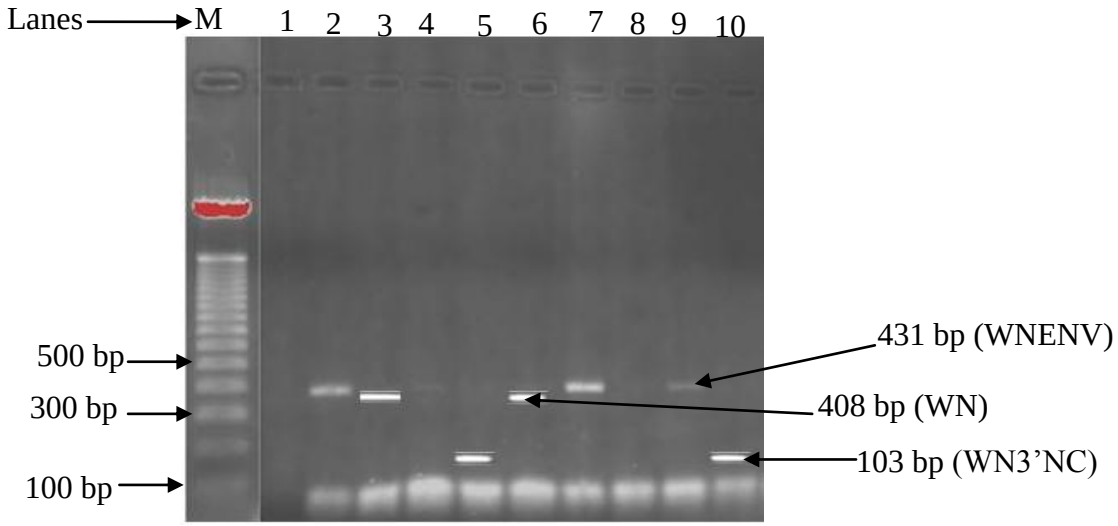
*- Statistically significant

Table 4.5: Seroprevalence of WNV in Relation to some Clinical Symptoms among Febrile Patients attending some Hospital in Kaduna state.

Symptoms	No Examined	No. positive for WNV (%)	Prevalence (%)	p-value(Fisher)/ OR	CI
Headache					
Yes	109	7(6.42)	5.2	0.345/0.797	0.730-0.870
No	26	0.(0.0)	0.0		
Stomach ache					
Yes	82	3(3.65)	2.2	0.432/2.150	0.461-10.015
No	53	4(7.54)	3.0		
Drowsiness					
Yes	51	3(5.88)	3.7	0.104/0.224	0.042-1.203
No	84	2(2.38)	1.5		
Body Weakness					
Yes	95	6(6.31)	4.4	0.674/0.380	0.044-3.266
No	40	1(2.5)	0.7		
Vomiting					
Yes	82	5(6.09)	3.7	0.704/0.604	0.113-3.233
No	53	2(3.77)	1.5		
Diarrhoea					
Yes	44	3(6.81)	2.2	0.682/0.628	0.134-2.938
No	130	6(4.61)	4.4		

KEYS: OR- odd ratio

CI- Confidence Interval



Lane M 100bp DNA ladder, lane 1- negative, lanes 2-10 pcr amplicons

Plate I: Amplicons of West Nile virus serotypes WNENV (431bp), WN (408bp) and WN3'NC (103bp).

CHAPTER FIVE

5.0 DISCUSSION

The study revealed the presence of WNV in the hospitals studied with the prevalence of 5.2%. This prevalence is an increase from the 1.2% reported by Baba *et al*. (2006) in semi-arid zones in Nigeria and 0.0% that was reported in Osun state Nigeria (Opaleye *et al*., 2014). This shows that the disease is on the increase, but consistent with the study carried out in Sudan that reported the prevalence of 6.4% (Khalid *et al*., 2014) and 5.2% reported in North Dokata USA (Micheal *et al*., 2006). However, Omilabu *et al.* (1990) reported an unusual high prevalence of 53% in the south western city of Ibadan in 1990.

This study investigated the prevalence of WNV in the senatorial districts of Kaduna state and found that Kaduna central had the highest prevalence of 3.0%. This could be due the nature of the settlement of the area which is densely populated with poor drainage systems which can serve as possible breeding sites for the mosquito vector or as a result of vector exposure to species of mosquito vector which is a major variable vehicle of transmission. This result of this study agrees with previous report by Monath (1980), Jonousek and Kramer (1999).

The prevalence of WNV in this study was higher in male population (3.7%) than in female population (1.5%) which is consistent with the report of Omilabu *et al.* (1990) in Ibadan and Ogbomosho but in contrast to the report of Olaleye *et al.* (1990) in Ibadan who reported higher prevalence in female population than in male population, another study in South Africa also reported a higher prevalence in female population than in the male population (Charmaine *et al*., 2014). The prevalence in this study could be attributed to the fact that male population are at higher risk of exposure to the mosquito vector as a result of their involvement in out-door

activities at peak biting time. However, there was no statistical significant between WNV infection and gender (P>0.05).

The study did not establish any association between WNV and age (p>0.05). Studies have reported age as a factor in susceptibility to infection (Hayes 1989; Margue *et al*., 2001). However, the absence of association in this study could be due to the size of the study population. A larger study population would be needed to really study the factor of age and WNV. Notwithstanding, in this study a higher prevalence of 2.2% was recorded in 31-40 age group which agrees with the studies of Olaleye *et al*. (1990) and Khalid and Shamsoun. (2014) in Ibadan and Sudan respectively that reported high prevalence in patients within the same age group. Contrary to this study, other studies (Omilabu *et al*., 1990; Mease *et al*., 2011) have reported higher prevalence in older age group. But surprisingly a higher prevalence was reported in children by Wang *et al.* (2009) in Ghana.

The report from this study could also be as a result of high rate of exposure to mosquitoes' bite as patients within age 31-40 are adult and so involve in out-door activities at peak biting time and are also involve in activities such as farming, fishing and social gathering which exposes them to the vector.

Education related studies had indicated that people with lower educational levels (primary education) were more likely to be infected (Idah *et al*., 2015). Though this population is considered literate, the poor educational system in the country leave much to be desired and may not have impacted positively on them, leading to the desired transformation. This study could not establish any statistical significant association between WNV infection and educational level.

This finding contradicts studies in Arizona which indicated that those who did not attend school were more at risk of the infection than those who are educated. (Gibney *et al.,* 2012).

Occupation has been reported to play a role in WNV infection (CDC, 2002[a]: 2003). The study established an association between WNV and occupation in the study population with farmers having the highest prevalence ($P<0.05$). However, this study contradicts a previous study conducted in Zambia which reported that farmers were less likely to have the infection compared to students (Idah *et al*., 2015). In most developing countries (Nigeria inclusive), poor vector control often lead to ubiquity of these infectious agent carriers and their bites occur all through the year as well as everywhere, which could be the reason for the result obtained in this study.

Blood transfusion is considered as a main risk factor in the transmission of WNV as reported by Pealer *et al.* (2003) and Iwamoto *et al.* (2003). From this study those that have been transfused with blood before the samples were collected from the patients had the higher prevalence of 3.0% and this agrees with the report of Idah *et al.* (2015) in Zambia in which blood transfusion was statistically significant to WNV infection but contradict the study of Wang *et al.* (2009) in Ghana. The low risk of infection through blood transfusion could be because infection occurs early in life and the window for infection through transfusion is quite short. However blood transfusion was not a statistically significant factor in this study.

Patients with cancer and those undergoing cancer chemotherapy both had prevalence of 0.7% which is low compared to a previous report that certain medical conditions such as cancer and compromised immune system as increased risk factors to WNV (Petersen, 2015). Report from a study carried out in Ghana also reported that the risk of pathogenicity of WNV in immunocompetent recipient appears to be limited (Wang *et al*., 2009). However in this study,

there was no statistical significant association (P>0.05) between WNV infection and those with cancer and undergoing any kind of chemotherapy. This implies that cancer and chemotherapy may not neccessarily predispose the patients to WNV infection.

Previous literature reported hypertension and diabetes as risk factors for WNV infection (Bode *et al*., 2006). However, in this study both hypertension and diabetes had lower prevalence of 1.5% each which contradict the above report. Cook *et al*. (2010) also reported the there was persistent symptoms of WNV >6months in patients with hypertension and diabetes and West Nile neuroinvasive disease. This study did not also agree with the report of Lyle *et al*. (2013) who reported that hypertension and diabetes are risk factors for WNV infection.

Symptoms such as stomach ache, headache, drowsiness, body weakness and vomiting shown high prevalence indicating symptoms characteristic of the disease. The result obtained from this study is in agreement with the CDC fact sheet report that stated that about 1 in 150 people infected with WNV will develop severe symptoms such as headache, body weakness and up to 20 percent of the people who become infected with WNV will have body and/or stomach ache, vomiting (CDC, 2003), the result from this study is also in agreement with that of Watson *et al.* (2004), Hayes *et al. (*2005) and Zou *et al.* (2010) that all reported that symptomatic persons experience an acute systematic febrile illness that often include headache, gastrointestinal pains, drowsiness and body pains.

WNV IgM positive sera were further subjected for the detection of RNA to WNV by RT-PCR using some primers which is in agreement with Lianciotti *et al* (1992) that reported RT-PCR as been useful for detecting RNA to WNV.

Out of the 7 IgM positive sera, 4 sera had RNA to WNV which might have accounted for the febrile illnesses in the study area and this result agreed favorably with previous reports of Nasci *et al* (2002) and Miagostovich *et al* (1997) that RT-PCR is an important tool for WNV diagnosis and virological surveillance. It is then probable that cases of WNV infection were missed among those patients (128 IgM negative sera) tested by serological technique alone, since testing all the IgM negative sera by RT-PCR was not feasible due to financial constraint at the time this study was carried out.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

From the result obtained in this study, there was serological evidence of WNV infection in the study area. The prevalence obtained from this study was 5.2% with Kaduna central senatorial district having the highest prevalence of 3.0%,

This study has reported that farming play a role as a socio-demographic factor in WNV infection. Other socio-demographic factors such as marital and educational status were not statistically significant. The result from this study has also reported that the use of mosquito nets is protective against WNV infection. Certain risk factors such as Blood transfusion, Organ transplant, chemotherapy, cancer, hypertension and diabetes were not statistically significant in this study.

The PCR result detected the presence of WNV in Kaduna metropolis and so the evidence of WNV in the study area was established through the findings in this study and it is also an indication of an ongoing infection in the study area

Further studies are needed to determine the disease burden of this important arbovirus in this state and other states of the country as this will help in designation of appropriate preventive measures to avoid contact with the vector and appropriate diagnostic technique put in place for the routine diagnosis of this infection which are mostly mistaken as malaria, typhoid or pyrexia of unknown origin (PUO). However, it is essential to maintain a sustained surveillance for this re-emerging infection in order to protect its outbreak in the future.

6.2 RECOMMENDATIONS

1. Occupational precaution should be put in place to avoid mosquitoe bite during farming process, mosquito repellent and insecticide should be used when necessary.
2. Prevention measures and recommendation should include;
    - The use of mosquito nets for sleeping should be encouraged as this will go a long way to avoid contact with the vector that transmits WNV.
    - Windows and doors should be ‘bug tight’ and broken screens repair or replaced
    - People should stay indoors at dawn and dusk, if possible when mosquitoes are most active.
    - Wear long sleeve shirts, long trousers and hat when going into infested area such as wetlands or woods.
3. Screening for this condition should be included in the routine screening nationwide especially for febrile patients with unknown origin.
4. Reduce the number of standing water and clear bushes which can serve as breeding sites for the vector.
5. Blood and organs should be properly screened before they are transfused and transplanted respectively.

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APPENDIX 1

DEPARTMENT OF MICROBIOLOGY

SCHOOL OF POST GRADUATE STUDIES

AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

"Seroprevalence and Molecular Detection of West Nile Virus in Febrile Patients Attending some Hospitals in Kaduna State, Nigeria"

INFORMED CONSENT FORM

Serial No: ……. Hospital No: …… Phone No: ……..

Having been well educated by the researcher on the concept, importance as well as steps that will be taken to carry out this research; I……..... ……. hereby willingly accept to be enrolled in this study, being made to understand that;

1. The data I will provide is solely for the purpose of this research work and confidentiality is assured.
2. The blood sample collected will be used for Serological Analysis and WNV Detection respectively, and the result will be communicated to me if I so desire.

……

Signature/thumbprint of client with date

APPENDIX 2

Research Questionnaire

Topic: "Seroprevalence and Molecular Detection of West Nile Virus in Febrile Patients Attending some Hospitals in Kaduna State, Nigeria."

Introduction: I am **MA'AJI, JOSEPHINE ASHULEE** a student of the above institution and department wish to seek for your consent to fill this questionnaire appropriately to enable me get some socio-demographic and clinical data from you. It will stand to be a basic background for the consequent judgment and conclusion of my thesis. All the data you will provide is solely for the purpose of this research, and hence will be treated as confidential. Thanks for your co-operation

Instruction : Please tick as appropriate and provide answers where necessary.

Section 'A' Personal Data

1. Gender: Male [ ], Female [ ]
2. Age: <20 [ ], 21-30 [ ], 31-40 [ ], 41-50 [ ], >51 [ ]
3. Marital Status : Single [ ] Married [ ] Separated[ ]
4. Place of resident : Kaduna North [ ] Kaduna central [ ] Kaduna South [ ]
5. Highest level of education : Primary [ ] Secondary [ ] Tertiary [ ] Informal [ ]
6. Occupation : Civil servant [ ], Housewife [ ], Fishing [ ], Bucher [ ],Farming [ ]
7. **Section 'B' : Epidemiological Risk Factors**
8. Have you been transfused with blood before? Yes [ ] No [ ]
9. Have you taken any organ transplant before? Yes [ ] No [ ]

10. Are presently undergoing any form of chemotherapy? Yes [ ] No [ ]

11. Are you suffering from any type of cancer? Yes [ ] No [ ]

12. Are you Hypertensive? Yes [ ] No [ ]

13. Are you Diabetic? Yes [ ] No [ ]

14. Do you sleep with mosquito net? Yes [ ] No [ ]

15. Do you have mosquito nets on the doors or windows of your house? Yes [ ]

No [ ]

16. Are you having any of the following symptoms

i. Headache Yes [ ] No [ ]

ii. Stomach pain Yes [ ] No [ ]

iii. Drowsiness Yes [ ] No [ ]

iv. Body weakness Yes [ ] No [ ]

v. Vomitting Yes [ ] No [ ]

vi. Diarrohea Yes [ ] No [ ]

APPENDIX 3

Ethical Approval

MINISTRY OF HEALTH, KADUNA STATE

All Communication to be addressed to:
THE HON. COMMISSIONER
Quoting Reference and Date
Telephone: 234-248048
Website: ttp://www/moh.kd.gov.ng.
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Kaduna State, Nigeria.

MOH/ADM/744/VOL.1/315

27th July, 2015

NOTICE OF APPROVAL AFTER FULL COMMITTEE REVIEW

SEROPREVALENCE AND MOLECULAR DETECTION OF WEST NILE VIRUS AMONG FEBRILE PATIENTS ATTENDING SOME HOSPITAL IN KADUNA STATE, NIGERIA

Name of Principal Investigator: **MA'AJI JOSEPHINE ASHULEE**
Address of Principal Investigator: **Dept. of Microbiology, Faculty of Science, Ahmadu Bello University Zaria, Kaduna State**

Date of receipt of Application: **19th June, 2015**
Date of Ethical Approval: **15th July, 2015**

This is to inform you that the Research described in the submitted Protocol, the Consent forms, advertisements and other participant information materials have been reviewed and given full approval by the Health Research Ethics Committee(HREC).

If there is delay in starting the research or any change, inform the HREC so that the dates of approval can be adjusted accordingly.

However, Researcher is kindly requested to submit a copy of his/her findings to the State Ministry of Health, please.

DR. B. M. JATAU
Chairman,
Health Research Ethics Committee

KADUNA STATE MINISTRY OF HEAL
OPEN REGISTRY
DESPATCHER
SIGN _____ DATE 27/7/15

APPENDIX 4

ELISA IgM RESULT FOR GWAMNA AWAN GENERAL HOSPITAL, KAKURI, KADUNA

SAMPLE	ABSORBANCE (WRNA)	SAMPLE	ABSORBANCE (NCA)	WEST NILE IgM	RESULT
S1	0.216	S48	0.089	2.426966292	NEGATIVE
S2	**3.05**	**S49**	**0.104**	**29.32692308**	**POSITIVE**
S3	0.172	S50	0.053	3.245283019	NEGATIVE
S4	0.221	S51	0.099	2.232323232	NEGATIVE
S5	**0.391**	**S52**	**0.045**	**8.688888889**	**POSITIVE**
S6	0.282	S53	0.14	2.014285714	NEGATIVE
S7	0.226	S54	0.078	2.897435897	NEGATIVE
S8	0.578	S55	0.458	1.262008734	NEGATIVE
S9	0.206	S56	0.072	2.861111111	NEGATIVE
S10	0.181	S57	0.084	2.154761905	NEGATIVE
S11	**2.376**	**S58**	**0.289**	**8.221453287**	**POSITIVE**
S12	0	S59	0.056	0	NEGATIVE
S13	0	S60	0.042	0	NEGATIVE
S14	0	S61	0.071	0	NEGATIVE
S15	0	S62	0.032	0	NEGATIVE
S16	**2.549**	**S63**	**0.056**	**45.51785714**	**POSITIVE**
S17	0.363	S64	0.451	0.804878049	NEGATIVE
S18	0	S65	0.048	0	NEGATIVE
S19	0.548	S66	0.434	1.262672811	NEGATIVE
S20	0.041	S67	0.121	0.338842975	NEGATIVE
S21	0	S68	0.06	0	NEGATIVE
S22	0	S69	0.067	0	NEGATIVE
S23	0.035	S70	0.155	0.225806452	NEGATIVE
S24	0.125	S71	0.118	1.059322034	NEGATIVE
S25	0.135	S72	0.073	1.849315068	NEGATIVE
S26	0.076	S73	0.094	0.808510638	NEGATIVE
S27	0.11	S74	0.107	1.028037383	NEGATIVE
S28	0.042	S75	0.06	0.7	NEGATIVE
S29	0.137	S76	0.136	1.007352941	NEGATIVE
S30	0.115	S77	0.112	1.026785714	NEGATIVE
S31	0.134	S78	0.125	1.072	NEGATIVE
S32	0.337	S79	0.289	1.166089965	NEGATIVE
S33	0.183	S80	0.129	1.418604651	NEGATIVE
S34	0.216	S81	0.148	1.459459459	NEGATIVE
S35	**2.643**	**S82**	**0.158**	**16.7278481**	**POSITIVE**
S36	0.209	S83	0.054	3.87037037	NEGATIVE

S37	2.737	S84	2.604	1.051075269	NEGATIVE
S38	0.115	S85	0.059	1.949152542	NEGATIVE
S39	0.304	S86	0.219	1.388127854	NEGATIVE
S40	0.233	S87	0.139	1.676258993	NEGATIVE
S41	0.927	S88	0.772	1.200777202	NEGATIVE
S42	0.123	S89	0.041	3	NEGATIVE
S43	0.197	S90	0.036	5.472222222	NEGATIVE
S44	0.208	S91	0.159	1.308176101	NEGATIVE
S45	0.156	S92	0.035	4.457142857	NEGATIVE
S46	3.258	S93	3.522	0.925042589	NEGATIVE
S47	0.446	S94	0.243	1.835390947	NEGATIVE

APPENDIX 5

ELISA IgM RESULT FOR HAJIYA GAMBO SAWABA GENERAL HOSPITAL, ZARIA,KADUNA.

SAMPLE	ABSORBANCE (WNRA)	SAMPLE	ABSORBANCE (NCA)	WEST NILE IgM	RESULT
S1	0.214	S48	0.112	1.910714286	NEGATIVE
S2	**2.794**	**S49**	**0.097**	**28.80412371**	**POSITIVE**
S3	0.871	S50	0.712	1.223314607	NEGATIVE
S4	0.165	S51	0.107	1.542056075	NEGATIVE
S5	0.142	S52	0.121	1.173553719	NEGATIVE
S6	0.263	S53	0.119	2.210084034	NEGATIVE
S7	0.807	S54	0.671	1.202682563	NEGATIVE
S8	0.327	S55	0.134	2.440298507	NEGATIVE
S9	0.263	S56	0.105	2.504761905	NEGATIVE
S10	0.618	S57	0.433	1.427251732	NEGATIVE
S11	0.315	S58	0.217	1.451612903	NEGATIVE
S12	0.329	S59	0.466	0.706008584	NEGATIVE
S13	0.561	S60	0.673	0.833580981	NEGATIVE
S14	0	S61	0.08	0	NEGATIVE
S15	0	S62	0.056	0	NEGATIVE
S16	0	S63	0.075	0	NEGATIVE
S17	0	S64	0.086	0	NEGATIVE
S18	0.091	S65	0.082	1.109756098	NEGATIVE
S19	0.024	S66	0.091	0.263736264	NEGATIVE
S20	0.02	S67	0.089	0.224719101	NEGATIVE
S21	0.086	S68	0.216	0.398148148	NEGATIVE
S22	0.066	S69	0.14	0.471428571	NEGATIVE
S23	0.075	S70	0.122	0.614754098	NEGATIVE
S24	0.051	S71	0.069	0.739130435	NEGATIVE
S25	0.144	S72	0.139	1.035971223	NEGATIVE
S26	0.115	S73	0.071	1.61971831	NEGATIVE
S27	0.078	S74	0.079	0.987341772	NEGATIVE
S28	2.685	S75	2.621	1.024418161	NEGATIVE
S29	0.654	S76	0.601	1.088186356	NEGATIVE
S30	0.091	S77	0.063	1.444444444	NEGATIVE
S31	0.293	S78	0.252	1.162698413	NEGATIVE
S32	0.405	S79	0.352	1.150568182	NEGATIVE
S33	0.171	S80	0.163	1.049079755	NEGATIVE
S34	0.204	S81	0.145	1.406896552	NEGATIVE
S35	0.251	S82	0.126	1.992063492	NEGATIVE

S36	0.176	S83	0.152	1.157894737	NEGATIVE
S37	0.108	S84	0.16	0.675	NEGATIVE
S38	0.091	S85	0.075	1.213333333	NEGATIVE
S39	0.167	S86	0.069	2.420289855	NEGATIVE
S40	0.13	S87	0.085	1.529411765	NEGATIVE
S41	0.215	S88	0.121	1.776859504	NEGATIVE
S42		S89	0.053	0	NEGATIVE
S43	0.152	S90	0.095	1.6	NEGATIVE
S44	0.17	S91	0.124	1.370967742	NEGATIVE
S45	0.193	S92	0.124	1.556451613	NEGATIVE
S46	0.185	S93	0.103	1.796116505	NEGATIVE
S47	0.266	S94	0.108	2.462962963	NEGATIVE

APPENDIX 6

ELISA IgM RESULT FOR GENERAL HOSPITAL, KAFANCHAN, KADUNA.

SAMPLE	ABSORBANCE (WRNA)	SAMPLE	ABSORBANCE(NCA)	WEST NILE IgM	RESULT
S1	0.207	S48	0.11	1.881818182	NEGATIVE
S2	**2.075**	**S49**	**0.136**	**15.25735294**	**POSITIVE**
S3	0.171	S50	0.103	1.660194175	NEGATIVE
S4	0.171	S51	0.201	0.850746269	NEGATIVE
S5	1.913	S52	1.955	0.978516624	NEGATIVE
S6	1.835	S53	1.818	1.009350935	NEGATIVE
S7	0.182	S54	0.111	1.63963964	NEGATIVE
S8	0.188	S55	0.076	2.473684211	NEGATIVE
S9	0.149	S56	0.049	3.040816327	NEGATIVE
S10	0.259	S57	0.069	3.753623188	NEGATIVE
S11	1.842	S58	1.963	0.938359654	NEGATIVE
S12	0	S59	0.041	0	NEGATIVE
S13	1.592	S60	1.043	1.526366251	NEGATIVE
S14	0.955	S61	1.311	0.728451564	NEGATIVE
S15	0	S62	0.031	0	NEGATIVE
S16	0.027	S63	0.053	0.509433962	NEGATIVE
S17	0.35	S64	0.544	0.643382353	NEGATIVE
S18	0	S65	0.04	0	NEGATIVE
S19	0	S66	0.08	0	NEGATIVE
S20	0	S67	0.089	0	NEGATIVE
S21	0.016	S68	0.258	0.062015504	NEGATIVE
S22	0.099	S69	0.187	0.529411765	NEGATIVE
S23	0	S70	0.087	0	NEGATIVE
S24	0.072	S71	0.045	1.6	NEGATIVE
S25	0.069	S72	0.059	1.169491525	NEGATIVE
S26	0.073	S73	0.094	0.776595745	NEGATIVE
S27	0.063	S74	0.054	1.166666667	NEGATIVE
S28	0.174	S75	0.188	0.925531915	NEGATIVE
S29	0.066	S76	0.02	3.3	NEGATIVE
S30	0.338	S77	0.27	1.251851852	NEGATIVE
S31	0.151	S78	0.143	1.055944056	NEGATIVE
S32	0.303	S79	0.329	0.920972644	NEGATIVE
S33	0.35	S80	0.326	1.073619632	NEGATIVE
S34	0.15	S81	0.126	1.19047619	NEGATIVE
S35	0.136	S82	0.057	2.385964912	NEGATIVE
S36	0.155	S83	0.102	1.519607843	NEGATIVE
S37	0.135	S84	0.088	1.534090909	NEGATIVE
S38	0.153	S85	0.075	2.04	NEGATIVE

S39	0.406	S86	0.266	1.526315789	NEGATIVE
S40	0.1	S87	0.027	3.703703704	NEGATIVE
S41	0.087	S88	0.049	1.775510204	NEGATIVE
S42	0.089	S89	0.041	2.170731707	NEGATIVE
S43	0.08	S90	0.047	1.70212766	NEGATIVE
S44	**0.089**	**S91**	**0.015**	**5.933333333**	**POSITIVE**
S45	0.258	S92	0.2	1.29	NEGATIVE
S46	**0.123**	**S93**	**0.022**	**5.590909091**	**POSITIVE**
S47	**2.008**	**S94**	**0.189**	**10.62433862**	**POSITIVE**