THE UNIVERSITY OF NAIROBI

DETERMINATION OF THE MAIN RESERVOIR HOSTS OF WEST NILE VIRUS AMONG WILD BIRDS IN TANA RIVER COUNTY, KENYA

By:

Nyamwaya Kemunto Doris (B.sc Honours)

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

Doris Kemunto Nyamwaya

(I56/69668/2013)

Signature..... Date.....

This thesis has been submitted for examination with my approval as a supervisor

Dr. Virginia Wang'ondu

The University of Nairobi,

School of Biological Sciences,

Email: vwwangondu@yahoo.com

Signature..... Date.....

Dr. Bernard Bett

International Livestock Research Institute,

Department of Food Safety and Zoonoses,

Email: b.bett@cgiar.org

Signature..... Date.....

DEDICATION

I dedicate this research dissertation to my loving father the late Dr. Zablon Ondari Ondieki who was always optimistic about my success but didn't live to read this. Rest in peace.

To my best friend and son Dan-Frank Motari: "you gave me a reason to work harder and I pray that you will follow my footsteps".

To my mother Mrs Alice Nyabate: "The far I have come, you started it and held my hand all the way. Thank you!"

To my sisters Betty, Phylis, Elizabeth, Faith and Brother Brian.

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Above all, it was by the grace of the almighty God and to him be the glory.

ABBREVIATIONS

RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
WNF	West Nile fever
WNV	West Nile virus
PCR	Polymerase chain reaction
dNTP	Deoxynucleoside triphosphate
RT	Reverse transcriptase
cDNA	complementary DNA
DTT	Dithiothreitol
DEPC	Diethylpyrocarbonate
EDTA	Ethylenediaminetetraacetic acid
NaOH	Sodium hydroxide
NH_4OAc	Ammonium Acetate
NCBI	National Center for Biotechnology Information
µg	Microgram
µL	micro litre
g×	sedimentation gravity

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ABSTRACT

Re-emerging infectious diseases can cause serious health and economic effects in a society. West Nile virus fever is a zoonotic arboviral infection maintained in nature within a cycle between mosquito vectors and birds. This virus was first isolated in Uganda with subsequent reports of epidemics globally. In order to establish effective monitoring and surveillance measures, knowledge on the ecological and transmission patterns is necessary. This study aimed at determining the main reservoir hosts of West Nile virus. Blood samples were obtained from 361 randomly sampled wild birds in Tana River County, Kenya, in the months of October and December 2014. The samples were subjected to nucleic acid based screening for West Nile virus using the virus specific primers in real time polymerase chain reaction after total ribonucleic acid extraction. The amplification was carried out against a standard curve generated using serial dilutions of a synthetic positive control. A total of 65 samples exhibited positive amplification with a high cycle threshold value of 30. Visualization of the amplified fragments on agarose gel revealed bands of targeted 445 base pair fragments. Sanger sequences of 5 of the samples indicated genetic relationship to West Nile virus *XJ11141*, *XJ11129, XJ11148* and *Ast-986* strains initially isolated from China and Russia. Phylogenetic analysis clustered the isolates with described lineage 1 strains in Genebank. A regression analysis indicated that the sampling location influenced the occurrence of West Nile virus while species, age, weight and sex of the birds did not have any effect. This study provides baseline information on the existing circulation of the virus in this region among wild birds that could spill into the human population and points to the need for implementation of surveillance programs. Therefore, there is need to enhance awareness in the public health department of this region to contain its circulation.

Keywords: Arbovirus, Polymerase chain reaction, Reverse transcription, West Nile Virus, Zoonosis.

CHAPTER 1

INTRODUCTION

1.1: Background

Re-emerging pathogens are novel disease agents in a population, infections with previously negligible incidences in the global population that begin to resurge, broadening their geographic and host ranges, old microbes causing new diseases, persistent organisms in the environment transmitted indirectly to humans through human modified environments or well adapted pathogens to non-human hosts that spill to humans (Morens *et al*., 2004; Smolinski *et al*., 2003; Wilcox and Colwell, 2005). These pathogens are significant to the public health sector as they may result in fatal outbreaks that cause massive economic and social losses. The emergence is favoured by several factors including close contact between human and livestock populations, high human and animal density, globalization of trade, geographical position, warm and humid climate, richness in wildlife host species, socio-economic situation and the socio-cultural practices (Jones *et al*., 2008; Smolinski *et al*., 2003). More than 60% of emerging diseases are zoonoses of which more than 70% of emerging epidemics are caused by pathogens which originate from wild animals (Jones *et al*., 2008).

Zoonoses are diseases that are transmissible from animals to human beings either directly through animal – human contact, contact with contaminated substances, or indirectly through ingestion of contaminated food/water or via various vectors such as ticks and mosquitoes. West Nile virus (WNV) is one of the re-emerging zoonotic mosquito-borne pathogens. It amplifies in various species of wild birds, and WNV antibodies have been detected in some mammals, reptiles and amphibians (Glaser, 2004). Humans and equine hosts are incidental

hosts as they produce insignificant viremia, and do not contribute to the transmission cycle (Kilpatrick *et al*., 2007).

West Nile virus was first isolated in Uganda with its occurrence being sporadically detected in various countries with fatal epidemic outbreaks (England *et al*., 2001; Petersen *et al*., 2013; Reisen, 2013). In most cases the infection is asymptomatic; however, it can cause severe central nervous system manifestations especially in elderly and immuno-compromised individuals (Diamond *et al*., 2003). The virulence of WNV could also be enhanced in malnourished individuals or when it occurs as a concurrent infection. Some birds develop a high and lasting viremia after infection that facilitates virus transfer to the mosquito vector. It is therefore important to understand the ecology and transmission patterns of WNV in order to implement valid monitoring and epidemic prevention measures.

WNV is a single strand RNA virus. It can be detected in a host using various serological and molecular techniques such as polymerase chain reaction (PCR). Reverse transcription PCR (RT-PCR) is used to first convert RNA into DNA using reverse transcriptase enzyme, a sensitive technique in which a very low copy number of RNA molecules can be detected (Bustina and Nolan, 2004). Standard protocols for WNV screening have been developed among which using real time PCR and WNV specific primers has been proven to be reliable and effective .

1.2: Problem statement

Re-emerging infectious diseases can result in unexpected epidemics when proper surveillance and preventive measures are not enforced. Sporadic outbreaks of the neurotropic WNV have been reported from many regions since its isolation from Uganda some of which have resulted in significant deaths among humans and avifauna (Petersen *et al*., 2012; Smithburn *et al*., 1940). Efforts to establish an effective vaccine or curative regime against West Nile fever (WNF) have not yet been successful. Migratory birds have been known to transfer the virus to non-endemic localities. Tana River County is a destination of many species of migrating wild birds that are potential reservoir hosts of WNV. Knowledge on sero-prevalence of the virus among birdlife is crucial in determining the presence and magnitude of the circulating pathogen and promoting decision making on appropriate measures to prevent outbreaks that could be of intense public health impact.

1.3: Project justification

Research carried out by the International Livestock Research Institute on human blood samples from Tana River County, revealed presence of antibodies against WNV. This is an indication of occurrence of the pathogen in this region. This study will enhance knowledge on the ecology of WNV by generating information on the amplifying reservoir hosts and factors that keep the pathogen in the enzootic transmission cycle hence contribute to establishment of surveillance and monitoring strategies in containment of its effects.

1.4: Objectives

1.4.1: Broad objective

To determine the main reservoir host of West Nile virus among wild bird species in Tana River, Kenya

1.4.2: Specific objectives

1. To isolate and determine the prevalence of the West Nile virus in local and migratory birds in Tana River District
2. To determine the effect of habitat characteristics and host factors on the risk of WNV infection in birds
3. To determine the diversity and phylogeny of West Nile virus in Tana River County

1.5: Research hypothesis

West Nile virus occurrence in Tana River County is influenced by prevailing habitat characteristics and various bird host factors including age, sex and species

CHAPTER 2

LITERATURE REVIEW

2.1: Aetiology

West Nile virus has been taxonomically grouped into the Japanese encephalitis antigenic complex within the flaviviridae family and flavivirus genus (Kuno *et al*., 1998). This complex includes Koutango, Usutu and Yaonde viruses from Africa, Brazil's Cacipacore virus, Alfuy virus in Australia, Saint-louis encephalitis virus in the Americas, Murray valley encephalitis in Australia and Japanese encephalitis virus in Asia.

Structurally, the virion is spherical with a diameter of 50nm. It has an icosahedron core 30 – 35 nm in diameter composed of repeated copies of a 12-kDa capsid protein that encloses a single stranded positive sense RNA of 11000 – 12000 nucleotides containing a single open reading frame encoding 10 proteins. Seven of these proteins are non-structural (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) and comprise the intracellular replication machinery of the virus. The other three proteins are structural and include core (C), membrane (M) and envelope (E) that recognizes the viral receptor on cell surfaces and induces humoral immunity (Duebel *et al*., 2001). Different strains of WNV have been isolated in different geographical locations with 8 distinct lineages based on phylogenetic analyses (Vazquez *et al*., 2010).

2.2: Ecology and transmission

The abundance and global distribution of WNV is dependent on interactions with its vectors, hosts and various abiotic factors. Climatic conditions play a significant role with increased temperatures, rainfall frequencies and humidity raising the incidence rate of the fever (Soverow *et al*., 2009).

Several factors influence dispersal of WNV including endemicity that promotes immunity among the local population, urbanization, abundance of competent vectors and hosts, age structure with the elderly being more susceptible and landing sites of migratory birds (Hubálek, 2001). Transmission intensity of WNV is directly dependent on reservoir competence and rate contact between hosts and vectors (Simpson *et al*., 2012). Mosquitoes form the vectoral component of the transmission cycle. In Europe and Africa, *Culex pipiens, Cx. modestus, Cx. Antennatus and Cx univittatus species* are the main vectors. Mosquitoes mainly get infected when taking a bloodmeal from a viraemic host. However, some vectors acquire the infection through transovarial route (Dohm *et al*., 2002). WNV is able to persist in the vector through unfavourable climatic conditions and emerge later to re-establish an infection cycle (Nasci *et al*., 2001)

WNV amplifies in avian hosts, as reported by Komar *et al*., (2003), passerines are competent reservoirs as demonstrated by their high susceptibility and ability to maintain high viremia for a prolonged duration. An epidemic in New York was accompanied by substantial avian mortality. Although massive crow deaths were observed, investigations have shown that the virus is a threat to most bird species that succumbed to the infection (Eidson *et al*., 2001). Co-evolution of the virus with avian hosts has been evidenced by a case study on house sparrows that showed reduction in mortality rates when infected with an earlier WNV strain compared to the high magnitude and prolonged viremias obtained following infection with evolved WNV strains (Duggal *et al*., 2014). Migratory birds have been demonstrated to participate in the process of transmitting the virus (Bessell *et al*., 2014). Fledgling migrating storks from Europe to the middle East were diagnosed with the virus on landing in Eilat (Israel) (Malkinson *et al*., 2002).

Dogs have been used as sentinels for WNV surveys but are unlikely to be reservoirs due to short term and low level viraemia (Kile *et al*., 2005). During surveillance for WNV in Africa, all dogs sampled from Chad were seropositive .This area is located at a confluence of two rivers and it has a high density of birds. In contrast, dog samples from wet forests of North-eastern Gabon were all seronegative as the region does not support viral circulation (No and Ratovonjato, 2014).

2.3: Epidemiology and geographical distribution

Geographically WNV is extensively distributed in Africa, Middle East, southern Europe, Western Russia, Southern Asia and Australia (Gubler, 2007; Petersen *et al*., 2013). After initial isolation of WNV by intra cerebral inoculation of serum from a febrile patient in the West Nile district of Uganda (Smithburn *et al*., 1940), there have been subsequent reports of sporadic occurrences and major epidemics globally. In 1951, a case was reported in Israel, a country that forms a migratory route for birds between Europe and Africa and this was followed by small intermittent outbreaks until 2000 when the country experienced a major epidemic with high incidences along the coastal plains and few cases in arid areas (Anis *et al.*, 2014). In 1999, WNV was detected for the first time in the western hemisphere at New York metropolitan area (England *et al*., 2001). Reports show that over 3 million people have been infected in the United States with irrigated farmlands in the central plains and some western states recording highest incidences (Petersen *et al*., 2012). There is extensive circulation of WNV in the central, Northern and southwestern regions of Iran among human and equine population as suggested by Chinikar *et al*., (2013). Timely information on possible WNV outbreaks can be obtained by regular serologic and molecular surveillance of wild birds. Prior to the 2010 epidemic in Greece, investigations had shown high virus neutralization titers in the avian population (Valiakos *et al*., 2012). Consequently, the outbreaks have reoccurred and spread to new localities (Danis *et al*., 2011).

A study in Albania revealed that seroprevalence of WNV-specific antibodies was regionally clustered with significant high rates being experienced near the Mediterranean sea and diminished towards inland locations (Berxholi *et al.,* 2013). In North West Kenya, WNV was isolated from male *Culex univittatus* complex mosquitoes trapped from Turkwel gorge with a genetic resemblance to a Romanian strain reported in 1996 (Miller *et al*., 2000).

2.4: Pathogenesis

West Nile virus mainly affects the central nervous system and may result to severe illness in humans. The infection will begin with inoculation of the virus by a bite of an infected mosquito vector. The virus undergoes replication at the Langerhans cells of the skin at this site (Johnston *et al*., 1996) after which infected cells access the lymphatic system (Johnston *et al*., 2000). Replication takes place again at secondary lymphoid tissues and the virus gets access to the bloodstream through the efferent lymphatic system and thoracic duct from where it disseminates to visceral organs, the brain and spinal cord (Diamond *et al*., 2003). The virus may cross the blood-brain barrier by passive transport across the endothelium of the choroid plexus or by being carried in infected inflammatory cells (Solomon *et al*., 2002). In certain conditions, the virus invades the central nervous system directly from the olfactory mucosa with subsequent spread to other sites by retrograde axonal transport (Nir *et al*., 1965). Studies on mice revealed that the kinetics of virus dissemination from lymphoid tissue, to serum and the central nervous system is impeded by development of antibodies. Severe human encephalitis due to West Nile virus infection is biased to the elderly due to decreased antibody production (Diamond *et al*., 2003).

2.5: Clinical features

Cases of WNF in humans have manifestations ranging from subclinical to clinical features. In most instances the infection is mild and asymptomatic (Dauphin and Zientara, 2007). Symptoms observed among some patients during WNV outbreaks were mostly fever, headache, muscle aches, fatigue, nausea, joint pain and few cases exhibited myocarditis, hepatosplenomegaly, ocular pain, conjunctivitis, skin rash, lymphadenitis, cough, sore throat, diarrhoea and abdominal pain. Less than 1% of patients developed meningoencephalitis that mostly resulted to death (Hubálek, 2001).

2.6: Diagnosis

Presence of WNV can be detected using various serological techniques. Immunoglobulin G (IgG) enzyme- linked immunosorbent assay (ELISA) has been developed using monoclonal antibodies (MAbs). Due to non-specificity of IgG, significant cross-reactivity between antisera and related heterologous antigens was observed (Johnson *et al*., 2000). Standardized Immunoglobulin M antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) was developed to enable early detection of antibodies following arboviral infections (Martin et al., 2000). This technique was useful in detection of early cases during 1999 WNV outbreak in the United States. It is intended for use in tandem with the IgG ELISA to produce clear antibody profile for each case. In Danis *et al.* (2011), WNV–specific antibody response was demonstrated among patients by screening their serum and cerebrospinal fluid samples using MAC-ELISA. Reverse transcriptase nested PCR (RT-nPCR) has been demonstrated to be rapid, reliable and specific in detecting WNV in animal tissues during the viremic phase (Johnson *et al*., 2001).

2.7: Prevention and control

Efforts to prevent occurrence of West Nile fever have led to the ideology of developing a valid vaccine against the pathogen which is however under development (Monath *et al*, 2001). Other methods to reduce the infection pressure (Jones *et al*., 2008) include intense surveillance both epidemiological, laboratory, ecological and anthropological. It would be a good practice to test mosquitoes; sample sentinel caged chicken flocks periodically and dead birds for WNV as they are good indicators of increased virus activity. Vector control can be done by eliminating larval habitats or making them unsuitable for larval development, bio-control can be used either as larvicides or adultcides. Behavioral measures that will reduce contact with the vector can be effective like use of insecticide treated nets, wearing protective clothing and use of window screens.

2.8: Treatment

Specific treatment for WNV infections has not yet been developed. Most patients rely on supportive care. As indicated in Ben-Nathan *et al*. (2003), acquisition of virus specific immuno-globulins not only protects the host from infection but also confers therapeutic effect on an already established infection. Administration of intravenous hyper immune gamma globulin during the early stage of the infection could improve neurological symptoms (Makhoul *et al*., 2009). Aguanosine analogue (Ribavirin) is a broad-spectrum antiviral therapy that can improve prognosis in WNV patients when administered at high doses (Jordan *et al*., 2000). So far there is no registered specific WNV infection drug or vaccine and there is need to identify a regime with high selectivity and therapeutic indices.

CHAPTER 3

MATERIALS AND METHODS

3.1: Description of the study area

The study was carried out in Bura, Hola and Ijara in Tana River County, Kenya. The County is located at $1^0$30'S $40^0$0'E and covers 35,376km^2 of the coast province (Fig.1) with a population density of 240,075 as per 2009 human population and housing census. The County is subdivided into Galole, Garsen and Bura constituencies, with its administrative headquarters located in Hola. It is a semi-arid area with annual relief rainfall of 400-750mm and mean annual temperatures varying from 30°C to 33°C. Challenges facing the County include food security, insecurity due to sporadic terrorist activities, flooding during rainy seasons and limited water access during dry spells.

Habitat diversity is unique ranging from forests, woodlands, grasslands, riverine, mangroves, sand dunes and bushlands. The main economic activities in the County are farming and pastoralism. The natives are mostly the Pokomo community who are farmers, Orma and Wardey communities who are nomads. The County has irrigation projects that produce cereals like maize and green grams, bananas among other food crops. Due to water pools in the irrigation schemes and the hot and humid climatic conditions, there is a high mosquito density and during harvesting period the avian diversity is high.

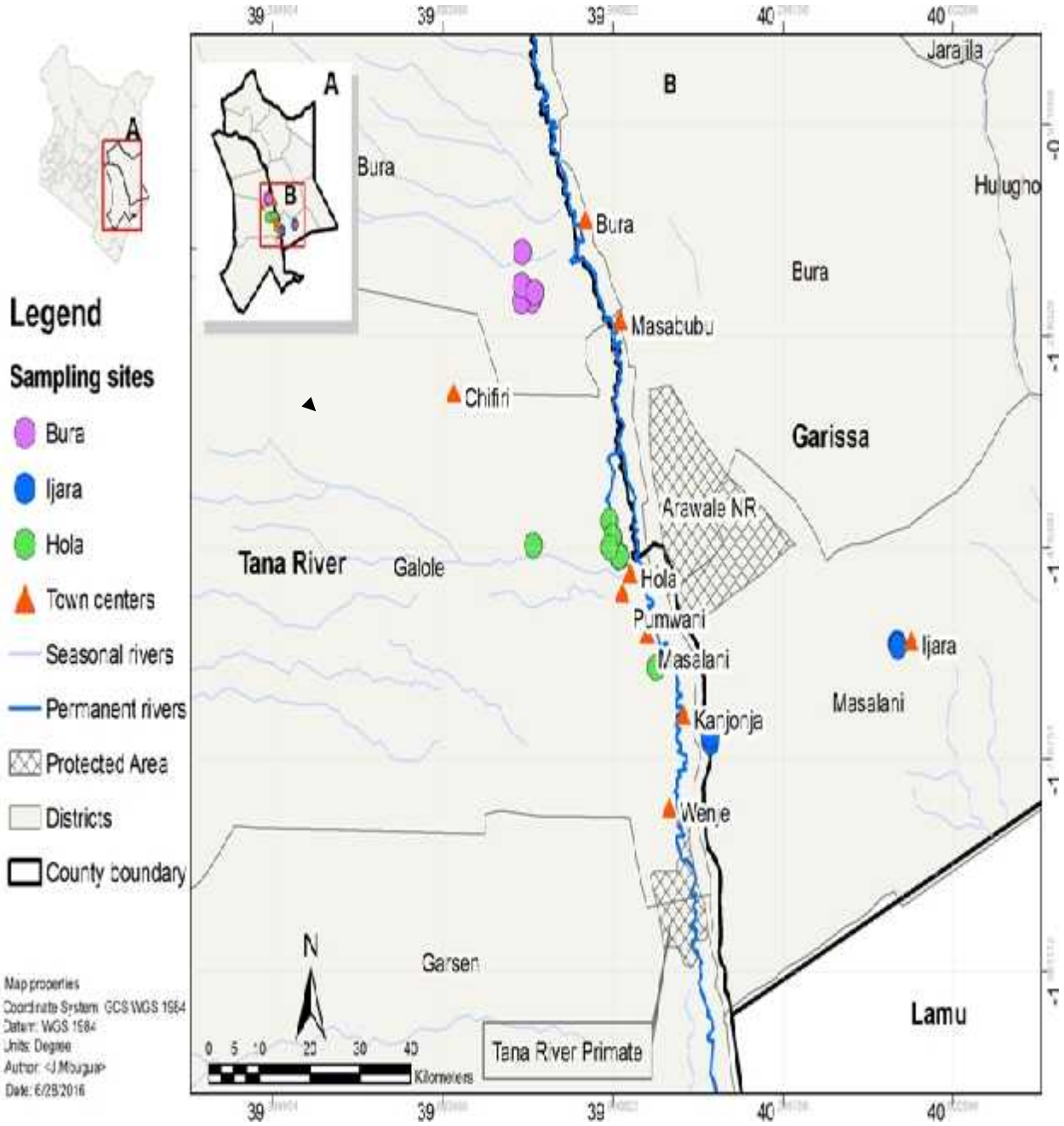

Fig 1: Map of Kenya indicating bird trapping sites in Tana River County (*source: James Mbugua*)

3.2: Sampling design

Sampling was done in October (22-28) and December (9-18), 2014. Bird trapping sites were selected randomly in irrigated and non-irrigated areas with varied distances from human settlements. In Bura, the sampling spots were located at Block A7, Fahari primary in village 9, farms in village 1, pond in village 8, BCC hotel compound, and at the agricultural research station. In Hola nets were set at Maendeleo graveyard, Matanya research fields, Kungu fish pond, Bondeni forest, Kyarikungu primary, National youth service (NYS) bushes and National irrigation board farms (NIB). From Ijara birds were trapped at the police camp field, Er-maan homestead and Gherille waterpan.

3.3: Bird trapping

Birds were trapped as described in (Randall *et al.*, 2013) using mist nets (12m length by 2m width,38 mm mesh and 6 panels) suspended 30cm from the ground to the net's maximum height using bamboo poles 3 metre long (Fig. 2). A total of 4 nets were set in the late evenings to enable consistency in opening time (0630 hours) and furled for a night.

Fig. 2: Mist nets used for trapping flying wild birds (*photo by Alice Madicott*)

Once the nets were opened, captured birds were extracted at an interval of 30 minutes. However, random extractions were done in some cases as soon as birds were observed to be netted to minimize traumatic stress on the trapped bird and the corresponding time was indicated. Extracted birds were placed in clean, cotton bird bags, under a shade.

Trapped birds were sampled according to standard ornithological bird handling and processing procedures (Komar *et al*., 2012). This included species identification, determination of age and sex, and biometric measurements including weight and taking the tail, tarsus, wing and head lengths. Each bird was banded using a labelled ring from the National Museums of Kenya for follow up purpose and to avoid re-sampling.

3.4: Blood sample collection

Bleeding of birds was done under aseptic conditions by a qualified and licensed ornithologist from the National Museums of Kenya. To obtain blood samples, a bird was held using the classic 'ringer's/ bander's grip' where it is held in the left hand and its head placed between the base of the index and middle fingers. The body was rotated towards the thumb with legs tucked under the body and against the palm. The left wing was extended and held between the tip of the index and middle fingers to locate the ulnar-humoral joint and expose the brachial vein. For small bird species, the wing vein was punctured with a 26 gauge needle and blood collected directly into micro hematocrit capillary tubes. For larger species, a syringe and needle were appropriate. A cotton wool ball immersed in 70% ethanol was used to exert pressure on the site to prevent haemorrhage for a minute before the bird was released.

3.5: Blood Sample handling

During the first round of sample collection, blood was dispensed into sterile barcoded vials that were racked and stored in solid carbon dioxide. Due to the technical, logistical and cost challenges arising from the use of dry ice, a decision was made to switch to Whatman® FTA® card (Fig. 3) technology for blood sample collection.

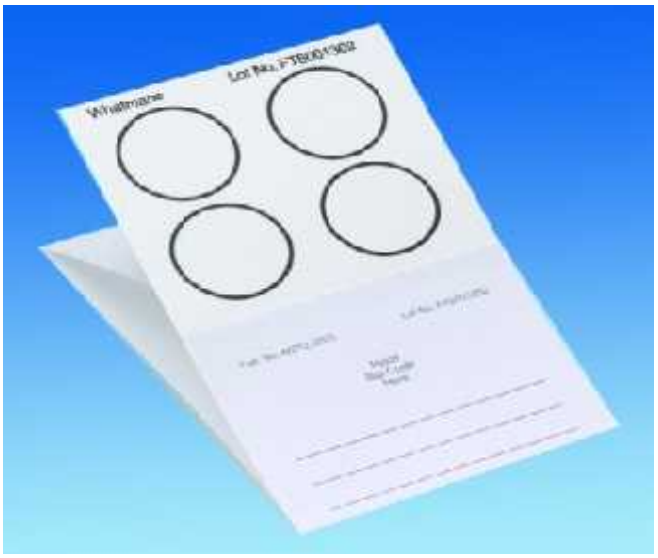

Fig 3: Whatmann® FTA® card used for blood sample collection

This technique has been shown to be effective in collecting samples for diagnostics and virus detection in human, animal and plant samples (Ndunguru *et al*., 2005). In the second round of sampling, blood was spotted on Whatman®FTA® classic cards with 4 sample areas per card. These cards contain chemicals that lyse cells, denature proteins and inactivate microorganisms. Released nucleic acids are entrapped in the fibers of the card matrix and are protected from nucleases, ultraviolet ray damage and oxidation. The cards were secured in a box and kept at room temperature for transportation to the laboratory.

3.6: RNA extraction

RNA was extracted using MagNA® Pure LC RNA Isolation Kit- High Performance (Roche Applied science). The kit comprised of Wash Buffers I and II, lysis/binding buffer, magnetic glass particles, DNase I, DNase Incubation Buffer, Isopropanol, Proteinase K, Proteinase K Buffer II and Elution Buffer (www.magnapure.com).

FTA®card blood spots were punched out in small pieces, transferred into 1.5ml sterile eppendorf tubes unto which 70µl phosphate buffered saline (PBS) was added. After incubation for 5 minutes at room temperature, 200µl of TriZol reagent was added and the contents vortexed for 3 seconds then incubated at room temperature for 3 hours before transferring to a sample cartridge. Blood samples in vials were thawed in ice and 200µl transferred into a sample cartridge after a brief vortex.

Automated Extraction of RNA from samples in the catridge was done in a MagNa® Pure LC 2.0 instrument (Roche Diagnostics, Manneheim, Germany) that relies on the principle of magnetism. Samples were lysed by incubation with a lysis/binding buffer that contains chaotropic salt. Proteins and nucleases were destroyed by proteinase K digestion and incubation with DNase degraded DNA. On addition of Magnetic Glass Particles (MGPs), RNA was bound to their surfaces. Unbound substances were removed by several washing steps and the purified RNA was eluted.

3.7: Quantitative analysis of RNA: concentration and purity

The quantity and purity of eluted extracted RNA was determined using NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Walton, Massachusetts, USA). Sterile nuclease-free water was used in standardization of the instrument, blanking was done using 2 µl of MagNA® Pure elution buffer and 2µl of the RNA from each sample was used in the quantification.

This instrument employs the convention of light absorbance molecules. The ratio of absorbance at 260nm/280nm was determined, with 1.8-2.0 considered indicative of high purity standards. Lower ratios would be indicative of protein contamination or partial dissolving of the pellet

3.8: Complementary DNA (cDNA) synthesis

Extracted total RNA was converted into cDNA using SuperScript™ II (SS II) first strand synthesis system for reverse transcriptase-PCR (Invitrogen). For each reaction, 4µl of sample total RNA was combined with 2µl of random hexamers and heated at 65°C for 5 minutes then placed on ice immediately for 1 minute to anneal the primers to the 3' terminal sequences of the RNA. SuperScript™ II reverse transcription mix was prepared according to the manufacturer's instructions [4 µl, 5X First strand buffer (250mM Tris-HCL, pH 8.3 at room temperature, 375Mm KCL, 15mM $MgCL_2$), 2 µl DTT (0.1M), 2 µl Bovine Serum Albumin (10mg/ml), 1 µl dNTPs(10Mm), 0.2 RNase™ OUT(40U/µL) and 0.5 µl of SS II] which was then incubated for 2minutes at 25°C. The volume of the mix components used was a product of the number of reactions plus two extra reactions to account for pipetting losses. To each of the reaction wells containing heated RNA and primer mix, 9.7 µl of the prepared mix was added ,gently pipette up-down to mix contents and centrifuged at 12000 rpm for 30 seconds.

The mix was annealed at 25°C for 10minutes, extended at 42°C for 15minutes and then the reverse transcription enzyme was inactivated by incubating for 15 minutes at 70°C then chilled at 4°C.

3.9: Conventional Polymerase chain reaction

PCR was carried out using 2X Biomix Red® premix [contains: BIOTAQ DNA Polymerase (5U/µl), 2mM dNTPs, 32mM (NH4)2SO4, 125mM Tris-HCl (pH 8.8 at 25°C), 0.02% Tween 20, 3mM mgCl2 Stabiliser, Inert Dye] (Bioline Reagents Ltd.) according to the manufacturer's instructions. Twenty five microliters of the premix was mixed with 19µl of nuclease free water, 0.5 µl of each primer (forward and reverse) and 5µl of the cDNA template. PCR cycling conditions were initial denaturation at 95°C, 3 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, an annealing step at 49°C for 45 seconds and extension step at 72°C for 45 seconds and a final extension of 72°C degrees for 10 minutes on GeneAmp®PCR system 9700 (ABI-Applied Biosystems, Veriti, USA).

3.10: Primer optimization

WNV- specific primers (Johnson *et al*., 2001) were purchased from Bioneer®. The reverse primer '1845' of sequence 5'TTCCATCTTCACTCTACACT -3' (mw-5937.8, Tm- 42.7°C, 11.5 nmoles) and the forward primer ' 1401' of sequence 5'ACCAACTACTGTGGAGTC -3' (Mw-5483.5,Tm-39.8°C,11.5 nmoles) were diluted to a final working concentration of 20 pmoles in sterile water. To optimize the primers, a 20µL PCR reaction was set up using 2µl of a synthentic WNV positive control (GenScript®) as a template, 0.5µl of each primer, 7µl of PCR water and 10µl of Bioline® mastermix. In a gradient PCR machine, the following program was used: initial denaturation (95°C for 3minutes), 30 cycles of denaturation (95°C for 30 seconds), annealing under gradient temperatures (54, 52,50,47,46, 45) °C for 45 seconds and extension (72°C for 30 seconds) and held at 4°C. The PCR products were visualized on a 1.5% agarose gel and the temperature that resulted in the best band was determined for use in Real-time PCR screening.

3.11: DNA Electrophoresis

Agarose gel (1.5% w/v) was made by mixing 1.5g agarose with 100ml 1x Tris-Acid EDTA (TAE) buffer [0.04 M Tris, 1mM EDTA pH 8.0, 0.1142 % (v/v) glacial acetic acid] and heating for 2 minutes in a microwave to ensure total dissolution. Four microliters of 3x GelRed™ (Biotium) was added to enable visualization of DNA and the solution allowed to cool before casting on a gel plate with a secured comb. After solidification each tooth of the comb formed a well on the gel. In a Mupid®-eXu submarine electrophoresis system (Eurogentec), the gel plate was submerged in 1x TAE buffer. The wells were loaded with 3 µl of DNA amplicons mixed with 2 µl of 6x orange DNA loading dye (Thermoscientific). O'GeneRuler 1kb plus (0.1µg/ µl) (Thermoscientific) ladder was used to facilitate size estimation. A power of 100volts was applied for 60 minutes. Gels were examined under UV illumination on the GelDoc-It® Imager (Ultra-Violet Products Ltd**).**

3.12: Real Time Polymerase Chain Reaction (RT-PCR)

RT-PCR incorporates traditional PCR thermal cycler technology with integrated fluorimeters and detectors that provide the ability to both excite a fluorochrome and detect the emitted light. In this study SyBR®Green 1 fluorescent dye was used as it undergoes 20-to-100 fold increase in its fluorescence upon binding to double stranded DNA. WNV specific primers 1845 and 1401 were used. The SYBR Green master mix (SYBR® Green 1 Dye, AmpliTaq Gold DNA Polymerase LD, dNTPs with dUTP/dTTP blend, passive reference 1 based on proprietary ROX™ dye, and optimized buffer component), primers and samples were first thawed on ice. A PCR master mix was prepared in a sterile 2ml micro centrifuge tube on ice. For each reaction well, 12.5µL SyBr® Green, 0.5µl of each primer (20pmoles) and 10 µl of PCR water (Bioline®) were added.

In each well of a sterile labelled MicroAmp™ fast 96 well reaction plate placed in an ice block, 1.5µl of synthesized cDNA of the sample was dispensed and 23.5 µl of prepared mastermix was added then gently mixed by pipetting up and down to result in a PCR preparation of 25µl. The plate was sealed using MicroAmp™ clear adhesive films (ABI) and loaded into 7900HT fast Real-time PCR system (Applied Biosystems). The following program was used: initial denaturation at 95°C (10minutes), 35cycles of denaturation at 95°C (30seconds), annealing at 49°C (45seconds) and extension at 72°C (45seconds), a dissociation cycle 95°C (15seconds),60°C(15 seconds),95°C(15 seconds) and a hold at 10°C(∞). Standard curve was created by serial dilution of a synthetic WNV positive control (10ng/µl) to attain concentrations of 5, 2.5, 1.25 and 0.625(ng/µl) which were included in the Real-time PCR run. Sterile PCR water was used for a negative control.

3.13: Purification of amplified PCR products

Samples whose real-time amplification curve resembled that of the positive control standard curve were purified using a mini-elute purification kit (Qiagen) for subsequent sequencing. During the cleanup all enzymes, salts and oligomers are removed resulting in high yields of pure nucleic acids in minimal elution volume. The technique employs the spin-column technology and relies on the selective binding properties of silica membrane. DNA adsorbs to the silica membrane in presence of high concentration of salt as contaminants pass through the column after which it is eluted under basic conditions and low salt concentration.

A volume equal to that of the sample (25µl) of buffer PE was added to the wells on the PCR plate containing positively amplified products and mixed by gently pipetting up and down. The 50µl content were applied on labelled sterile spin columns and centrifuged at 7500RPM for 2 minutes.

The flow-through was discarded; 750µl of buffer PB was added to the column and centrifuged at 7500rpm for 2 minutes. Flow-through was again discarded then centrifuged again at maximum speed for one minute. The spin columns were transferred to sterile 1.5 ml microcentrifuge tubes and 30µl of elution buffer was added and allowed to settle at room temperature for 1 minute before centrifugation at 7500rpm for 2 minutes. The spin columns were discarded and the eluted volume was quantified using a nanodrop.

3.14: Data analysis

Raw sampling metadata was entered in an excel spreadsheet. Initial data exploration was done by generating pivot tables and charts on Microsoft Excel to evaluate general relationships among the various variables .This data was then imported into R-software for subsequent analysis. The frequency of the sampled bird species was determined.

Using the principal discrimination analysis, the species were grouped in accordance to similarities in morphological characteristics. Cluster analysis was performed to show differences in the obtained aggregates.

Obtained nucleotide sequences were analyzed using the CLC bio software from which consensus of the forward and reverse sequences were formed and exported to Mega6 software (Tamura *et al*., 2013) for alignment and phylogenetic analysis of the isolated strains.

In order to establish the relationship between the occurrences of WNV, the region of sampling and bird characteristics (age, sex and species), a generalized linear model with a logit link was used. The Hosmer Lemeshow test was then used to test for the goodness of fit for this logistic regression model.

Hosmer–Lemeshow test statistic:

$$H = \sum_{g=1}^{G} \frac{(O_g - E_g)^2}{N_g \pi_g (1 - \pi_g)}.$$

Where,

O_g -observed events

E_g - expected events

N_g - observations

π_g - predicted risk for the g^{th} risk decile group

G - Number of groups

CHAPTER 4

RESULTS

4.1: Bird species sampling

A total of 449 birds were trapped of which 361 were blood sampled and the rest either escaped after capture or were too weak to be bled. Most birds were captured in Bura (207(46.10%)) in comparison to Hola (183 birds). The least number of birds (59(13.14%)) were trapped from Ijara. The most frequent of sampled bird species were the red-billed *Quelea* (n=81) and lesser masked weaver birds (n=57). All age groups of birds were represented in the sample. Using morphological characteristics, sex of some birds could not be defined due to lack of distinguishing features and were grouped under 'undefined' (Fig. 4). However, considering the birds with defined gender (n=257), most of the captured birds were adult males (n=145). Trapped immature birds were few (n=4) and none were captured in Ijara.

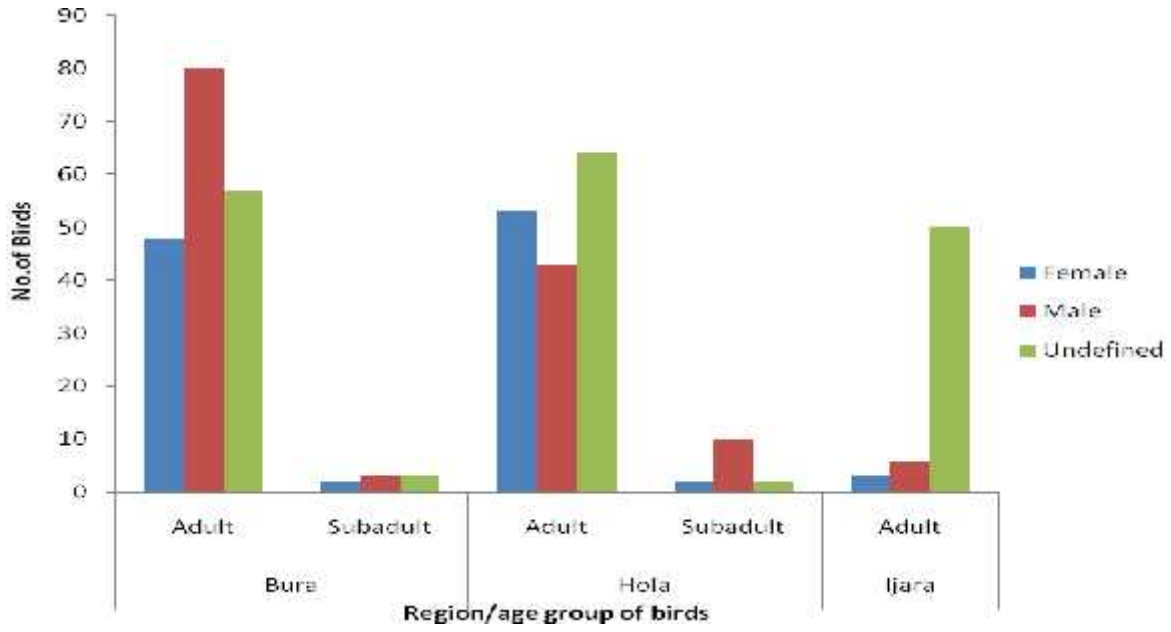

Fig.4: Bird frequency of different age groups and sexes trapped in Bura, Hola and Ijara

The bled birds recorded different weights. The heaviest were laughing doves with a mean weight of 98.25 grams whereas the lightest were violet backed sunbirds with a mean weight of 8.00 grams. The morphology of the sampled birds was diverse with wing, head and tarsus lengths varying among species. The means of the physical parameters measured (weight, tarsus, wing and head) are shown in Table 1.

Table 1: Mean weight, tarsus, wing, and head lengths of sampled birds

Region	**Weight (g)**	**Tarsus (mm)**	**Wing (mm)**	**Head (mm)**
Bura	48.45 ± 25.80	23.25±7.54	88.60±29.55	33.58±8.80
Hola	48.01±22.70	24.23±5.05	77.72±21.84	33.29±6.20
Ijara	49.15±36.50	33.05±8.42	128.71±22.93	45.61±10.14

The above variables were used to generate clusters of species. This was necessitated by the large species diversity among the sampled birds. The cluster analysis generated 5 distinct clusters (Fig. 5) by grouping the birds according to the closeness of similarities in their characteristics defined by wing, head and tarsus lengths as well as weight. The cluster dendrogram contains red line cuts that comprises of a complete group.

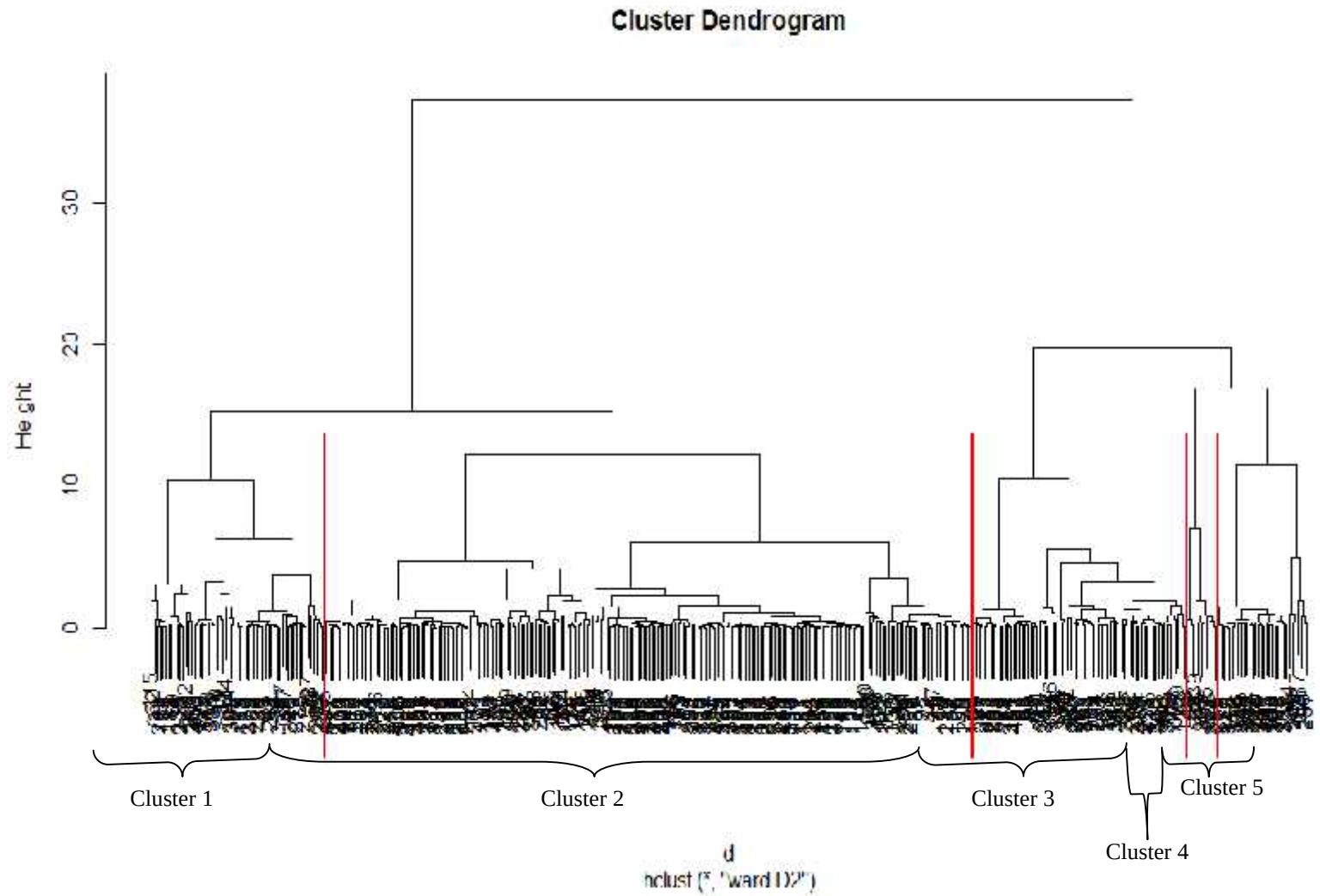

Fig. 5: A dendrogram indicating 5 cluster groups of trapped birds

From the cluster summary statistics (Table 2), cluster 1 and 2 generally consisted of small birds that had long beaks of approximately 40mm and 90mm respectively. Cluster 2 birds however, were slightly larger when compared to cluster 1 birds. Cluster 3 and 4 comprised of medium sized birds with short beaks measuring below 20mm but long tarsus of up to a mean length of 53.22mm. Cluster 5 comprised of large birds that had short wings and tarsus and recorded the highest mean weight of 351.34 grams.

Table 2: Summary statistics of the generated cluster groups

Groups	Weight (g)	Head length (mm)	Tarsus length (mm)	Wing length (mm)
Cluster 1	141.36	42.31	30.92	22.08
Cluster 2	220.86	88.20	42.83	31.88
Cluster 3	310.64	15.82	52.94	53.22
Cluster 4	331.41	20.59	46.89	24.05
Cluster 5	351.34	45.96	30.36	22.48

Cluster 1 incorporated most of the sampled birds including 28 different species with a total of 228 birds. Cluster 4 had the least number of birds compared to other clusters (Tables 3,4,5,6 and 7).

Table 3: Frequency and mean (±sd) of weight, tarsus and wing length measurements of bird species in cluster 1

Bird species	Frequency	Weight (g)	Tarsus (mm)	Wing (mm)
3 streaked tchagra	1	55.00 (0.00)	27.80 (0.00)	71.00 (0.00)
African golden weaver	8	43.12 (4.85)	23.90 (1.42)	70.13 (7.66)
Brown throated barbet	3	39.00 (3.46)	21.70 (0.53)	64.33 (3.21)
Chestnut weaver	2	52.00 (5.66)	24.75 (1.20)	84.50 (7.78)
Common bulbul	3	47.00 (1.73)	23.30 (1.04)	80.67 (1.15)
D'arnaud's barbet	3	53.00 (2.00)	26.77 (0.25)	75.00 (1.00)
Eastern violet backed Sunbird	2	40.67 (55.2)	20.47 (0.89)	43.00 (32.2)
Fire fronted bishop	31	18.58 (6.64)	20.84 (0.98)	60.97 (4.54)
Golden pipit	8	41.63 (3.50)	28.64 (3.56)	84.88 (5.08)
Green winged pytilia	4	29.50 (6.45)	21.75 (3.73)	59.50 (4.43)
Grey headed sparrow	2	63.00 (1.41)	26.65 (0.92)	93.50 (3.54)
House sparrow	22	39.23 (15.6)	20.75 (1.42)	72.58 (0.73)
Lesser honey guide	2	46.00 (4.24)	16.40 (0.28)	86.50 (9.19)
Lesser masked weaver	43	44.61 (4.01)	24.05 (0.32)	70.47 (1.85)
Northern brown bul	4	44.50 (4.04)	27.30 (4.38)	75.25 (4.65)
Northern wheat ear	1	53.00 (0.00)	31.00 (0.00)	96.00 (0.00)
Olivaceous warbler	1	27.00 (0.00)	25.50 (0.00)	72.00 (0.00)
Pale wren warbler	4	15.50 (5.74)	24.10 (0.2)	60.00 (1.63)
Pink breasted lark	1	52.00 (0.00)	28.00 (0.00)	93.00 (0.00)
Red billed quilea	67	35.78 (8.11)	21.84 (0.03)	67.04 (0.62)
Red tailed shrike	2	47.50 (6.36)	27.45 (0.64)	94.50 (2.12)
Upcher's warbler	1	16.00 (0.00)	24.10 (0.00)	72.00 (0.00)
Violet backed sunbird	1	8.00 (0.00)	20.50 (0.00)	63.00 (0.00)
Violet breasted sunbird	1	86.44 (0.00)	19.79 (0.00)	58.89 (0.00)
White browed scrub robin	7	34.86 (2.54)	27.80 (1.04)	68.43 (2.30)
White browed sparrow weaver	2	61.00 (5.23)	28.17 (0.43)	99.33 (1.50)
White eared bulbul	1	43.00 (0.00)	33.30 (0.00)	77.00 (0.00)
Willow warbler	1	48.00 (0.00)	23.95 (0.00)	70.00 (0.00)
Total	**228**			

Table 4: Frequency and mean (±sd) of weight, tarsus and wing length measurements of bird species in cluster 2

Bird species	Frequency	Weight (g)	Tarsuslength (mm)	Winglength (mm)
Barn swallow	5	33.60 (4.22)	13.92 (0.98)	119.20 (3.90)
Blue naped mousebird	1	75.00 (0.00)	23.50 (0.00)	89.00 (0.00)
Diederick cuckoo	1	54.00 (0.00)	20.00 (0.00)	118.00 (0.00)
Eastern violet backed sunbird	1	40.67 (0.00)	20.47 (0.00)	43.00 (0.00)
Emerald spotted wood dove	9	76.00 (24.33)	22.55 (0.92)	106.40 (5.23)
Harlequin quail	3	95.00 (9.54)	29.87 (1.19)	102.00 (7.94)
Namaqua dove	24	58.67 (5.87)	17.37 (0.84)	104.83 (3.14)
Northern crombec	3	88.00 (0.00)	18.50 (0.50)	45.00 (7.94)
Red fronted warbler	2	94.00 (0.00)	20.30 (0.42)	44.00 (0.00)
Speckled mousebird	3	69.00 (2.00)	25.87 (0.90)	92.00 (2.65)
Violet breasted sunbird	8	86.44 (32.1)	19.78 (4.24)	58.89 (3.48)
Willow warbler	1	48.00 (0.00)	23.95 (0.00)	70.00 (0.00)
Total	**61**			

Table 5: Frequency and mean (±sd) of weight, tarsus and wing length measurements of bird species in cluster 3

Bird species	Frequency	Weight (g)	Tarsuslength (mm)	Winglength (mm)
Fischer's starling	10	67.80 (1.54)	35.50 (0.42)	108.40 (4.58)
Golden breasted starling	4	79.25(16.03)	35.68 (1.87)	127.25 (5.56)
Laughing dove	23	98.25 (20.7)	24.45 (0.97)	136.92 (4.53)
Magpie starling	1	73.00 (0.00)	32.20 (0.00)	106.00 (0.00)
Northern-white-crowned shrike	1	71.00 (0.00)	26.90 (0.00)	116.00 (0.00)
Nubian wood pecker	6	74.33 (5.57)	24.83 (2.07)	105.50 (1.38)
Red and yellow barbet	1	82.00 (0.00)	30.60 (0.00)	96.00 (0.00)
Red billed buffalo weaver	3	91.33 (6.81)	37.57 (1.50)	112.33(3.21)
Red billed quilea	1	35.78 (0.00)	21.84 (0.00)	67.04 (0.00)
Rosy patched bush shrike	1	76.00 (0.00)	37.20 (0.00)	90.00 (0.00)
Rufous chatterer	2	68.00 (0.00)	33.15 (0.07)	87.00 (2.81)
Ruppel's long tailed starling	3	38.00 (43.1)	46.78 (2.14)	155.50 (10.2)
Superb starling	4	79.25 (4.03)	37.38 (1.11)	113.75 (6.13)
White headed buffalo weaver	15	87.87 (29.5)	35.58 (1.29)	113.18 (4.45)
Total	**75**			

Table 6: Frequency and mean (±sd) of weight, tarsus and wing length measurements of bird species in cluster 4

Bird species	Frequency	Weight (g)	Tarsuslength (mm)	Winglength (mm)
African mourning dove	1	117.67 (8.32)	31.83 (6.97)	149.00 (2.64)
Black headed plover	2	122.00 (0.01)	70.40 (0.02)	189.50 (0.01)
Ruppel's long tailed starling	7	38.00 (43.1)	46.78 (2.14)	155.50 (10.2)
Spur winged plover	1	133.00 (0.00)	70.00 (0.00)	196.00 (0.00)
Grand Total	**11**			

Table 7: Frequency and mean (±sd) of weight, tarsus and wing length measurements of bird species in cluster 5

Bird species	Frequency	Weight (g)	Tarsuslength (mm)	Winglength (mm)
African mourning dove	2	117.67 (8.32)	31.83 (6.97)	149.00 (2.64)
Grey headed king fisher	3	64.33 (32.25)	17.43 (1.68)	105.00 (8.60)
Laughing dove	1	98.25 (0.00)	24.45 (0.00)	136.91 (0.00)
Malachite king fisher	2	32.5 (3.54)	11.45 (1.06)	56.5 (0.71)
Ring necked dove	22	114.39 (6.53)	26.97 (1.52)	141.40 (3.86)
Somali bee eater	1	27.00 (0.00)	11.10 (0.00)	77.00 (0.00)
White throated bee eater	1	50.00 (0.00)	12.40 (0.00)	101.00 (0.00)
Total	**32**			

4.2: Quantification and purity of extracted RNA

Extracted RNA from bird blood samples stored in vials was of higher concentration compared to the extractions from WhatMann®FTA® cards using spectrophotometry estimations made on the nanodrop (Table 8). The mean concentrations were found to be significantly different, ($t = 6.59$; $df = 388$, $p < 0.0001$). Mean absorbance ratio 260: 280 nm for extracts from blood in vials was 2.05 ±1.07 while that from FTA® cards gave a mean of 1.94 ±0.69. A two sample t-test showed no significant difference in the quality of obtained RNA from FTA and vial blood ($t = 1.49$, $df = 358$; $p = 0.14$)

Table 8: Spectrophotometric concentrations (±sd) of extracted RNA from blood samples stored in sterile cryovials and Whatman®FTA® cards

Sample storage	Mean conc. (ng/µl)	Maximum conc.	Minimum conc.
Vials	39.77±62.66	376.30	0.02
FTA® cards	10.17±3.81	22.39	0.99

4.3: Real- time amplification of RNA

Serial dilutions of the positive control produced a standard curve reflecting a straight line correlation of the quantity of the template and the cycle for fluorescence detection (cycle threshold (ct) with a slope of -3.82. The higher the template concentration the lower the ct value recorded (Fig. 6). The correlation coefficient was significant (R^2=0.998).

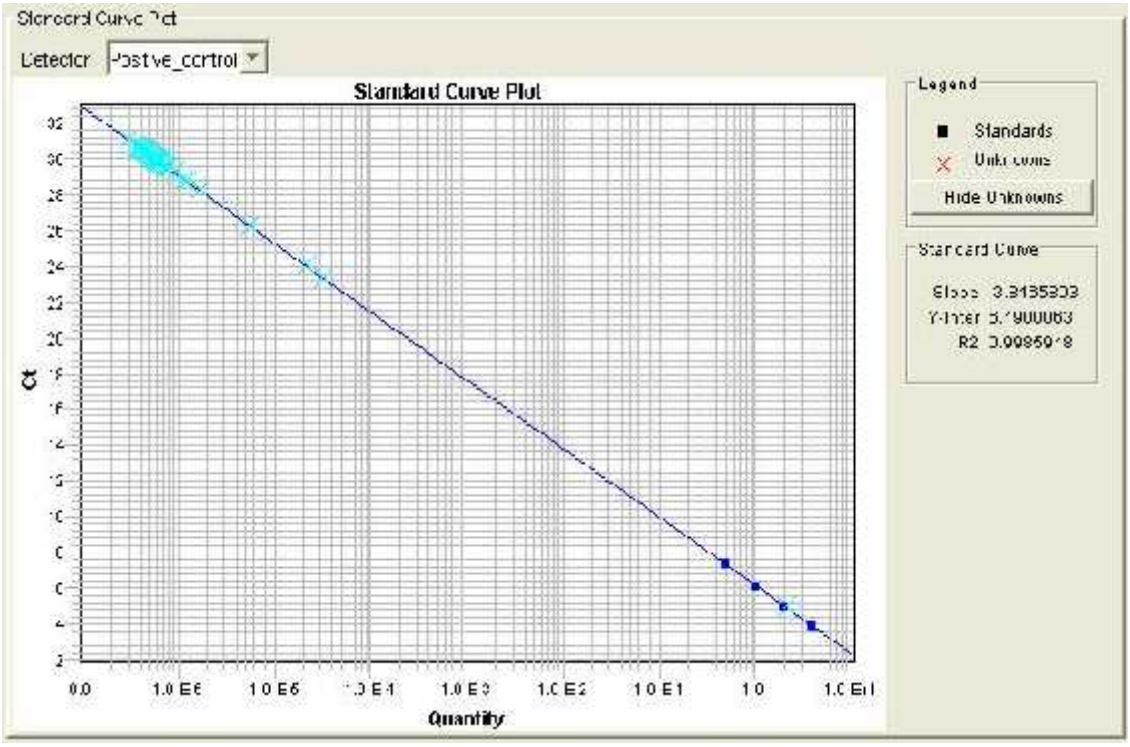

Fig. 6: A Real Time PCR standard curve plot of the template quantity against cycle threshold

The initial PCR cycles produced low fluorescence signal that could not be detected hence defined the baseline of the reaction. In the exponential phase (where the products double with each cycle and the accumulated template can be detected) the amplification curves in reference to the standard serial dilutions were parallel to each other.

A plot of ΔRn (delta Rn**) (**magnitude of the signal generated) against the PCR cycles indicate a delayed signal detection for the samples being screened as compared to the positive control (Fig. 7). The cycle threshold for signal detection increased with reduction in template concentration. The negative controls did not generate a signal above the threshold and its curve was limited to the baseline.

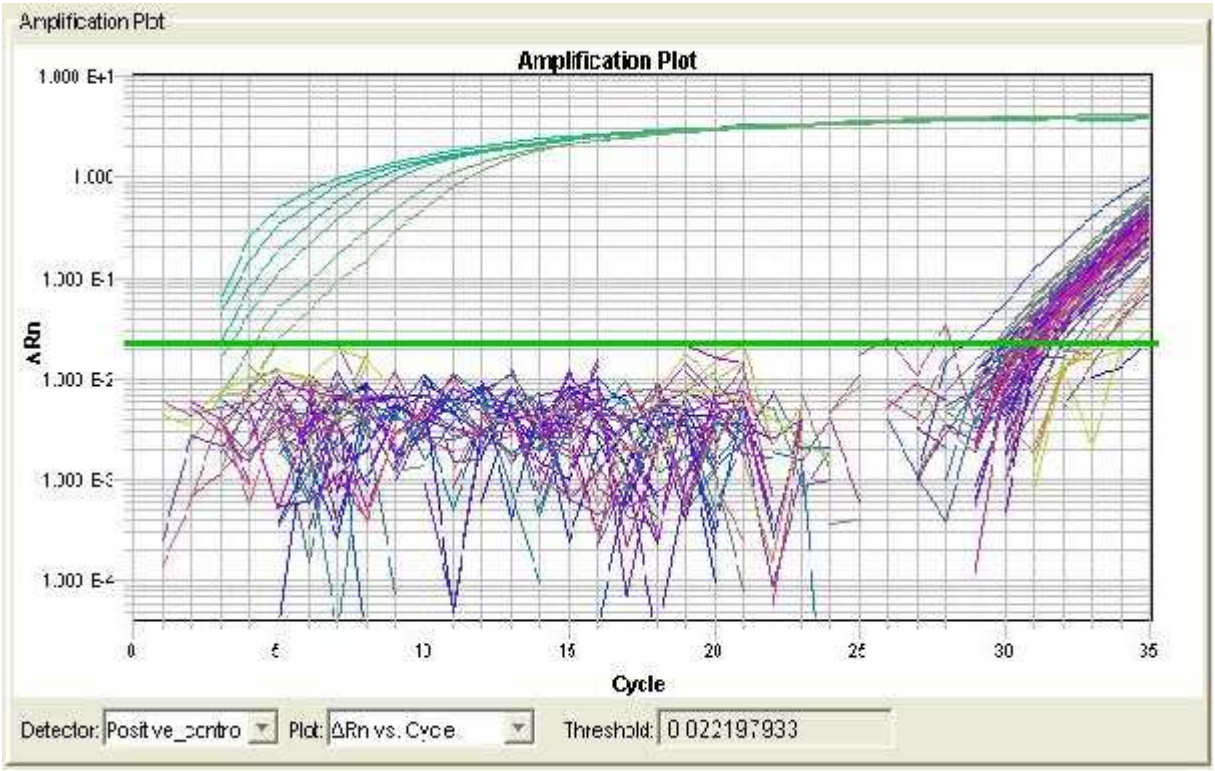

Fig. 7: Fluorescence signal magnitude plot against PCR cycles generated from the Real- time PCR

Thermal ramping program was able to determine the melting temperatures (the temperature at which the DNA strands separate into single strands) of the obtained PCR products. The obtained peaks for positive samples were similar to the peaks generated from the positive control and this acts as part of the confirmation for the specificity of the PCR reaction.

The height of the peak was directly proportional to the concentration of the template with the standards generating the highest peaks (Fig. 8). In PCR runs where no positive sample was detected only the positive control serial dilutions generated peaks. The negative control did not generate any peak during the melt curve analysis.

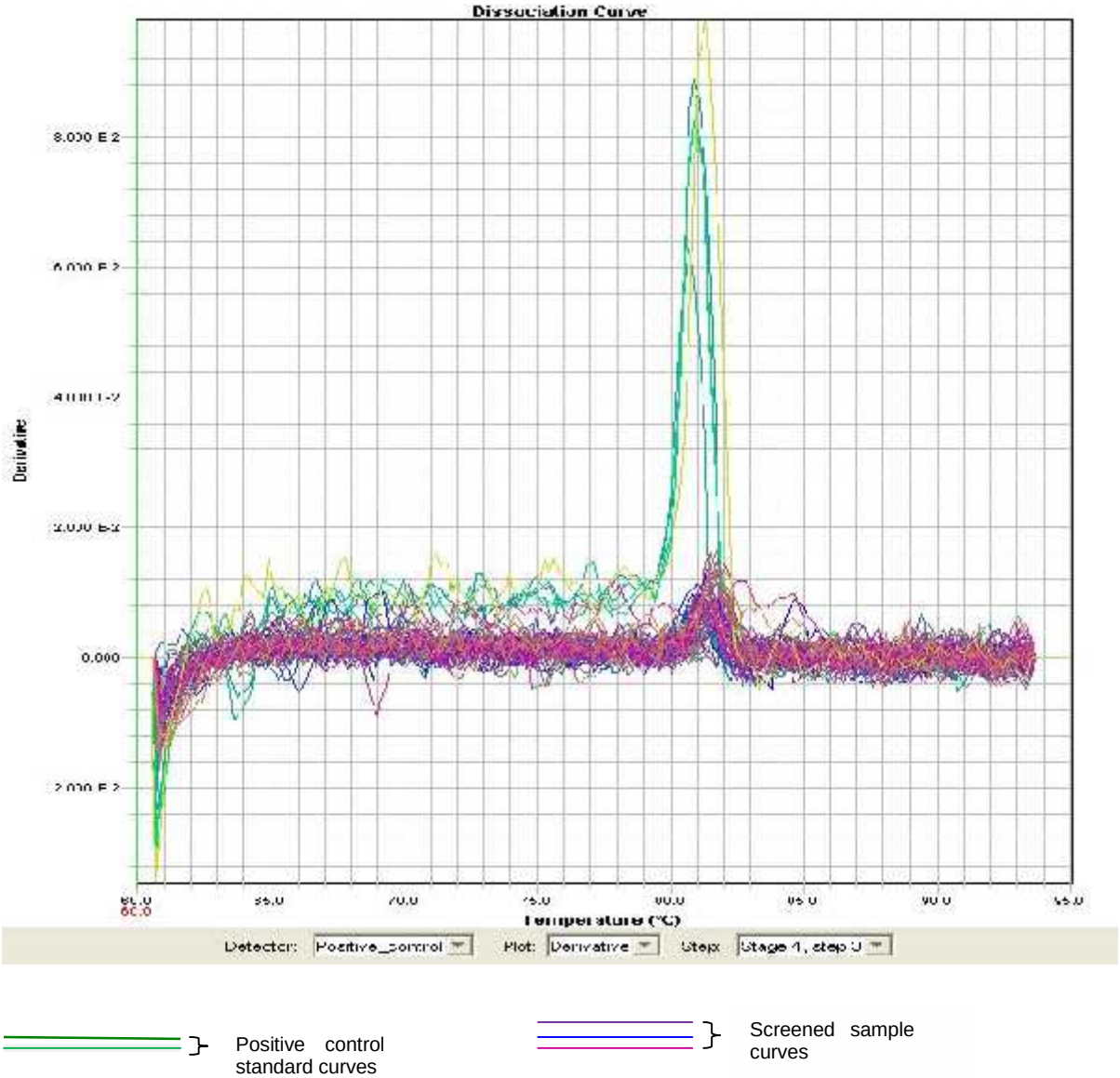

Fig.8: Derivative melting curve for the standards and some positive samples in a Real-time PCR

4.4: Gel electrophoresis

Visualization of positive products from Real time PCR screening revealed fragments lying between 400 and 500 bp region. The positive control amplification also resulted in a product of the same range but its band was denser than bands of the samples. Each loaded sample resulted on only one visible band. The negative controls did not produce any band confirming absence of contamination during the PCR setup (Fig. 9).

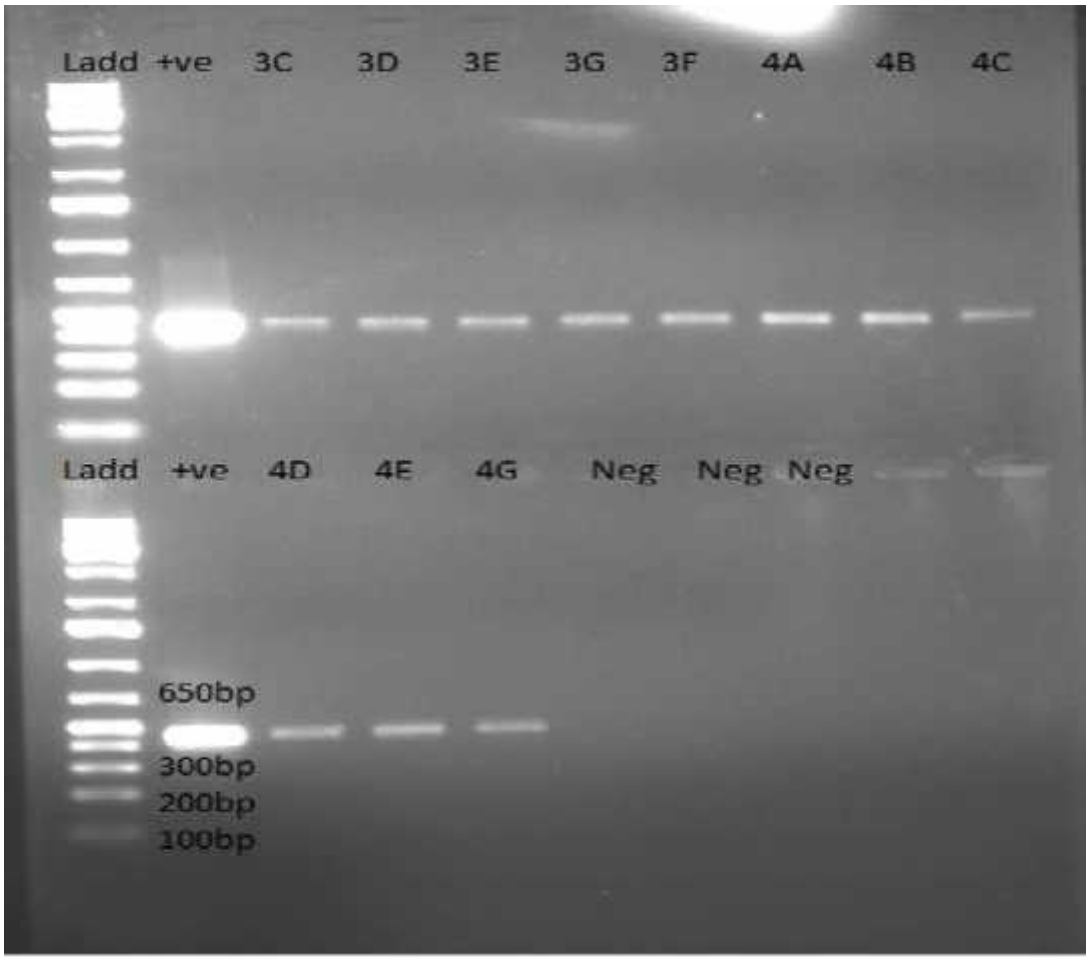

Fig. 9: Agarose gel image of some of the positive samples from Real time PCR: **Key**: **3C- 4G** – Samples, **Neg** – Negative control, **+ve –** Positive control,

Ladd - O'GeneRuler 1kb plus (0.1µg/ µl) (Thermoscientific)

4.5: Sequencing of positive samples

All 65 positively amplified samples in the real time PCR screening were purified and 35 had a concentration above 20ng/µl that was suitable for di-deoxy (Sanger) sequencing. The reverse and forward sequences of most of the samples could not be assembled into contigs due to short read lengths. Sequences from 5 samples (4A, 4B, 4E, 2G and 2D) were successfully assembled forming consensus sequences of length 305, 345, 291, 332 and 341 bases respectively. Sequence 4A was amplified from a blood sample drawn from a ring-necked dove, 4B and 4E from *Namaqua* doves, 2D from a red-billed *Quelea* and 2G from a lesser-masked weaver bird. The synthetic positive control (SS) was also sequenced yielding a consensus sequence of 403bp. The obtained sequences were searched in the nucleotide database of NCBI using a megablast optimized to search for highly similar sequences. There was high similarity of both the sample sequences and the positive control to various isolates and strains of WNV. The positive control sequence highly matched with 3 WNV strains (*XJ11141, XJ11129* and *XJ11148)* and a WNV isolate *Ast 02-3-717*. The sample sequences also matched with the same strains but had a stronger match to WNV strain *Ast-986* as opposed to *Ast 02-3-717.* The identity of the sequences was varied as indicated on the blast output (Table 9).

Table 9: NCBI blast output of the obtained sequences

Sample	Matching organisms	% Query coverage	% identity	E-value
SS	WNV strain *XJ11141* envelope protein E gene	100	100	0.0
	WNV strain *XJ11129* complete genome	100	100	0.0
	WNV strain *XJ11148* envelope protein E gene	100	100	0.0
	WNV isolate *Ast 02-3-717* complete genome	100	99	0.0
4A	WNV strain *XJ11141* envelope protein E gene	100	100	4e-157
	WNV strain *XJ11129* complete genome	100	100	4e-157
	WNV strain *XJ11148* envelope protein E gene	100	100	4e-157
	WNV strain *Ast-986* complete genome	100	99	2e-155
4E	WNV strain *XJ11141* envelope protein E gene	100	99	8e-144
	WNV strain *XJ11129* complete genome	100	99	8e-144
	WNV strain *XJ11148* envelope protein E gene	100	99	8e-144
	WNV strain *Ast-986* complete genome	100	99	4e-142
2G	WNV strain *XJ11141* envelope protein E gene	100	99	4e-167
	WNV strain *XJ11129* complete genome	100	99	4e-167
	WNV strain *XJ11148* envelope protein E gene	100	99	4e-167
	WNV strain *Ast-986* complete genome	100	99	2e-165
2D	WNV strain *XJ11141* envelope protein E gene	100	99	2e-175
	WNV strain *XJ11129* complete genome	100	99	2e-175
	WNV strain *XJ11148* envelope protein E gene	100	99	2e-175
	WNV strain *Ast-986* complete genome	99	99	1e-172
4B	WNV strain *XJ11141* envelope protein E gene	100	99	1e-177
	WNV strain *XJ11129* complete genome	100	99	1e-177
	WNV strain *XJ11148* envelope protein E gene	100	99	1e-177
	WNV strain *Ast-986* complete genome	99	99	7e-175

Key: SS – positive control, **4A, 4E, 4B, 2D** and **2G –** Sample sequences

Of all the isolates, 13 isolates had nucleotide sequences that were between 180 and 341 base pairs and they were used to estimate and visualize the diversity of WNV in the study region. A sequence identity matrix was generated incorporating retrieved sequences of WNV isolates from the Genebank (NCBI). The matrix indicated similarity of the isolates to other WNV isolated strains. There was a higher percentage identity to isolates that were grouped under lineage one ranging from 81 to 96 when compared to those grouped under lineage 2 that ranged between 67 and 75. Human immunodeficiency virus was used as an out group and its identity was less than 45% on all isolates (Table 10).

Table 10: Sequence identity matrix indicating the percentage similarity of Tana River WNV strains (bold) with some WNV isolates in the GenBank

WNV Strains	Sample-4B	Sample-4A	Sample-3F	Sample-4E	Sample-3G
goose-Hungary/03	93.6	92.0	88.4	78.3	87.7
KJ786934_NY2001-6263_Homosapiens	93.6	92.7	88.8	77.8	88.2
68856-ICDC-4	92.0	91.0	88.4	78.7	87.2
XJ11129_Culex_pipiens_pipiens	95.9	94.7	91.2	80.2	90.6
JX442281_XJ11141	95.9	94.7	91.2	80.2	90.6
DQ374650_Ast02-3-717	95.5	94.7	91.2	80.2	90.6
DQ411031_Ast01-187	95.5	94.7	91.2	80.2	90.6
DQ411030_Ast01-182	95.5	94.7	91.2	80.2	90.6
HM538818_4893	94.3	93.0	89.3	78.3	88.7
KJ501417_WNV-1/US/BID-V6684/2006	94.3	93.0	89.3	78.3	88.7
EF631149_CpWw21	94.3	93.0	89.3	78.3	88.7
DQ786573_France407/04	92.3	91.4	87.9	77.3	87.2
AY712947_Bird1461	93.9	93.0	89.3	78.3	88.7
AY052409_ISR98-GooKha	93.3	92.4	88.4	77.3	87.7
HM147822_WNV_SAfrica	72.5	71.4	69.8	63.8	69.5
EF429199_SA381/00	74.1	72.8	71.2	63.8	70.0
Sample-4B	100.0	97.3	95.8	86.0	95.6
Sample-4A	97.3	100.0	95.8	84.5	95.6
Sample-3F	95.8	95.8	100.0	99.4	94.6
Sample-4E	86.0	84.5	99.4	100.0	98.2
Sample-3G	95.6	95.6	94.6	98.2	100.0

A phylogenetic tree (Fig.10) was generated by maximum likelihood analysis to illustrate the relationship between the isolates in this study and those available in Genebank. The tree revealed 2 distinct clades of lineage 1 and 2. Isolates from Tana River indicated closeness in similarity with branches showing bootstrap values of less than 50 and all of them clustered with described lineage 1 strains in Genebank.

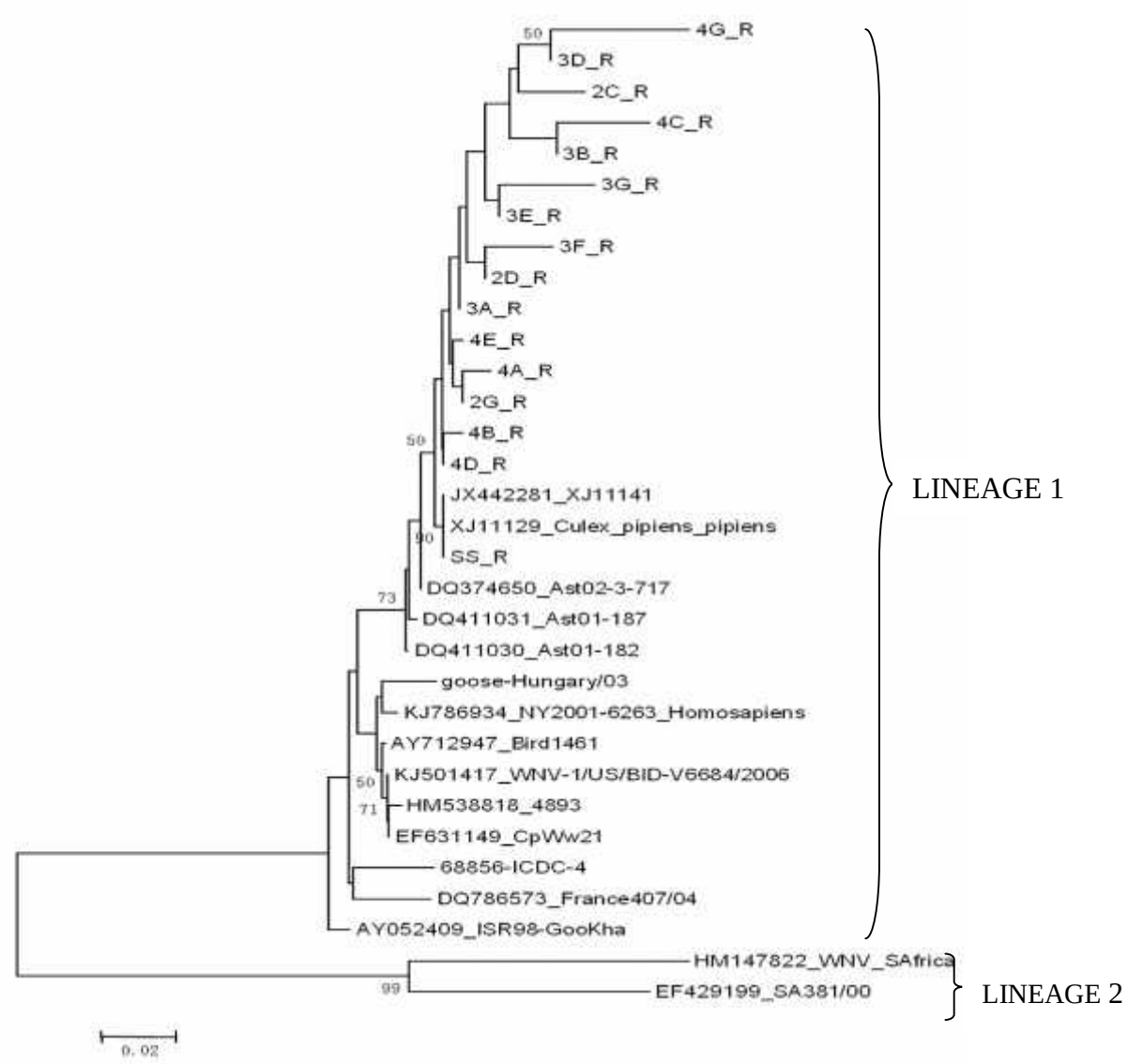

Fig.10: Phylogram showing the relationship of WNV isolates

4.6: West Nile virus distribution

Real time PCR screening of bird blood yielded a total of 65 samples infected with WNV. Of this, most were from the lesser masked weaver birds and red billed *Quelea*. Comparison of the proportion of the positives to the total samples of each species resulted in no difference in prevalence of WNV among the different species. Bura recorded a higher frequency of infected birds while Hola had the lowest (Table 11).

Table 11: The WNV positive samples and the frequency ratio to the total number of sampled birds per species

	WNV Positive samples				Positive/total
SPECIES	**Bura**	**Hola**	**Ijara**	**Total**	**sampled(%)**
African golden weaver	2	-	-	2	0.25
African mourning dove	1	-	1	2	0.67
Barn swallow	2	-	-	2	0.40
Emerald spotted wood dove	-	-	2	2	0.20
Golden breasted starling	-	-	1	1	0.25
Golden pipit	2	-	-	2	0.25
Grey headed king fisher	1	-	-	1	0.07
House sparrow	4	-	-	4	0.15
Laughing dove	3	1	-	4	0.17
Lesser masked weaver	4	10	-	14	0.25
Namaqua dove	4	1	-	5	0.21
Nightjar	-	-	1	1	1.00
Nubian wood pecker	-	-	1	1	0.17
Red billed quelea	9	3	-	12	0.15
Ring necked dove	1	-	2	3	0.14
Ruppel's long tailed starling	-	-	2	2	0.20
Violet backed sunbird	1	-	-	1	1.00
White browed sparrow weaver	1	-	-	1	0.25
White headed buffalo weaver	1	-	3	4	0.24
White throated bee eater	1	-	-	1	1.00
Total	**37**	**15**	**13**	**65**	

A regression analysis in a generalized linear model factoring the effects of the bird's age, sex, region of capture and species as grouped in various clusters to infection was done and gave the result in Table 12.

Table 12: Variables included in the regression analysis using the generalized linear model

Variables	**Parameter estimate**	**Standard error**	**Z -value**	**P - value**
Intercept	-2.11	0.38	-5.62	0.00***
Age subadult	0.47	0.47	0.99	0.32
Region. Hola	0.92	0.37	-2.48	0.01*
Region. Ijara	0.22	0.45	0.48	0.63
Region.Bura	0.00	-	-	-
Sex. Male	0.37	0.41	0.91	0.36
Sex. Undefined	-0.05	0.47	-0.10	0.92
Sex.female	0.00	-	-	-
Group 2	0.19	0.42	0.45	0.65
Group 3	0.18	0.49	0.38	0.70
Group 4	0.70	0.77	0.90	0.37
Group 5	0.24	0.65	0.37	0.71
Group 1	0.00	-	-	-

Likelihood ratio test on the models used in the regression analysis showed that the variable Region was significant ($P < 0.05$). The Hosmer and Lemeshow test determined the goodness of fit of the model ($P = 0.19$) .The null hypothesis (H_O) for this test is that the mean predicted by the model is not different from the observed while the alternative hypothesis (H_A) is that the predicted mean is different from the observed mean.

A plot of the standardised residuals versus fitted values (Fig. 11) was used to test the validity of the model. The figure shows that the standardised residuals ranged between -1.0 and 2.0

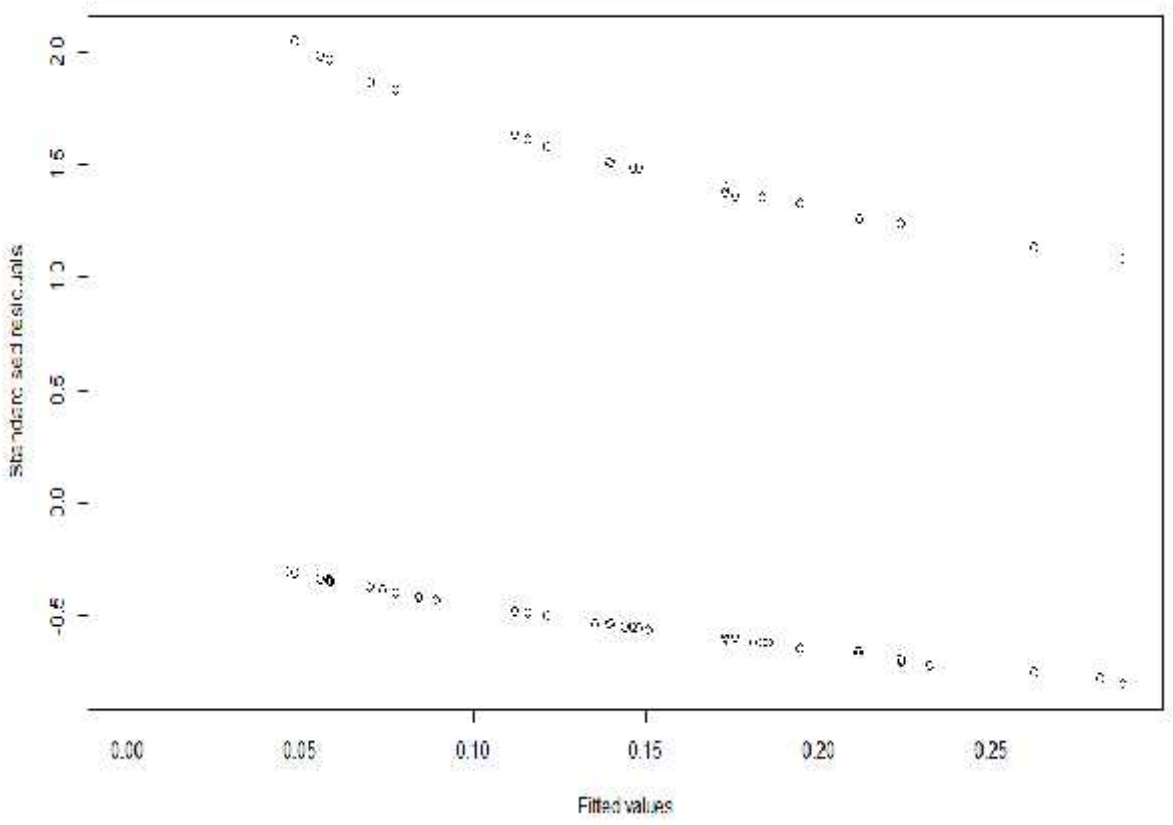

Fig 11: Standardised residuals plot versus the fitted value

CHAPTER 5

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1: Discussion

5.1.1: Bird sampling

Tana River County is a rich avifaunal destination supporting both local and migratory birds as revealed by the high diversity of sampled birds. Owing to the similarity of land use patterns in Hola and Bura where irrigation is a major practise, these regions attracted similar avian groups. Land use practices tend to play a major role in bird distribution within this region. Irrigated farms attracted a large number of crop pests including red-billed *Quelea* that was reported as the most abundant bird species globally (Fry and Keith, 2004) and the lesser masked weaver birds that are characteristic territorial colonial nesters. In this study these two groups recorded a notably high number in comparison to other species because of an increased sampling chance as they move in swarms. In addition, Small- sized birds had an increased probability to be captured compared to large birds as it is reflected in table 4 where cluster 1 incorporated the highest number of bird species compared to other clusters. Large birds like doves had a tendency of hitting the nets and escaping before extraction as they were not fully entangled within the mesh hence reducing the frequency of their capture unlike the small birds which hardly escaped once entangled.

Results indicate a relatively low number of sampled immature birds in comparison to sampled adults. From Ijara however, no juveniles were sampled. Studies by O'Brien *et al.* (2010) revealed that nestling and juvenile birds exhibit a potential contribution to arbovirus transmission cycles including WNV. The low number of this age group among samples in this study would have limited the actual virus prevalence estimation and it could be important to modify the trapping method used to enable sampling of this group of birds.

None of the trapped birds demonstrated clinical signs for encephalitis including those that tested positive in Real time PCR screening. This collaborates the study done by Valiakos *et al*. (2012) where they isolated a WNV strain with genetic important virulence markers from a magpie that did not exhibit any disease symptoms. This has been linked to presence of an active innate immunity brought about by ancestral co-evolution and long association between birds and WNV.

5.1.2: Blood sample storage and quality of extracted RNA

Fresh blood samples can be effectively stored in sterile cryovials inside compressed solid carbon dioxide and be successfully used in molecular application as shown in this study. For better nucleic acid extraction in downstream molecular applications, there is a crucial need to include anti-coagulating agents like acid citrate dextrose and ethylenediaminetetraacetic acid (EDTA) to get higher yields (Lee *et al*., 2010). The choice of these agents would be crucial as some agents like heparin have been shown to interfere with magnesium ions and polymerases that are used in downstream PCR reactions (Kaudewitz *et al*., 2013). In addition, Holland *et al* (2003) reinforced the inclusion of commercially available RNase inhibitors to secure the integrity of the RNA component in the sample.

High quality RNA extraction is not easily achieved due to instability (Holland *et al*., 2003). In this study there was very low concentration of extracted RNA from some samples.

To improve on this, it would be necessary to mix the sample aliquots for RNA analysis with RNA stabilizing buffer containing β-mercaptoethanol (Holland *et al*., 2003).

The Whatmann®FTA® cards provide a convenient and economical alternative to sample storage in terms of portability and ability to maintain samples intact at room temperature. Holland *et al* (2003) also advocated for the use of pre-treated cards to collect samples as this inactivates PCR inhibitors that are naturally contained in whole blood.

Downstream application for these samples was however challenged with the low concentration of RNA that was extracted from most of these samples. This would have been as a result of the automated extraction method used. This result agrees with studies by Niversitesi *et al*. (2015) where they found that automated extraction produced low quantity and quality of nucleic acids when compared to manual extraction that would however be prone to contamination and was only effective for few samples. It is suggested that effective RNA extraction can be achieved from isolated lymphocytes after Ficoll® gradient separation of whole blood. This approach is more cost effective and produces better RNA yield than direct isolation from whole blood (Holland *et al*., 2003).

Results indicate that the purity of extracted RNA was independent of sample storage method used ($P > 0.05$). The ratio between the absorbance at 260 nm and 280 nm is used to evaluate the purity of the RNA that is expected to be around 2.0. A lower ratio could be due to contamination by peptides, phenols, aromatic compounds or carbohydrates (Pinto *et al*., 2009). It is clear that most of the extractions were pure in this study and could be used in subsequent molecular analyses. The purity would be attributed to the use of automated extraction for all samples. Kalmar *et al.* (2015) found that automated MagNapure technique was 50 % efficient when compared to the manual method but advocated for its use in studies involving high sample throughput.

5.1.3: Real-time PCR amplification

Utilization of PCR to detect nucleic acids from varied organisms is an important tool in most molecular based scientific research. In this study, real time PCR provided a homogeneous system that did not necessitate any post amplification manipulations. Nucleic acid based screening using SYBRgreen^{TM} -Real time PCR for WNV was able to detect some positive samples in this study and could be adopted for use in routine surveillance practices since it is economical.

Papin *et al*. (2010) suggested that this method not only has the capability to detect WNV strain variants with mutations in the target site but also can be used with 3'-proof-reading polymerases and those that are active at high extension temperature. The sensitivity of this technique could be increased through nested amplification. In Johnson *et al.* (2001) first amplification using primers used in this study only produced few positives compared to the actual number obtained after a nested amplification.

The high cycle threshold of 35 observed in the positive samples indicates presence of low amount of viral particle template. Similarly, Wheeler *et al* (2012) considered samples with a cycle threshold score of less than 40 to be positive. However, since the primers used were designed to amplify a genome section of a specific WNV strain, there could be false negative results as described in Papin *et al*. (2010) due to strain variation in primer target sequences. To account for this, multiple primers could be used against different target sequences in the WNV genome as demonstrated in Wheeler *et al* (2012) who proceeded to confirm positive samples by screening using a different set of primers that targeted non-structural protein 1 region of the viral genome after initially screening with primers targeting the envelope region of the viral genome. Alternatively, special PCR conditions like` step- down' could be used to enable annealing to variant targets.

5.1.4: Gel electrophoresis

The visualised amplified fragments indicated bands specific only to a region lying between 400 and 500 base pairs. This confirmed that the primers used were specific and only amplified target genome regions that they were designed to amplify. The same bands of 445 base pairs were obtained by Johnson *et al.* (2001). The positive control produced a denser band than samples implying that the template was present in a relatively high concentration. From the results, all negative controls did not show a sign of any amplified product and this was an indication that there was no contamination during PCR setup.

5.1.5: Occurrence of WNV

From the regression analysis, the host factors and taxa did not affect the occurrence of WNV except for the location that had a significant influence. The generalized linear model indicated that birds from Hola were less likely to have WNV when compared to birds from Bura and Ijara as indicated by the respective P-values. All other factors, besides the region, did not have an influence on WNV occurrence. However, research by Gómez *et al*. (2008) on mammals' exposure to WNV revealed that species, age and urbanization had a significant influence on WNV sero-prevalence. In this study, sampled juveniles were few compared to adults and it could be necessary to investigate the effect of age on WNV occurrence using a sample with a proportional representation of various age groups. Studies by O'Brien *et al*. (2010) indicated that nestlings played a role in WNV amplification.

The likelihood ratio test indicates that the variable region was significant in the regression model. The results given in Table 13 give a more detailed analysis of this variable looking at specific levels with Bura being used as a reference category. They suggest that birds sampled at Hola had a significantly lower prevalence of WNV infection compared to those from Bura ($p = 0.01$). There was no significance difference in the prevalence of WNV between birds sampled at Ijara and those from Bura.

The Hosmer Lemeshow test estimated a P value of 0.19 suggesting that the null hypothesis stating that the mean predicted by the model is not different from the observed should be accepted. The model therefore fits the data. Standardised residuals were within the expected range of ± 3. Residuals outside this range are considered as being outliers. The scatter plot shows that these residuals formed a uniform band around the mean value and suggests that the assumption of common variance was also met hence the model was valid in this particular study.

5.1.4: WNV strain diversity

Analysis of the obtained sequences indicated a high similarity of the WNV isolates to the strains in Genebank classified under lineage 1. However they exhibited a higher similarity among themselves as opposed to those isolated from different geographical localities. This would be an indication that a particular strain of WNV was introduced to this locality either by an infected migratory bird as reported in Duggal *et al*. (2015) and its existence has been maintained through amplification in local resident bird species.

In reference to the blast result, there is an indication that WNV circulating in Tana River county would be genetically closely related but not similar to WNV strains *XJ11141, XJ11129 and XJ11148* isolated from mosquitoes in Xinjiang Uyghur, western China (Lu *et al*., 2014) and *Ast-986* strain isolated from a human patient's blood in Volgograd region, Russia (Prilipov *et al.*, 2000). Phylogenetic comparisons of complete nucleotide sequences of E gene from the Xinjiang isolates showed a high degree of genetic identity of lineage 1 with other highly pathogenic WNV strains, such as WNV NY99 and isolates from Russia (Lu *et al*., 2014).

The sequences obtained in this study were shorter than the expected 445bp gene limiting conclusive analysis and inference on exact strain diversity. Most of the reverse and forward sequences did not reach a common region that could enable overlap to form a longer sequence for phylogenetic studies. This challenge could be due to inadequacy of the amount of template available for sequencing or usage of insufficient primers. Further studies can be done to enable genetic classification of Tana River county WNV isolates using the baseline data obtained from this study.

5.2: Conclusions

From this study it can be concluded that WNV is in circulation within Tana River County among the diverse wild bird species. Its choice of an amplification host has been shown to be independent of the species and most of the resident birds would serve as reservoir hosts. Land use patterns especially irrigation practices influence the bird population groups, hence WNV transmission patterns. The virus has a low specificity to the host species but its occurrence in this study has been shown to be influenced by location. Availability of good breeding grounds for mosquito vectors and adequate food base for birds in this county are among factors that would enhance propagation of this pathogen.

It is clear that WNV genome exists in multiple variants but there is increased identity similarity of a strain in a given locality. Since the virus has been reported to rapidly expand its geographical range and epidemic development, there is need to promote awareness in the public health departments and among local residents in order to prevent fatal outbreaks.

There is inadequate data on surveillance for active WNV infection in birds and this study can therefore provide a baseline for further research to enable adequate comprehension of ecology of this pathogen in Kenya and assist in statement of public health measures to avoid incidents of morbidity and mortality due to sudden outbreaks and conserve wild birdsl.

5.3: Recommendations

It would be recommended that a similar study to be carried out and the detected positive samples to be sequenced using the next generation sequencing technique to enhance the quality of obtained sequence data in terms of length and clarity. This technology has been described to have a high throughput, longer read length and enhanced accuracy (Lin *et al*., 2012) for a better analysis.

Unlike Sanger, obtained longer sequences could allow substantial analysis and determination of the phylogeny of the amplified fragment hence showing clearly the genetic relationships between Tana river county isolates and those isolated from other geographical locations.

Further studies are needed to establish the persistence duration of the virus in infected wild birds, virulence factors and the dynamics of pathogenicity potential. In addition, the vectoral competence of the mosquito species in this county should be established to enhance innovation of prevention and control strategies.

Birds are known to be prone to infection by other arboviruses beside WNV like Chikungunya, Usutu, Sindbis and Tahyna virus. This study strengthens the need to carry out metagenomic studies on bird blood samples from this locality to establish other pathogens that would be in circulation to enable installation of various monitoring and preventative measures so as to avoid abrupt epidemics.

It would be necessary to consider sampling and screening of the large birds like crows which recorded the highest mortality rates during the 1999 New York outbreak and domestic birds so as to establish a holistic picture of WNV prevalence.

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