CHARACTERIZATION OF AVIPOXVIRUSES FROM CHICKENS AND DOMESTIC PIGEONS IN TANZANIA

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ABSTRACT

Avipoxviruses (APVs) are double stranded DNA viruses belonging to the family *Poxviridae*, sub-family *Chordopoxvirinae* and genus *Avipoxvirus*. APVs cause pox in birds; in chickens the disease is known as fowl pox, caused by *Fowlpox virus* (FWPV). In the recent years the incidence and prevalence of fowl pox in chickens has increased in Tanzania, characterized by high mortalities of chicks and growers. The main research question among researchers has been "are there variant strains of FWPV or other APVs circulating in domestic birds that pose a threat to chickens in Tanzania?'' The general objective of this study was to investigate the characteristics of APVs from chickens and domestic pigeons in Tanzania, focusing on development of appropriate fowl pox vaccine. The specific objectives were to determine genetic characteristics of APV isolates from chickens and domestic pigeons in Tanzania, to investigate integration of *Reticuloendotheliosis virus* (REV) genes in the Tanzanian FWPV field isolates and the imported commercial FWPV vaccines currently used in the country, and to evaluate virulence characteristics of Tanzanian strains of FWPV and pigeonpox virus (PGPV) in chickens. Samples of cutaneous nodular lesions were collected from chickens (n = 154) and pigeons (n = 17) suspected to have pox, followed by virus isolation. Genetic characterization involved DNA extraction; polymerase chain reaction (PCR) amplification of FWPV and PGPV P4b gene, REV envelope (*env*) gene and REV 5' long terminal repeat (LTR); gel electrophoresis of PCR products; purification of PCR products; sequencing of purified PCR products and analysis of the sequence data using standard procedures. Biological characteristics, particularly virulence characteristics of Tanzanian strain(s) of FWPV and PGPV in chickens, were investigated by inoculating 10-day old embryonated

chicken eggs and susceptible chickens with either REV-integrated Tanzanian FWPV strain, REV-free Tanzanian FWPV strain, or Tanzanian PGPV strain. Sixty six (66) out of the 154 samples (42.86%) analyzed for the presence of FWPV were found to contain FWPV, indicating that the 66 chickens from which the samples were collected had fowl pox as a result of FWPV infection. Analysis of sequences of the P4b gene (open reading frame [ORF] 167) revealed that the Tanzanian FWPV isolates were 99.65 – 100% identical to each other and 99 – 100% identical to several published sequences of FWPV isolates from various countries in different continents of the world, including Europe and Asia. Phylogenetic analysis revealed that all Tanzanian FWPV isolates belong to clade A subclade A1. This implies that based on sequences of the P4b gene the FWPVs currently prevalent in Tanzania are genetically and phylogenetically closely related. However, analysis of selected FWPV isolates in other genomic regions (between ORFs 201 and 203) revealed integration of various genomic fragments of REV in the genome of FWPV. Out of 55 field isolates of FWPV analyzed for integration of REV *env* gene and REV 5'LTR, 96.36% (n = 53) were found to have genomic fragments of REV. Most of them, 69.09% (n = 38) contained REV *env* gene and REV 5'LTR fragments, 18.18% (n = 10) contained fragments of REV *env* gene only, and 9.09% (n = 5) contained fragments of REV 5'LTR only. Two isolates (3.64%) were found not to be integrated with either REV *env* gene or REV 5'LTR. None of the screened vaccine strains from the imported commercial fowlpox virus vaccines was found to be integrated with REV *env* gene and/or REV 5'LTR. Analysis of sequences of a PCR product with fragment size 807 bp showed 95 – 100% identity to sequences of several REV *env* gene obtained in the GenBank and 100% identity to sequence of *env* gene of REV provirus integrated in a FWPV isolate. The sequence of a PCR product with fragment size 370 bp showed 88 – 99% identity to sequences of several

REV LTR obtained in the GenBank, and 99 – 100% identity to sequences of LTR of REV provirus integrated in several FWPV isolates from other countries. Of the 17 samples of cutaneous nodular lesions collected from pigeons, two (both from Morogoro region) were found to contain PGPV. Sequence analysis revealed that the Tanzanian PGPV isolate derived from this study was 90 - 99% identical to several APV isolates from birds belonging to different species from several countries. The Tanzanian PGPV isolate showed 91% identity to each of the Tanzanian FWPV isolates, also derived from the present study, and 99% identity to all three PGPV isolates obtained in the GenBank. Phylogenetic analysis revealed that the Tanzanian PGPV isolate belongs to clade A, subclade A2, sharing a recent common ancestor with APVs belonging to subclade A3. Biological characterization revealed that unlike a PGPV isolate from a Norwegian wild pigeon, *Palumbus palumbus,* that could infect and cause pox in chickens; the Tanzanian strain of PGPV can infect but does not cause pox in chickens. The study also revealed high mortality rate (57%) in chickens inoculated with REV-integrated Tanzanian FWPV strains as compared to zero mortality observed in chickens inoculated with REV-free Tanzanian FWPV strains, or a Tanzanian PGPV strain currently circulating in Morogoro region, and chickens in the control group. Based on the findings from this study the following conclusions are drawn: (a) Currently there is a heterogeneous population of FWPV in Tanzania comprising of REV-integrated FWPV strains and REV-free FWPV strains. (b) REV-integrated FWPV strains are more virulent in susceptible chickens than REV-free FWPV strains. (c) The increased incidences and prevalence of fowl pox currently experienced in Tanzania, characterized with high mortality rates of chicks and growers, could be attributed to the emergence of variant strains of FWPV which are REV-integrated. (d) The imported commercial fowl pox vaccines currently used in Tanzania are not contaminated with REV, therefore the vaccines

can safely continue to be used in the country. (e) Integration of genomic fragments of REV in the genome of field strains of FWPV currently prevalent in Tanzania is not attributed to imported commercial fowl pox virus vaccines currently used in the country. It could be attributed to recombination of field strains of REV and field strains of FWPV. (f) As opposed to a PGPV isolate from a Norwegian wild pigeon, *Palumbus palumbus*, that could infect and cause pox in chickens; this study has revealed that the Tanzanian strain of PGPV currently circulating in Morogoro region is not pathogenic in chickens, therefore it does not pose a threat to chickens in the country. On basis of the findings from this study the following future studies are recommended: (a) More studies aiming at detection and characterization of PGPV isolates from other regions and geographical locations of Tanzania should be conducted in order to establish genetic and antigenic characteristics of PGPV currently circulating in the country. This recommendation is based on the fact that in the present study only two isolates of PGPV could be obtained from pigeons in Morogoro region, Eastern Tanzania. (b) Studies to determine pathogenicity and lethality of PGPV and FWPV in different host systems are required. (c) Epidemiological features and risk factors for FWPV and PGPV transmission ability and spread should be investigated. (d) Evolutionary characteristics of FWPV, PGPV and REV should be systematically studied to unravel possible factors that could be linked with their genetic and antigenic diversity.

DECLARATION

I, SELEMAN NASIB MASOLA, do hereby declare to the Senate of Sokoine University of Agriculture, that this thesis is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

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(Supervisor)

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LIST OF ABBREVIATIONS

ABPV	Albatrosspox virus
AGPV	Agapornispox virus
ALSV	Avian leukosis sarcoma virus
ANOVA	Analysis of varience
ANT	Antarctica
APV	Avipoxvirus
APVs	Avipoxviruses
ARG	Argentina
ARU	Animal Research Unit
AWHN	Australian Wildlife Health Network
BEL	Belgium
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CAM	Chorioallantoic membrane
CAMs	Chorioallantoic membranes
CFSPH	Center for Food Security and Public Health
CHI	Chile
CMPV	Camelpox virus
CNPV	Canarypox virus
co	Nucleocapsid core
COSTECH	Tanzania Commission for Science and Technology
CSV	Chick syncytial virus
CWPV	Cowpox virus
DIAV	Duck infectious anaemia virus
DNA	Deoxyribonucleic acid

DVMP	Department of Veterinary Microbiology and Parasitology
ECEs	Embryonated chicken eggs
ECTV	Ectomelia virus
ECU	Ecuador
EDTA	Ethylenediaminetetraacetic acid
EEV	Extracellular enveloped virions
EID_{50}	Mean (50%) embryo-infectious dose
e.g	For example
ELISA	Enzyme-linked immunosorbent assay
env	Envelope
ES	Spain
Fig	Figure
FLPV	Falconpox virus
FVM	Faculty of Veterinary Medicine
FWPV	Fowlpox virus
gag	Group-specific antigen
GER	Germany
GIS	Geographical information system
GTPV	Great titpox virus
HU	Hungary
ICTV	International Committee on Taxonomy of Viruses
IMV	Intracellular mature virions
ISSN	International Standard Serial Number
ITA	Italy
ITRs	Inverted terminal repeats
JNPV	Juncopox virus
kbp	Kilobase pair
KOR	South Korea

lb	Lateral bodies
LTR	Long terminal repeat
LTRs	Long terminal repeats
MCPV	Macawpox virus
MEGA	Molecular Evolutionary Genetics Analysis
MKPV	Monkeypox virus
MNPV	Mynahpox virus
MOCV	Molluscum contagiosum virus
NIH	National Institutes of Health
NL	Netherlands
NOR	Norway
ORF	Open reading frame
ORFs	Open reading frames
OIE	Office International des Épizooties
OSPV	Ostrichpox virus
PASS	Private Agricultural Sector Support
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCRs	Polymerase chain reactions
PGPV	Pigeonpox virus
pi	Post-inoculation
pol	Polymerase
POR	Portugal
pro	Protease
PRPV	Parrotpox virus
PSPV	Psittacinepox virus
QUPV	Quailpox virus
REA	Restriction enzyme analysis

REV	Reticuloendotheliosis virus
REV-A	Reticuloendotheliosis virus strain A
REV-T	Reticuloendotheliosis virus strain T
REVs	Reticuloendotheliosis viruses
RFLP	Restriction fragments length polymorphism
RLDC	Rural Livelihood Development Company
RNA	Ribonucleic acid
SLPV	Starlingpox virus
SNV	Spleen necrosis virus
SRPV	Sparrowpox virus
SUA	Sokoine University of Agriculture
TALIRI	Tanzania Livestock Research Institute
TBE	Tris-Borate-EDTA
TK	Thymidine kinase
TKPV	Turkeypox virus
TVLA	Tanzania Veterinary Laboratory Agency
UAE	United Arab Emirates
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VACV	Vaccinia virus
VARV	Variola virus
VIC	Veterinary Investigation Centre

LIST OF SYMBOLS FOR UNITS

nm	Nanometer
µm	Micrometer
ml	Milliliter
mg	Milligram
mm	Millimeter
µl	Microliter
g	Gram
mg	Milligram
V	Volt
%	Percent
ºC	Degree Celsius

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background Information

Avipoxviruses (APVs) are double stranded DNA viruses belonging to the family *Poxviridae*, sub-family *Chordopoxvirinae* and genus *Avipoxvirus*. APVs are economically important because they cause a disease known as pox in birds. In chickens the disease is known as fowl pox, caused by *Fowlpox virus* (FWPV) (Tripathy, 2005). Fowl pox is controlled by vaccinating susceptible chickens with appropriate vaccines of FWPV or PGPV-origin (Tripathy, 2005)

1.2 Problem Statement and Justification of the Study

1.2.1 Problem statement

Poultry diseases is one of the major constraints in the development of the poultry industry in Tanzania (Msami, 2007; Coppolilo *et al.*, 2009; Goromela, 2009; RLDC, 2012; PASS Trust, 2013). High chicken mortality rates of 40 to 80% have been recorded in local chickens in Tanzania and other African countries (Goromela, 2009). Fowl pox is among endemic poutry diseases which have been affecting chickens in Tanzania (Msami, 2007; Coppolilo *et al*., 2009; Goromela, 2009). As opposed to the past decades where incidences and prevalence of the disease in the country were low, currently the disease incidence and prevalence in the country have increased (Oxfam, 2009; Wambura and Godfrey, 2010; RLDC, 2012). In areas where Newcastle disease has been controlled using I-2 thermostable vaccine, fowl pox outbreaks have been occurring (Oxfam, 2009; Wambura and Godfrey, 2010); often characterized with high mortalities of chicks and growers (Oxfam, 2009) leading to great economic losses to poultry keepers and the nation at large.

Currently commercial fowl pox vaccines are imported from some European countries, some poultry keepers vaccinate their chickens with the imported vaccines in order to control fowl pox. However, in the United States, Fadly *et al*. (1996) reported an outbreak of lymphomas in broiler chickens caused by vaccination of the chickens with REV-contaminated fowlpox virus vaccine. Vaccination of chickens with imported commercial fowl pox vaccines might have led to introduction of reticuloendotheliosis in Tanzania if the vaccines are contaminated with REV, and this could have led to increased chicken mortality.

1.2.2 Justification of the study

Prior to this study factors attributing to the increased incidences and prevalence of fowl pox currently experienced in Tanzania were not known. However, it was speculated that the increased incidence and prevalence of the disease currently experienced in some countries, Tanzania being one of them, could be attributed to emergence of variant strains of FWPV or other APVs which are more pathogenic in susceptible chickens than strains which were circulating in the countries in the past decades (Singh *et al*., 2000; Singh *et al*., 2005). In order to confirm or refute this speculation, characterization of field strains of FWPV which affect chickens, and other APVs particularly pigeonpox virus (PGPV) which affect pigeons in Tanzania was needed.

Moreover, although to date no cases of reticuloendotheliosis outbreaks have been reported in Tanzania in flocks of chickens or turkeys previously vaccinated against fowl pox, examination of the vaccines to determine their REV contamination status was important so as to be certain with the safety of the vaccines.

Last but not least, for the past decades Tanzania has been using imported commercial fowl pox vaccines for control of fowl pox. Currently the country has the capacity of developing and producing fowl pox vaccines which may be sold to poultry keepers at lower prices compared to the imported vaccines .The understanding of genetic diversity and biological characteristics of field strains of FWPV and PGPV currently circulating in chickens and pigeons, respectively, in Tanzania was of paramount importance towards development of appropriate fowl pox vaccine for control of fowl pox in Tanzania, and other countries with populations of FWPV which are genetically and antigenically similar to the FWPV strains currently circulating in Tanzania.

1.3 Research Questions

This study sought to answer the following research questions:

1. Are there variant strains of FWPV or other APVs circulating in domestic pigeons which pose a threat to chickens in Tanzania?
2. Are the Tanzanian field strains of FWPV and the imported commercial fowl pox vaccines currently used in Tanzania contaminated with REV?
3. What are the virulence characteristics of Tanzanian strains of fowlpox and pigeonpox viruses in chickens?

1.4 Study Objectives

1.4.1 General objective

The general objective of this study was to investigate the characteristics of APVs from chickens and domestic pigeons in Tanzania, focusing on development of appropriate fowl pox vaccine.

1.4.2 Specific objectives

i) To determine genetic characteristics of APV isolates from chickens and domestic pigeons in Tanzania.

ii) To investigate integration of genomic fragments of REV in the Tanzanian field strains of FWPV, and the imported commercial fowl pox vaccines currently used in the country.

iii) To evaluate virulence characteristics of the Tanzanian strains of FWPV and PGPV in chickens.

1.5 Outline of the thesis

This thesis has been developed in published papers format according to Sokoine University of Agriculture (SUA) guidelines (SUA, 2007; SUA, 2011). The thesis has nine chapters; chapter 1 gives the general introduction that describes the background information of APVs and control of fowl pox, problem statement, study justification, research questions, study objectives which define the scope of this study, and an outline of the thesis.

Chapter 2 consists of a literature review. It gives a review on the taxonomy of poxviruses, morphology of poxviruses, replication of poxviruses, genome of poxviruses, *Reticuloendotheliosis virus* (REV), genome of REV, replication of REV, REV as a contaminant of field strains of FWPV, comparative virulence of REV-integrated FWPV and REV-free FWPV strains, REV as a contaminant of commercial FWPV vaccines, pox in birds, diagnosis of pox in birds, classification of APVs based on phylogenetic relationship (based on nucleotide sequences of the P4b and DNA polymerase genes), as well as genetic and biological characterization of

APVs. Chapter 3 consists of the general methodology. It describes the methodology that was used in order to achieve each of the specific objectives outlined in chapter 1.

Chapters 4, 5, 6 and 7 consist of four research papers i.e papers I, II, III and IV, respectively. The papers have been published in peer reviewed scientific journals namely British Microbiology Research Journal (paper I), British Biotechnology Journal (papers II and IV) and Advances in Research (paper III).

Chapter 4 describes the occurrence of fowl pox in various regions and geographical locations of Tanzania as revealed by virus isolation, PCR, and sequence blast results. It also explains the genetic characteristics and phylogenetic relationship of the Tanzanian FWPV isolates based on sequences of the P4b gene (ORF 167). Chapter 5 describes existence of variant strains of FWPV in Tanzania as a result of integration of various genomic fragments of REV in the genome of FWPV. It also points out that the imported commercial fowl pox vaccines currently used in Tanzania are REV-free. Chapter 6 describes the occurrence of pox in pigeons in Eastern Tanzania (Morogoro region) as revealed by virus isolation, PCR, and sequence blast results. Moreover, it explains the genetic and phylogenetic relationship of the Tanzanian PGPV isolate to PGPV and other APV isolates from other countries, based on sequences of the P4b gene. Chapter 7 explains biological characteristics of Tanzanian strains of FWPV and PGPV, particularly the virulence characteristics of field strains of FWPV and PGPV in chickens.

Chapter 8 consists of a general discussion. It outlines the the important findings from the present study, as well as their significance and implications. Chapter 9 outlines general conclusions and recommendations based on the findings reported in chapters 4, 5, 6 and 7; also outlined in chapter 8.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Taxonomy of poxviruses

Poxviruses are double stranded DNA viruses belonging to the family *Poxviridae*. Members of this family can infect both humans and animals (MicrobiologyBytes, 2007; Shanley, 2008). The family is divided into two sub-families namely *Chordopoxvirinae* and *Entomopoxvirinae*, members of these sub-families affect chordates (vertebrates) and non-chordates (invertebrates), respectively. The *Chordopoxvirinae* sub-family consists of eight genera namely *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Parapoxvirus*, *Suipoxvirux*, and *Yatapoxvirus*. Sub-family *Entomopoxvirinae* consists of three genera namely *Entomopoxvirus A*, *Entomopoxvirus B* and *Entomopoxvirus C* (MicrobiologyBytes, 2007). Some members of each genus are shown in Table 1.

2.2 Morphology of poxviruses

A mature poxvirus is oval or brick-shaped, 250 × 250 × 350 nm in size (Kulich *et al*., 2008). Thin sections in electron microscope reveal that the outer surface of poxviruses is made up of lipid and protein which surrounds the biconcave core, with two "lateral bodies" (Weli *et al*., 2004; MicrobiologyBytes, 2007; Kulich *et al*., 2008) (Figure 1). The core consists of a nucleoid containing the linear double-stranded DNA (Kulich *et al*., 2008). Poxviruses exist in two forms: the extracellular enveloped virions (EEV) which contain two membranes; and the intracellular mature virions (IMV) which have an inner membrane only (MicrobiologyBytes, 2007).

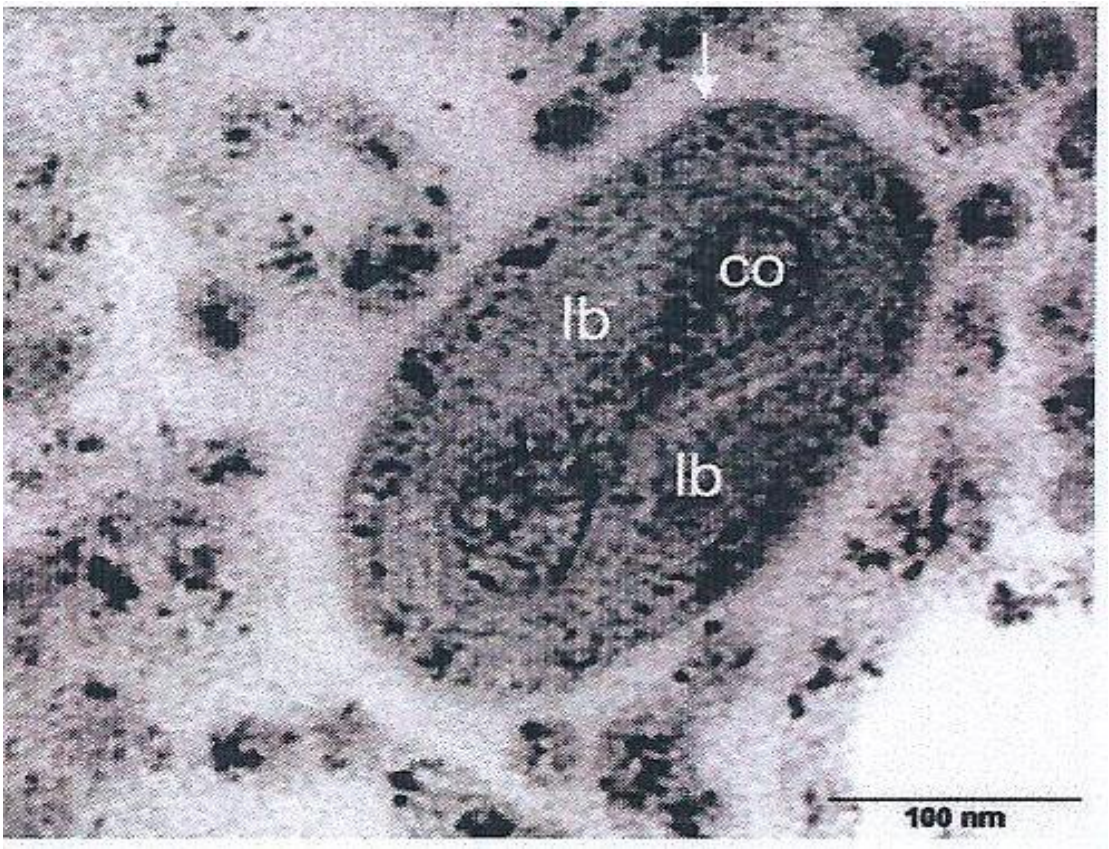

Figure 1. Electron micrograph of ultrathin section showing an oval mature avipoxvirus with an electron-dense biconcave nucleocapsid core (co), two lateral bodies (lb) and an outer lipoprotein coat or envelope (arrow). This Figure was adapted from Kulich *et al*. (2008).

Table 1. **Taxonomy of poxviruses**

Family	Subfamily	Genus	Some members
Poxviridae	*Chordopoxvirinae*	*Avipoxvirus*	*Fowlpox virus*, *Canarypox virus*, *Pigeonpox virus*, *Juncopox virus*, *Mynahpox virus*, *Psittacinepox virus*, *Quailpox virus* and *Turkeypox virus*
		Capripoxvirus	*Sheeppox virus*
		Leporipoxvirus	*Myxoma virus*
		Molluscipoxvirus	*Molluscum contagiosum virus*
		Orthopoxvirus	*Vaccinia virus*, *Smallpox (variola) virus*, *Monkeypox virus* and *Cowpox virus*
		Parapoxvirus	*Orf virus*, *Bovine papular stomatitis virus*, *Deerpox virus* and *Sealpox virus*
		Suipoxvirus	*Swinepox virus*
		Yatapoxvirus	*Yaba monkey tumor virus* and *Tanapox virus*
	Entomopoxvirinae	*Entomopoxvirus A*	*Melolontha melolontha entomopox virus*
		Entomopoxvirus B	*Amsacta moorei entomopox virus*
		Entomopoxvirus C	*Chirosonomus luridus entomopox virus*

Sources: Smith *et al*. (2002); MicrobiologyBytes (2007); Shanley (2008); Weli and Tryland (2011).

2.3 Replication of poxviruses

Poxvirus replication takes place in the cytoplasm. The virus is sufficiently complex to have acquired all the functions necessary for genome replication. The process of replication involves receptors, penetration into the host cell, uncoating, gene expression, genome replication and assembly. Receptors are not known; penetration is complex and may involve more than one mechanism. Uncoating occurs in two stages. Stage I involves removal of the outer membrane as the particle enters the cell. In stage II the particle (minus the outer membrane) is further uncoated and the core passes into the cytoplasm (MicrobiologyBytes, 2007).

Replication takes place in the so-called "virus factories", these are electron-dense zones in the cell cytoplasm containing no cellular organelles (Kulich *et al*., 2008). Gene expression is carried out by viral enzymes associated with the core and is divided into two phases. In phase I, early genes (about 50% of genome) are expressed before genome replication. In phase II, late genes are expressed after genome replication. Genome replication is believed to involve self-priming, leading to the formation of high molecular weight concameters (isolated from infected cells) which are subsequently cleaved and repaired to make virus genomes. Assembly occurs in the cytoskeleton; the events involved in putting together such a complex particle are not understood, but could involve interactions with the cytoskeleton such as actin-binding proteins (MicrobiologyBytes, 2007).

The first visible structure appearing in the course of virion replication is a membrane structure resembling a crescent. Then the crescent gets longer and begins to encircle

the fine, slightly electron-dense material (Figure 2A). The opposite ends of the crescent meet, creating a spherical structure that contains the core. These spherical structures are called intracellular virions or immature viruses (Figure 2B). The virion core begins to elongate to form an ellipse, flattened at the sides by two lateral bodies. The virion begins to mature to its characteristic brick-shape, called the IMV (Figure 2C), which is infectious (Kulich *et al*., 2008; Weli and Tryland, 2011). IMV leaves the virus factory and exits the cell by budding through the cytoplasmic membrane (Hatano *et al*., 2001; Kulich *et al*., 2008) or by cellular cytolysis (Kulich *et al*., 2008).

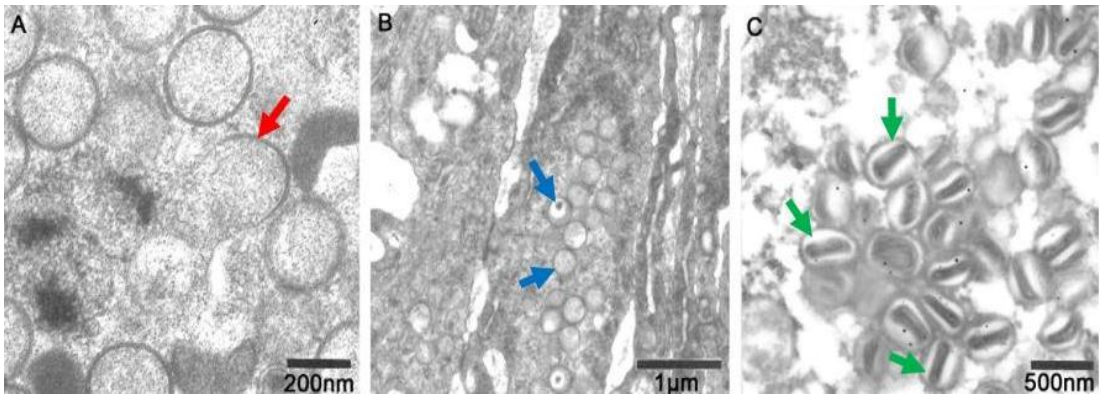

Figure 2A, B and C. Electron micrographs showing virion morphology of poxviruses in the host cells. (A) crescent-shaped structures consisting of a membrane with spicules on the convex surface (red arrow), (B) spherical non-infectious immature viruses (blue arrows), (C) oval-shaped IMV (green arrows). This Figure was modified from Weli and Tryland (2011).

2.4 Genome of poxviruses

A genome is the complete set of genetic material of an organism. According to Shanley (2008) poxviruses are the largest and most complex viruses. They are linear

double-stranded DNA viruses of about 130-365 kilobase pair (kbp) (MicrobiologyBytes, 2007; Shanley, 2008; Weli and Tryland, 2011). The genome of each poxvirus is flanked by inverted terminal repeats (ITRs) that typically contain a small number of genes whose positions and orientations are mirrored at the opposing ends of the genome. ITRs range in length from 58 base pair (bp) in *Variola virus* (*Variola virus*, strain India 1967) to 12,400 bp in *Shope fibroma virus* (*Shope fibroma virus*, strain Kasza). The number of open reading frames (ORFs) that map within the ITR range from zero in variola virus to 12 ORFs in *Myxoma virus* (Seet *et al*., 2003).

Genomes of some poxviruses have been sequenced. According to Smith *et al.* (2002) orthopoxviruses whose genomes have been sequenced include *Vaccinia virus* (VACV), *Variola virus* (VARV), *Monkeypox virus* (MKPV), *Camelpox virus* (CMPV), *Ectomelia virus (*ECTV) and *Cowpox virus* (CWPV). To date members of the genus *Avipoxvirus* whose genomes have been sequenced are *Fowlpox virus* (FWPV) (Afonso *et al*., 2000) and *Canarypox virus* (CNPV) (Tulman *et al*., 2004). Most of the essential genes are located in the central part of the genome, while non-essential genes are located at the ends. Genes that are centrally located in the genome are mostly conserved among all poxviruses and tend to be involved with common molecular functions such as replication or virion assembly, whereas terminally located genes tend to be more variable and are often involved in host range restriction or immune subversion (Seet *et al*., 2003).

With exception of the genome of CNPV which has 328 genes (also known as ORFs) (Tulman *et al*., 2004), the genomes of other sequenced poxviruses contain about 250 genes (MicrobiologyBytes, 2007) (Figure 3). Because of the large DNA genome, poxviruses have been evaluated as viral vector carrying foreign genes (Moore *et al*., 2000; Weli *et al*., 2004). Other reports (Hertig *et al*., 1997; Diallo *et al*., 1998; Singh *et al*., 2000; Garcia *et al*., 2003; Singh *et al*., 2003; Singh *et al*., 2005; Prukner-Radovčić *et al*., 2006; Davidson *et al*., 2008; Biswas *et al*., 2011) demonstrated that the genome of most field strains of FWPV and some vaccine strains of FWPV carry inserts of various genomic fragments of *Reticuloendotheliosis virus*.

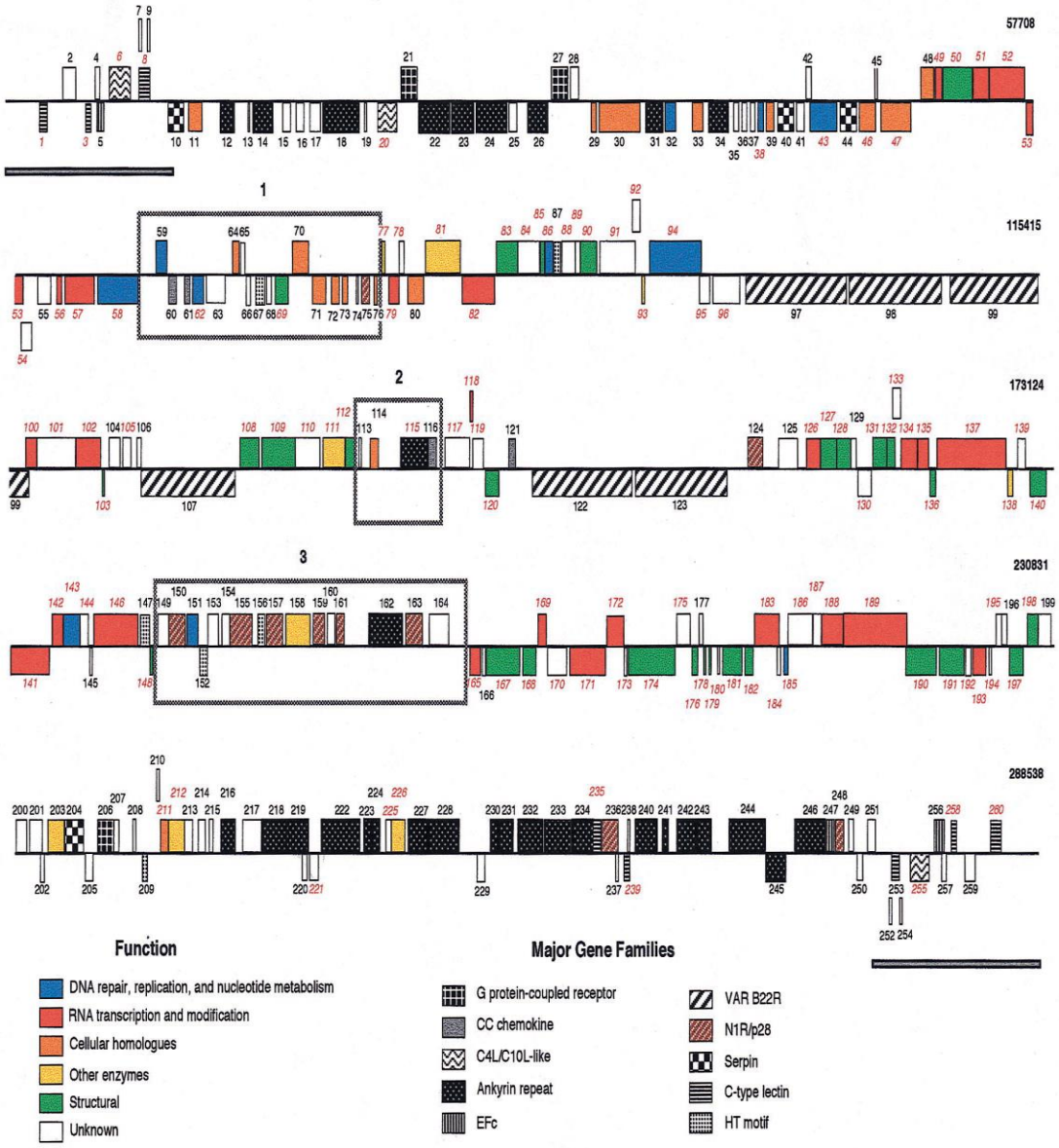

Figure 3. Linear map of the FWPV genome. Genes (or ORFs) are numbered from left to right based on the position of the methionine initiation codon. ORFs transcribed to the right are located above horizontal lines; ORFs transcribed to the left are below. VACV homologues are indicated with red italicized numbers. Genes with similar functions and members of gene families are colored according to the figure key. ITRs are represented as gray bars below the ORF map. Boxed regions 1 to 3 indicate novel coding regions at junction sites of major genome rearrangements. This Figure was adapted from Afonso *et al.* (2000).

2.5 Reticuloendotheliosis virus (REV)

REV comprise a group of avian RNA viruses that belong to the *Retroviridae* family (Payne and Venugopal, 2000; Büchen-Osmond, 2004). Currently this family is divided into three sub-families: *Orthoretrovirinae*, *Spumaretrovirinae* and the unclassified member of the *Retroviridae* family. Among these sub-families the most important is the *Orthoretrovirinae* sub-family, which consists of six genera: *Alpharetrovirus*, *Betaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Gammaretrovirus* and *Lentivirus* (Büchen-Osmond, 2006).

The recent classification of retroviruses has placed REV under the sub-family *Orthoretrovirinae* and genus *Gammaretrovirus* whereas the other avian retroviruses, the avian leukosis-sarcoma virus (ALSV) group, which have several morphological and biochemical features similar to REV (Payne and Venugopal, 2000) are classified in the genus *Alpharetrovirus* (Büchen-Osmond, 2004).

Based on the replication ability in the host cells REV isolates are classified as either defective or non-defective (replication competent). Defective REV refers to REV isolates which lack the ability to replicate in the cell cultures or naturally-infected cells of susceptible hosts while those which can replicate are referred to as non-defective (Payne and Venugopal, 2000; Fadly, 2005) . The group of defective REV comprises of strain T (REV-T) (Aly *et al*., 1993**)**; a laboratory strain of REV with deletions within the genome and possesses a viral oncogene, v-*rel*, inserted at some site within the genome (Stephens *et al*., 1983; Payne and Venugopal, 2000). This strain transforms haematopoietic cells and fibroblasts to tumourigenic cells in vitro (Herzog *et al*., 1986). Strains that comprise the non-defective group of REV include REV-A, *Spleen necrosis virus* (SNV), *Chick syncytial virus* (CSV), and *Duck*

infectious anaemia virus (DIAV) (Aly *et al*., 1993). Most field isolates of REV are non-defective (Fadly, 2005) and have been reported to cause runting disease syndrome in chickens and ducks, characterized by cellular and humoral immunosuppression, and chronic lymphoid neoplasms in chickens (Payne and Venugopal, 2000). REV-T causes acute cell neoplasia if experimentally infected to susceptible birds (Payne and Venugopal, 2000).

2.6 Genome of REV

Like other retroviruses non-defective REV have a simple genome that consists of four genes namely *gag*, which encodes for the synthesis of internal viral proteins which form the matrix, the capsid, and the nucleoprotein structure; *pol*, that encodes for an RNA-dependent DNA polymerase (also known as reverse transcriptase) and integrase enzymes; and *env*, which encodes for the viral envelope glycoproteins. In addition to these genes, a small gene known as *pro*, which encodes for viral protease is located within or adjacent to the *gag* gene (Weaver *et al*., 1990; Moore *et al*., 2000; Payne and Venugopal, 2000; Davidson *et al*., 2008). The structural genes (*env* and *gag*) are flanked by genomic sequences which regulate viral replication, which in the DNA provirus form the viral long terminal repeats (LTRs) (Figure 4) that carry promoter and enhancer sequences (Payne and Venugopal, 2000).

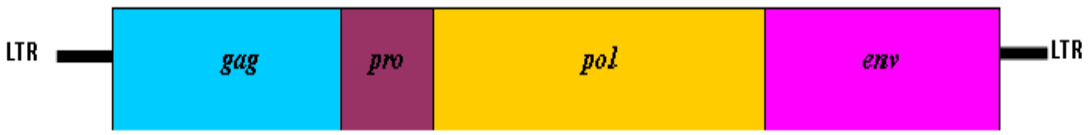

Figure 4. Linear map of the genome of non-defective REV. This Figure was modified from Bohls *et al*. (2006).

2.7 Replication of REV

Similar to other retroviruses, the replication cycle of REV involves reverse transcription of the virion RNA into linear double stranded DNA and the subsequent integration of the REV proviral DNA into the genome of the host cell or host virus (Moore *et al*., 2000). The two processes i.e reverse transcription of RNA to DNA and integration of the proviral DNA into the genome of the host cell or host virus are accomplished by an RNA-dependent DNA polymerase (also called reverse transcriptase) and an enzyme known as integrase, respectively (Vogt, 1997).

2.8 REV as a contaminant of field strains of FWPV

In some countries various genomic fragments of REV have been demonstrated to be integrated in the genome of field strains of FWPV by using polymerase chain reaction (PCR). The countries include Australia (Hertig *et al*., 1997; Diallo *et al*., 1998), Croatia (Prukner-Radovčić *et al*., 2006), India (Biswas *et al*., 2011), Israel (Davidson *et al*., 2008) and the United States (Singh *et al*., 2000; Garcia *et al*., 2003; Singh *et al*., 2003; Tadese and Reed, 2003; Singh *et al*., 2005). The type of REV genes and size of fragments amplified was determined by primer sets used. Tadese and Reed (2003) and Diallo *et al*. (1998) demonstrated the integration of REV LTR in the FWPV genome. Hertig *et al*. (1997) and Davidson *et al*. (2008) were able to demonstrate the integration of near-full length REV provirus in the genome of some of the screened field FWPV isolates. The rest of the authors demonstrated the integration of REV *env* and REV LTR in the genome of FWPV.

2.9 Comparative virulence of REV-integrated FWPV and REV-free FWPV strains, and development of an appropriate FWPV vaccine

According to Singh *et al*. (2005) field strains of REV-integrated FWPV are more virulent than REV-free FWPV strains. Furthermore, Singh *et al*. (2005) suggested that in a country where fowl pox is endemic caused by a heterogeneous population of FWPV comprising of REV-integrated FWPV and REV-free FWPV strains, an appropriate autogenous FWPV vaccine can be developed from a REV-integrated FWPV isolate after attenuating it by removing the REV provirus if the removal of the REV provirus from the FWPV genome does not alter its immunogenicity and antigenicity.

2.10 REV as a contaminant of commercial FWPV vaccines

REV is a potential contaminant of FWPV vaccines. This is supported by previous reports (Fadly *et al*., 1996; Hertig *et al*., 1997; Singh *et al*., 2000; Garcia *et al*., 2003; and Awad *et al*., 2010) in which commercial fowlpox vaccines were confirmed to be contaminated with REV by using various techniques, including PCR and enzyme-linked immunosorbent assay (ELISA).

Vaccination of chickens and turkeys with REV-contaminated fowlpox vaccine leads to dissemination of REV. This may cause outbreaks of reticuloendotheliosis in vaccinated birds if prior to vaccination the birds were susceptible to reticuloendotheliosis. For instance, in 1966 to 1969 cases of a neoplastic disease were reported in turkeys in Israel after vaccination of the turkeys with REV-contaminated fowlpox vaccine (Diallo *et al*., 1998). In the United States an outbreak

of lymphomas occurred in broiler chickens after vaccination with REV-contaminated fowlpox vaccine (Fadly *et al.*, 1996). Currently fowlpox vaccines used in Tanzania are imported from European countries. However, REV-contamination status of these vaccines is not known.

2.11 Pox in birds

Avipoxviruses (APVs) cause an acute contagious disease of birds generally known as avian pox. According to Kulich *et al.* (2008), Arathy *et al.* (2010) and Atkinson *et al.* (2010) clinically, the disease presents itself in three forms: the cutaneous, diphtheritic and systemic forms. The cutaneous form is characterized by proliferative skin lesions around the beak, on the eyelids, crest, feet and all other body areas relatively free of feathers. The diphtheritic form is characterized by fibrous necrotic proliferative lesions on the mucous membrane of the oral cavity, oesophagus as well as the upper section of the respiratory tract. In the systemic form various body systems and tissues of an infected bird are involved (Kulich *et al.*, 2008; Arathy *et al.*, 2010; Atkinson *et al.*, 2010). Infections due to APVs have been reported in more than 200 species of wild and domestic birds leading to substantial economic losses in commercial poultry (Weli *et al.*, 2004). Avian pox is worldwide distributed (van Riper and Forrester, 2007; Davidson *et al.*, 2008) (Figure 5).

For more than half a century vaccines of FWPV- or pigeonpox virus (PGPV)-origin have been used to prevent fowl pox in chickens in areas where the disease is endemic (Singh *et al.*, 2000). However, although in the past the vaccines conferred protection to the vaccinated birds against the disease (Fatunmbi and Reed, 1996;

Singh and Tripathy, 2000; Singh *et al*., 2000); in recent years outbreaks of fowl pox in previously vaccinated flocks have been reported in several countries including Australia (Diallo *et al*., 1998), the United States (Singh *et al*., 2003), Croatia (Prukner-Radovčić *et al*., 2006), and India (Biswas *et al*., 2011). The attributing factor to this phenomenon is the increased virulence of most field strains of FWPV due to integration of various genomic fragments of REV in their genomes (Singh *et al*., 2005). This fact is demonstrated by incidences of fowl pox outbreaks which were experienced in the United States despite routine vaccination against the disease (Singh *et al*., 2003).

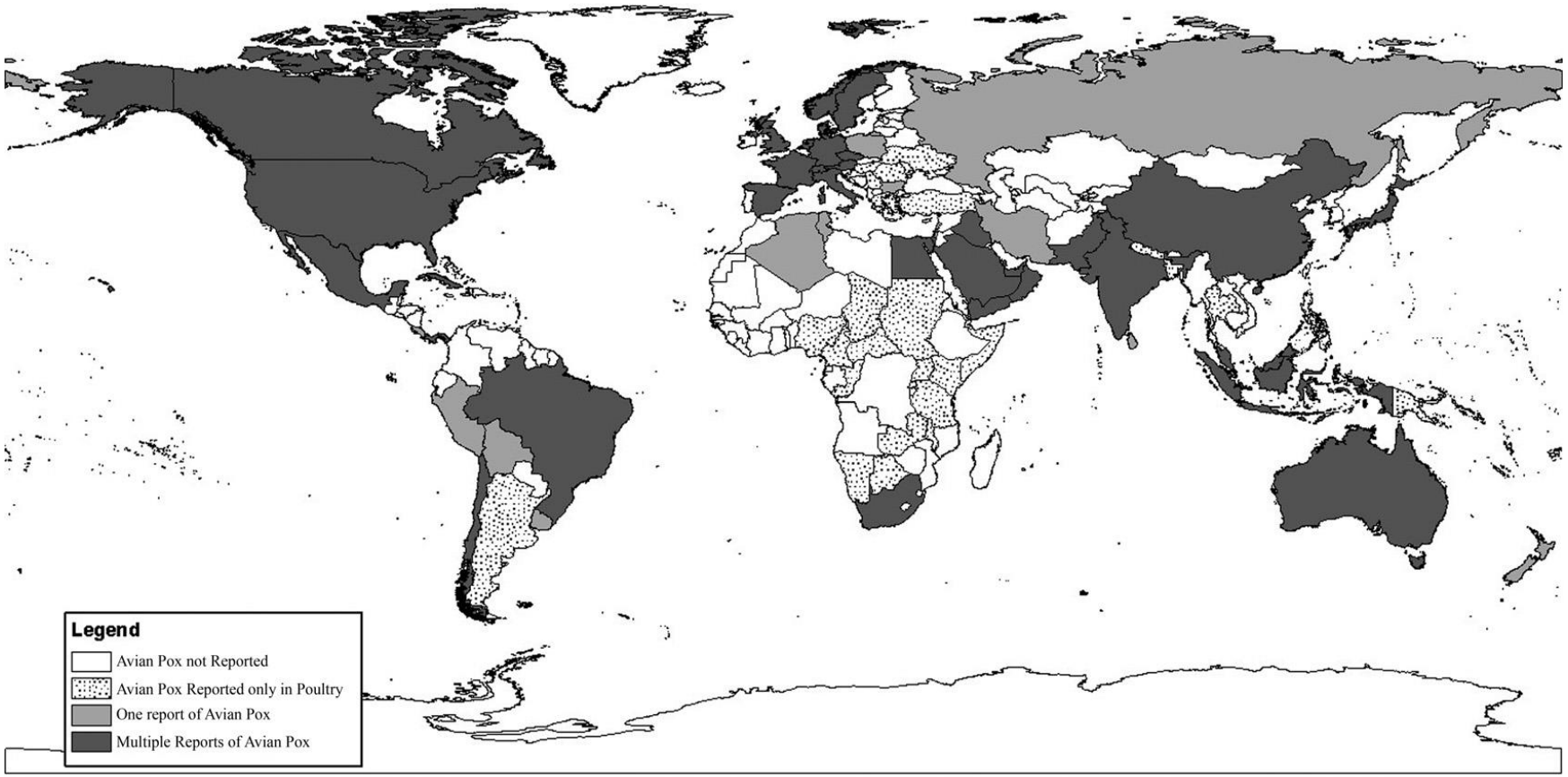

Figure 5. Map of the World showing distribution of pox in birds. This Figure was adapted from Van Ripper and Forrester (2007).

2.12 Diagnosis of pox in birds

Provisionally avian pox can be diagnosed based on characteristic gross lesions varying from papules to nodules (Tripathy, 2005; Weli and Tryland, 2011). However, the cutaneous form of the disease may be confused with diseases like papillomatosis (Literák *et al*., 2003) and mange (AWHN, 2012; CFSPH, 2012; OIE, 2013), which are characterized with gross lesions similar to those of avian pox. The diphtheritic form may be confused with conditions like candidiasis, capillariasis and trichomoniasis which are also characterized with fibrous necrotic proliferative lesions on the mucous membrane of the upper digestive tract (van Riper and Forrester, 2007); or infectious laryngotracheitis which is characterized with similar lesions on the mucous membrane of the trachea, larynx and mouth (OIE, 2008). Thus, it is important to confirm the viral aetiology of the condition. This can be attained by isolation of the virus from affected tissues, demonstration of either microscopic lesions or viral particles with typical poxvirus morphology in the affected tissue, and demonstration of viral DNA in the affected tissues (Tripathy, 2005).

The virus can be isolated by inoculating chorioallantoic membrane (CAM) of developing chicken embryos (Weli *et al*., 2004; Tripathy, 2005; Kulich *et al*., 2008), susceptible birds, or cell cultures of avian origin. Chicken embryos (9-12 days old) are the preferred and most convenient host for virus isolation (Tripathy, 2005).

Microscopic lesions include oesinophilic cytoplasmic inclusion bodies and elementary bodies. Oesinophilic cytoplasmic inclusion bodies can be demonstrated by fluorescent antibody and immunohistochemical methods or when affected tissues

stained with Haematoxylin and Eosin are examined microscopically. Elementary bodies can be demonstrated in the smears from lesions stained by the Gimenez method (Tripathy, 2005).

Viral particles with typical poxvirus morphology can be demonstrated by negative-staining as well as in ultrathin sections of the lesions when examined under electron microscope (Weli *et al*., 2004; Simon *et al*., 2005; Tripathy, 2005; Kulich *et al*., 2008). At molecular level the disease is confirmed by demonstration of the presence of APV DNA in the tissue specimen by using PCR (Tripathy, 2005; Kulich *et al*., 2008; OIE, 2008; Farias *et al*., 2010).

2.13 Classification of APVs based on phylogenetic relationship

Jarmin *et al*. (2006) reported that based on phylogenetic analysis of nucleotide sequences of the P4b gene APVs are grouped into three major clades: clade A (FWPV and FWPV-like viruses), clade B (CNPV and CNPV-like viruses) and clade C (Psittacinepox virus [PSPV] and PSPV-like viruses). According to these authors clade A was divided into four subclades: A1, A2, A3, and A4. Clade B was divided into two subclades: B1 and B2, and clade C had no subclades. Manarolla *et al*. (2010) indicated that APV strains from the same avian species can display great nucleic acid divergence. This is demonstrated by a significant genetic divergence of a quailpox virus (QUPV), strain QP-241 (GenBank accession number GQ180200), from another strain of QUPV (GenBank accession number DQ873809) which belongs to subclade A2. Moreover, the report indicated that two strains of CNPV, strain PA1-09-92 (GenBank accession number GQ221269) and strain PA295-93

(GenBank accession number GQ180208) clustered in clade A, instead of clade B. These results sustained what was reported previously for some APV strains from sparrows, falcons and pigeons (Lüschow *et al*., 2004; Jarmin *et al*., 2006; Rampin *et al*., 2007) and confirmed that sequences of APV isolates from the same species can be found in different subclades or even different clades. This supported what was reported earlier by Jarmin *et al*. (2006) that evolutionary taxonomy of the host does not appear to have a major role in driving evolution of APVs.

Recently Gyuranecz *et al*. (2013) reported four novel APV subclades; A5, A6, A7, and B3; based on phylogenetic analysis of nucleotide sequences of the P4b gene, DNA polymerase gene, and concatenated sequences of the P4b and DNA polymerase genes (Figures 6, 7 and 8).

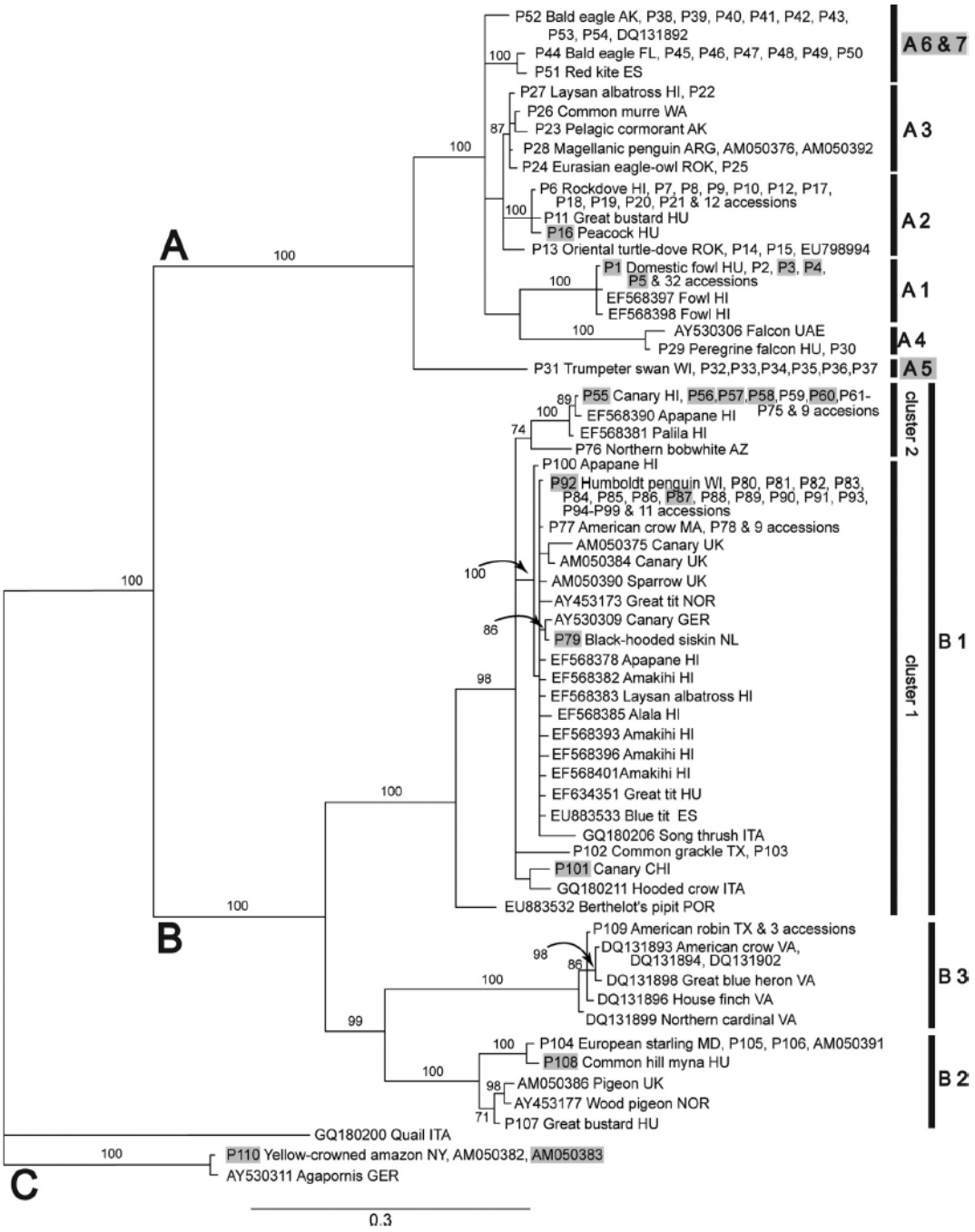

Figure 6. Bayesian phylogram of DNA sequences from genes encoding 4b core proteins of APVs. Posterior probability values of > 70 are shown. APV clades A to C, subclades, and clusters are labeled according to the nomenclature of Jarmin *et al*. (2006). Novel subgroups described by Gyuranecz *et al*. (2013) are highlighted by gray. Isolate origins are given either as U.S. state abbreviations or using the following location codes: Antarctica (ANT), Argentina (ARG), Belgium (BEL),

Chile (CHI), Ecuador (ECU), Germany (GER), Hungary (HU), Italy (ITA), Netherlands (NL), Norway (NOR), Portugal (POR), Spain (ES), South Korea (ROK), United Arab Emirates (UAE), and United Kingdom (UK). APVs that were isolated from captive birds (aviaries, zoos, etc.) are highlighted by gray. The scale represents the number of substitutions per site. Due to the large number of APV isolates in the 4b gene analyses (n = 226), the names for isolates with identical sequences from GenBank accessions are abbreviated as follows: (i) P1 genotype, AB292647, AF198100, AJ005164, AJ581527, AM050377, AM050378, AM050379, AM050380, AY453171, AY453172, AY530302, AY530304, AY530307, DQ873808, EF568377, EF634347, EF634348, M25781, GU108500, GU108501, GU108502, GU108503, GU108504, GU108505, GU108506, GU108507, GQ221269, GQ180212, GQ180207, GQ180201, GU108509, and GU108508; (ii) P6 genotype, AM050385, AM050387, AM050388, AY530303, AY530305, DQ873809, DQ873810, DQ873811, EF016108, GQ180210, GQ180208, and GQ180204; (iii) P55 genotype, EF568379, EF568384, EF568386, EF568387, EF568388, EF568389, EF568391, EF568399, and EF568400; (iv) P77 genotype, AM050381, AM050389, AY530308, GQ487567, GU108510, GQ180202, GQ180203, GQ180205, and GQ180209; (v) P92 genotype, AY530310, AY318871, AY453174, AY453175, EF568380, EF568392, EF568394, EF568395, EF634349, EF634350, and GQ180213; and (vi) P109 genotype, DQ131895, DQ131897, DQ131900, and DQ131901. This Figure was adapted from Gyuranecz *et al.* (2013).

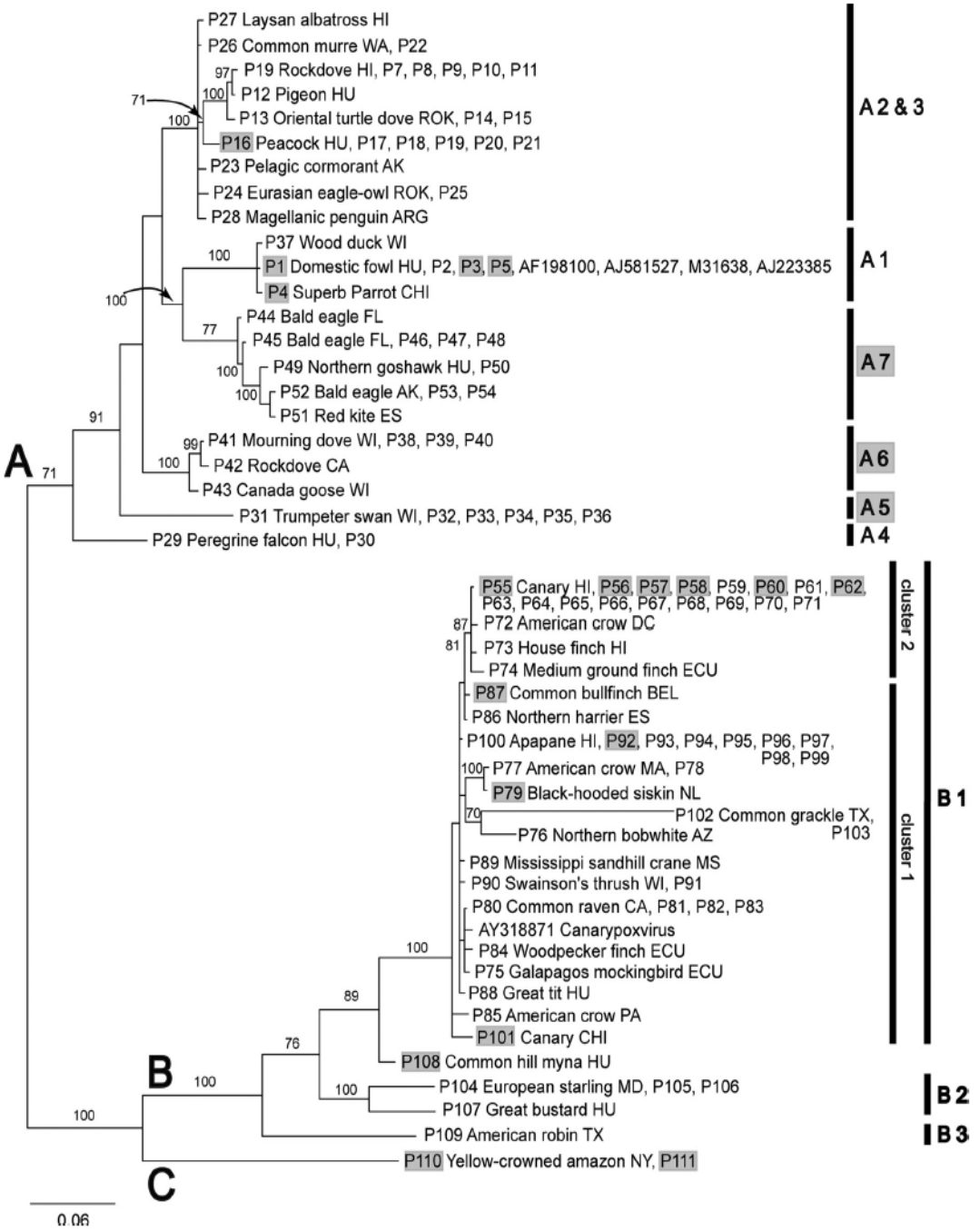

Figure 7. Bayesian phylogeny of DNA sequences from gene encoding DNA polymerase protein of APVs. Posterior probability values of > 70 are shown. APV clades A to C, subclades, and clusters are labeled according to the nomenclature of Jarmin *et al*. (2006). Novel subgroups described by Gyuranecz *et al*. (2013) are highlighted by gray. Isolate origins are given either as U.S. state abbreviations or

using the following location codes: Antarctica (ANT), Argentina (ARG), Belgium (BEL), Chile (CHI), Ecuador (ECU), Germany (GER), Hungary (HU), Italy (ITA), Netherlands (NL), Norway (NOR), Portugal (POR), Spain (ES), South Korea (ROK), United Arab Emirates (UAE), and United Kingdom (UK). APVs which were isolated from captive birds (aviaries, zoos, etc.) are highlighted by gray. The scale represents the number of substitutions per site. This Figure was adapted from Gyuranecz *et al*. (2013).

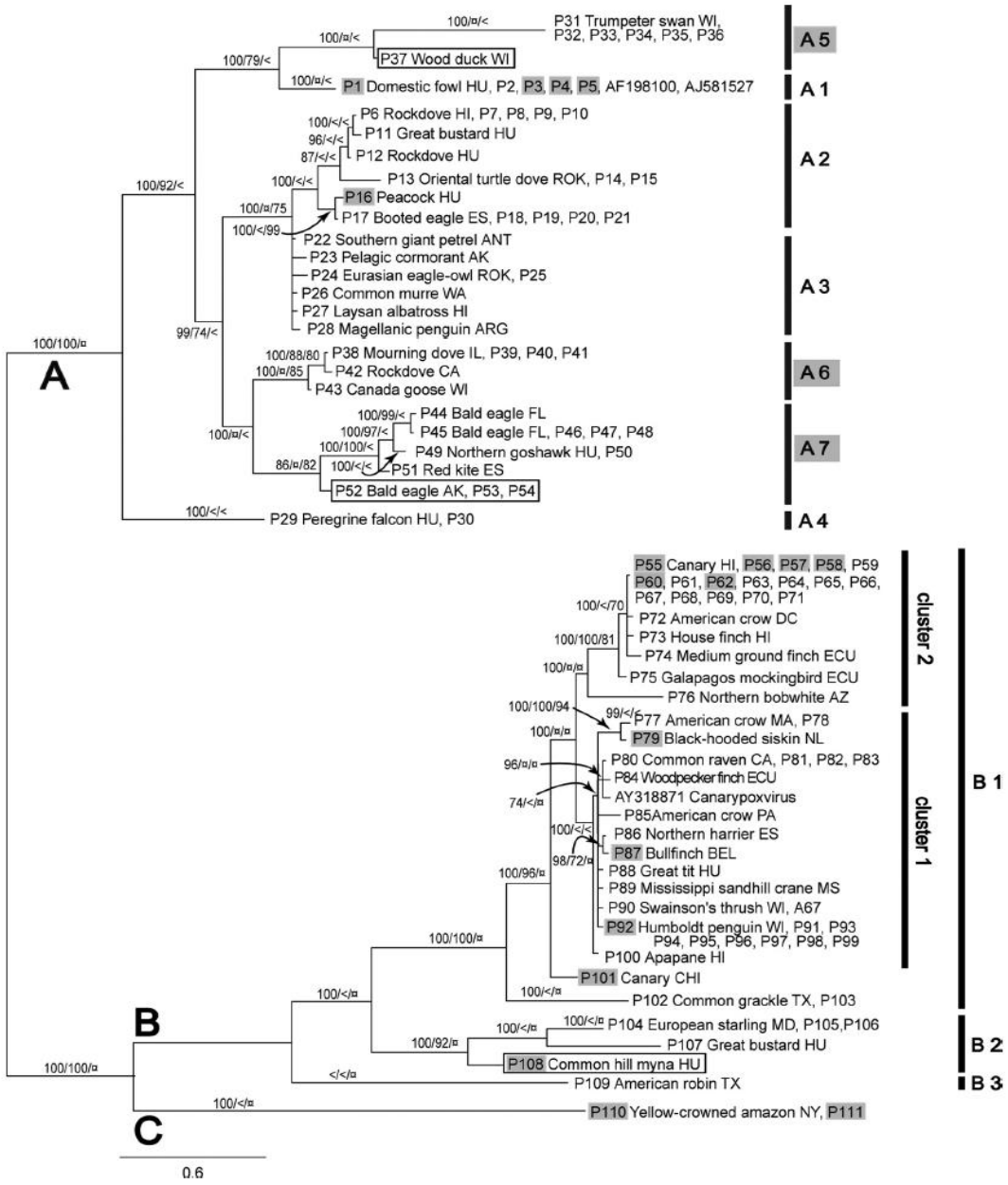

Figure 8. Bayesian phylogeny of concatenated DNA sequences from genes encoding 4b core and DNA polymerase proteins of APVs. Posterior probability values of the Bayesian trees (1,000 replicates) and neighbor-joining and maximum likelihood bootstrap values (1,000 replicates) of > 70 are indicated. Symbols: <, lower than 70; ¤, branch does not exist with that method. APV clades A to C, subclades, and clusters are labeled according to the nomenclature of Jarmin *et al*. (2006). Novel subgroups described by Gyuranecz *et al*. (2013) are highlighted by

gray. Isolate origins are given either as U.S. state abbreviations or using the following location codes: Antarctica (ANT), Argentina (ARG), Belgium (BEL), Chile (CHI), Ecuador (ECU), Germany (GER), Hungary (HU), Italy (ITA), Netherlands (NL), Norway (NOR), Portugal (POR), Spain (ES), South Korea (ROK), United Arab Emirates (UAE), and United Kingdom (UK). APVs which were isolated from captive birds (aviaries, zoos, etc.) are highlighted by gray, isolates containing potential recombinations are set in a box. The scale represents the number of substitutions per site. This Figure was adapted from Gyuranecz *et al*. (2013).

2.14 Characterization of APVs

Although before this study no single Tanzanian AVP isolate had been characterized, APV isolates from several countries have been characterized based on their antigenic, genetic and biological properties as described below:

In the Galápagos islands, Thiel *et al*. (2005) characterized APV isolates from chickens and wild birds [warblers (*Dendroica petechia*), small ground finches (*Geospiza fuliginosa*), medium ground finches (*Geospiza fortis*), cactus finches (*Geospiza scandens*), and Mockingbirds (*Nesomimus parvulus*)] by using PCR combined with restriction enzyme analysis (REA), nucleotide sequence analysis and phylogenetic analysis. Their report indicated that the sequences of the CAX and CA3-2 amplicons from five virus isolates from chickens from the Galápagos Islands and one from the United States were identical to the published sequence for FWPV at both loci. Based on this finding they concluded that chickens in Galápagos were

infected with an APV that is very similar, if not identical, to the strain that infects poultry in the United States. In contrast, the sequences of several CA3-2 amplicons from the passerine birds indicated different viruses; however, each of them was very similar to CNPV. One of these amplicons, Gal1 (sequenced for 10 strains), contained restriction sites for *Spe*I and *Age*I, and the other, Gal2 (sequenced for five strains) did not. The Gal1 strain was more similar to CNPV than was Gal2 and was also the more prevalent of the two strains, specifically in the finches. Evidence of APV infection was restricted to chickens at Santa Cruz that were infected with FWPV and the passerine birds that were infected with the two variants of CNPV.

In Norway, Weli *et al*. (2004) characterized APV isolates from two wild birds, a Norwegian wild sparrow (*Passer domesticus*) and a wood pigeon (*Palumbus palumbus*), as well as a FWPV (vaccine strain) by using REA. Comparison of the restriction fragments length polymorphism (RFLP) profiles generated by *Bam*HI digestion of the genomes revealed that Norwegian sparrowpox virus (SRPV) and wood PGPV are genetically different from each other and from FWPV. As far as biological characterization is concerned, pathogenicity study revealed that chickens infected with PGPV had higher antibody titers and more extensive lesions compared to those infected with SRPV and FWPV (vaccine strain) isolates. These findings suggested that the PGPV was more immunogenic and more virulent in chickens as compared to isolates of FWPV (vaccine strain) and SRPV. However, it was not clear whether the PGPV would cause severe loss of chickens in case of an outbreak since none of the PGPV-infected chickens died during the study.

In the Czech Republic, Kulich *et al*. (2008) characterized APV isolates from wild birds known as blackcaps (*Sylvia atricapilla*) by using PCR combined with nucleotide sequence analysis and phylogenetic analysis. Sequence analysis revealed that the analyzed isolates had the same sequences, suggesting that the analyzed viruses could belong to the same strain. Results of both nucleotide sequence analysis and phylogenetic analysis showed that the analyzed viruses were more closely related to CNPV than to FWPV. This finding was consistent with the fact that blackcaps belong to the same order as canaries (order *Passeriformes*), contrary to domestic chickens and turkeys (FWPV hosts) that belong to order *Galliformes*.

In the United Arabs Emirates, Lierz *et al*. (2007) characterized APV isolates from a collection of captive stone curlews (*Burhinus oedicnemus*) by using PCR combined with phylogenetic analysis. The sequence of the analyzed isolate clustered in the APV clade together with APV isolates from sparrows and canaries. This implied that the APV isolated from the captive stone curlews were phylogenetically closely related to CNPV.

In Croatia, Prukner-Radovčić *et al*. (2006) characterized APV isolates from chickens, a turkey and a pigeon by using PCR combined with REA. REA of PCR products carried out using *Eco*RV revealed the same cleavage patterns for APV isolates from all birds. Digestion with *Nla*III revealed identical cleavage patterns for APV isolates from chickens and a turkey and a different one for an APV isolate from a pigeon. Furthermore, by using multiplex PCR these authors demonstrated the integration of REV *env* gene and REV LTR in the genomes of APVs from chickens and a turkey.

In the United States, Shivaprasad *et al.* (2002) characterized APV isolates from ostriches that were kept in premises where turkeys which had shown clinical signs of poxvirus infection had been raised earlier. The isolates were characterized based on western blotting of virus antigen, RFLP of genomic DNA, pathogenesis, and cross-protection studies in chickens. Antigenic and genetic studies revealed no significant difference between the APV isolated from ostriches (OSPV) and FWPV. In addition to that, susceptible chickens immunized with the OSPV were protected when challenged with a virulent strain of FWPV. These findings indicated that the APV isolated from the ostriches had similar antigenic, genetic and biological properties to FWPV. Furthermore, Garcia *et al.* (2003) characterized field isolates of FWPV by using PCR combined with sequencing. Their findings revealed integration of nucleotide sequence of REV LTR in the genome of FWPV.

In Italy, Rampin *et al.* (2007) characterized APV isolates from common buzzards (*Buteo buteo*) by using PCR combined with sequence analysis and phylogenetic analysis. Molecular characterization based on the P4b gene indicated a 100% homology of the isolated APV with APVs belonging to subclade A2. However, analysis of *fpv*139 locus (corresponding to VACV H2R [TM]) did not reveal similarities of the isolate with other APVs.

In Hungary, Palade *et al.* (2008) characterized three APV isolates from great tits (*Parus major*) by using PCR combined with sequence analysis and phylogenetic analysis. Nucleotide sequence analysis of a 428 bp fragment of the P4b gene revealed that two Hungarian isolates (PM9 HUN and PM33 HUN) showed 100%

identity to each other and to two great tit poxvirus strains isolated in Norway in 1973 (GTPVA256 and GTPVA311). The third Hungarian isolate (PM34 HUN) was more closely related to a different Norwegian isolate (GTPVA310) than to the other Hungarian isolates. These Norwegian isolates belong to subclade B1 (Rampin *et al*., 2007). Nucleotide sequence analysis of a shorter fragment of the P4b gene (227 bp) revealed 100% identity between the Hungarian isolates, the Norwegian isolates and a great tit poxvirus strain detected in Austria in 2007. Phylogenetic analysis revealed the sequence of the amplicon to be clustered in the APV clade together with APV isolates from stone curlew (accession number AY530310) and canary (accession number AY318871), implying that the APV isolated from the great tits were phylogenetically more closely related to CNPV than to FWPV.

In Japan, Terasaki *et al*. (2010) characterized an APV isolate from Flamingo (*Phoenicopterus roseus*) by using PCR combined with sequence analysis and phylogenetic analysis. Sequence analysis revealed that the P4b gene sequence of an APV isolate from flamingo was closely related to those of APV isolates from pigeons, PGPVB7 (accession number AY453177) and PGPV950 (accession number AM050386), which belong to subclade B2 (Rampin *et al*., 2007; Carulei *et al*., 2009). This finding was reinforced by phylogenetic analysis.

In Great Britain, Lawson *et al*. (2012) characterized APV isolates from 20 great tits using PCR combined with sequence analysis and phylogenetic analysis. A single identical nucleotide sequence was obtained from all 20 great tit samples following amplification of the 4b core protein gene (accession number JQ067665). This

sequence was identical to the 4b core protein amplicon generated from great tit pox lesions in Norway (accession numbers AY453174 and AY453175), Austria (accession number DQ857759), Hungary (accession numbers EF634350 and EF634349) and the Czech Republic (accession numbers FJ863096 and FJ863095). This sequence corresponds to Canarypoxvirus subclade B1.

In West Indies, Arathy *et al*. (2010) characterized a FWPV isolate from Grenada using PCR combined with sequence analysis and phylogenetic analysis (based on the P4b gene), as well as PCR and sequence analysis (based on the thymidine kinase [TK] gene). Sequence analysis revealed 99–100% identity with other reported FWPVs P4b gene, and 100% nucleotide identity to the previously reported FWPV TK gene. Phylogenetic analysis indicated that the isolate belongs to clade A subclade A1. Further analysis revealed integration of REV *env* gene and REV LTR in genome of the FWPV isolate.

In Egypt, Abdallah and Hassanin (2013) characterized APV isolates from different species of domestic birds (i.e chickens, a turkey and a pigeon) using PCR combined with sequence analysis and phylogenetic analysis. All the studied isolates were characterized as members of clade A based on the amplicon length of *fpv140* gene locus. The phylogenetic analysis of the *fpv167* (P4b) gene clustered all four isolates from chickens and an isolate from a turkey in subclade A1. An isolate from a pigeon clustered in subclade A2 and showed 100 % nucleic acid identity with the Indian wood pigeon isolate. When the *fpv140* gene was used for the phylogenetic analysis, the isolate from the pigeon clustered within subclade A4 with the other PGPVs.

In Australia (Hertig *et al*., 1997), Israel (Davidson *et al*, 2008) and India (Biswas *et al*., 2011); characterization of field isolates of FWPV by using PCR combined with sequencing revealed integration of nucleotide sequence of near-full length REV provirus in the genome of the analyzed isolates.

According to Tripathy (2005) APVs isolated from Hawaiian crows (*Corvus hawaiiansis*), Hawaiian geese (*Branta sandvicensis*), Palila (*Loxiodes bailleui*), and Apapane species (*Himatione sanguinea*) are different from each other as well as from FWPV. Similarly, a poxvirus isolated from an Andean condor (*Vultur gryphus*) at the San Diego Zoo is antigenically, genetically, and biologically different from FWPV. Furthermore, Tripathy (2005) pointed out that poxviruses isolated from psittacines seem to be antigenically different from poxviruses of other avian species. Genomic profiles of CNPV, mynahpox virus (MNPV) and quailpox virus (QUPV) show marked differences from FWPV, when their DNA is compared after restriction endonuclease digestion. QUPV shows marked antigenic differences from FWPV and, although some cross-reacting antigens are present, provides limited or no cross-protection against FWPV.

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CHAPTER THREE

3.0 GENERAL METHODOLOGY

Different methodologies were used in order to achieve the objectives of this study. The methodologies used were as described below under each specific objective:

3.1 Determination of genetic characteristics of APV isolates from chickens and domestic pigeons in Tanzania

To achieve this objective, a cross sectional study design was used; where samples of proliferative cutaneous nodular lesions collected from featherless or poorly feathered parts of chickens and pigeons, between November 2011 and May 2014, were examined for the presence of FWPV and PGPV, respectively. The samples were collected in various geographical locations and regions of Tanzania. Samples from chickens were collected in Kigoma, Tabora, Mwanza, Mara, Singida, Dodoma, Lindi, Arusha, Kilimanjaro, Morogoro, Pwani, Iringa, Dar es Salaam, and Mbeya regions; and samples from pigeons were collected in Morogoro, Pwani and Dar es Salaam regions.

Nonprobability (purposive) sampling technique (Teddlie and Yu, 2007; Palinkas *et al*., 2013) was used; chickens and pigeons that had proliferative cutaneous nodular lesions in featherless or poorly feathered parts of the body, which are suggestive of the cutaneous form of pox (Kulich *et al*., 2008; Arathy *et al*., 2010; Atkinson *et al*., 2010), were selected. The samples were collected from the selected chickens (n = 154) and pigeons (n = 17) and stored in the deep freezer at -20ºC until required. Two

samples of imported lyophilized fowl pox vaccines (one representing a batch of FWPV vaccine manufactured in Spain, and the other sample representing a different batch of FWPV vaccine from a different manufacturer based in Israel) were purchased from some commercial sources in Morogoro Municipality, and stored at SUA in the refrigerator at 4ºC until required.

Laboratory work was conducted at the Faculty of Veterinary Medicine (FVM), Sokoine University of Agriculture (SUA), Morogoro, Tanzania. The FWPV and PGPV were isolated in specific-pathogen free (SPF) embryonated chicken eggs (ECEs) by using procedures described previously by Wambura and Godfrey (2010). DNA samples were extracted from samples of chorioallantoic membranes (CAMs) containing virus cultures after the 4th passage by using ZR Tissue and Insect DNA MiniPrep^{TM} Kit Catalog Number D6016 (Zymo Research Corp., USA) according to the manufacturer's instructions. Thereafter the DNA samples were subjected to conventional polymerase chain reaction (PCR) using specific oligonucleotide primers for amplification of the P4b gene, followed by gel electrophoresis of a sample (5 µl) of each PCR products in 1X Tris-Borate-EDTA (TBE) buffer for 45 minutes at 100V in order to identify the PCR products containing APV-specific DNA. Samples of PCR products found to have APV-specific DNA were purified using EXOSAP Amplicon Purification Kit (Affymetrix, USA) according to the manufacturer's instructions.

The sequencing reaction was cleaned using ZR-96 DNA Sequencing Clean-up Kit^{TM} Catalog Numbers D4052 and D4053 (Zymo Research Corp., USA) according to the

manufacturer's instructions. Thereafter, the purified PCR products were sequenced using ABI V3.1 Big Dye Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions, and the sequences of each PCR product were assembled using CLC Main Workbench version 6.7.1 software to get a consensus sequence.

To establish sequence homology, the sequences were analyzed using BLAST algorithm (Palade *et al*., 2008; Biswas *et al*., 2011); and similarities among the Tanzanian FWPV isolates were investigated using BLAST two sequences programme which gives alignment of two sequences of interest (Biswas *et al*., 2011).

Prior to phylogenetic analysis the nucleotide sequences reported in this study were aligned with reference APV sequences from the GenBank using Clustal Omega programme (Sievers *et al*., 2011). Thereafter phylogenetic analysis was conducted using MEGA version 5.2.2 software (Tamura *et al*., 2011) so as to establish possible phylogenetic relationships and grouping of the Tanzanian strains of FWPV and PGPV.

Finally, the findings from this work were published in papers I and III which are presented in chapters 4 and 6, respectively, of this thesis.

3.2 Determination of integration of genomic fragments of REV in the Tanzanian field strains of FWPV, and the imported commercial fowl pox vaccines currently used in the country

To achieve this objective, 55 samples of FWPV isolates from naturally infected chickens and two isolates of FWPV from samples of the imported commercial FWPV vaccines were examined for integration of REV envelope (*env*) gene and REV 5'long terminal repeat (5'LTR) using PCR and sequencing techniques.

The sampling units were samples of genomic DNA extracted previously and confirmed to contain FWPV-specific DNA (Masola *et al.*, 2014). Nonprobability (purposive) sampling technique (Teddlie and Yu, 2007; Palinkas *et al*., 2013) was used where 55 samples of genomic DNA (extracted from samples of cutaneous nodular lesions) confirmed to have FWPV-specific DNA, and two samples of genomic DNA (extracted from the commercial FWPV vaccines) also confirmed to have FWPV-specific DNA (Masola *et al.*, 2014) were examined for integration of REV *env* gene and REV 5'LTR using PCR and sequencing techniques.

The study involved amplification of FWPV P4b gene, REV *env* gene and REV 5'LTR by using conventional PCR; agarose gel electrophoresis of PCR products, where a sample (5 µl) of each PCR product was run in the gel in 1X TBE buffer for 45 minutes at 100V; purification of PCR products found to contain REV-specific DNA by using EXOSAP Amplicon Purification Kit (Affymetrix, USA), according to the manufacturer's instructions; sequencing of the purified PCR products using ABI V3.1 Big Dye Sequencing Kit (Applied Biosystems, USA), according to the

manufacturer's instructions; and analysis of the sequence data by using BLAST algorithm (Palade *et al*., 2008; Biswas *et al*., 2011). The findings from this work were published in paper II presented in chapter 5 of this thesis.

3.3 Determination of virulence characteristics of the Tanzanian strains of FWPV and PGPV in chickens.

To achieve this objective, an experimental study design and probability sampling technique were used. Virulence characteristics of Tanzania strains of FWPV and PGPV in chickens were evaluated both *in ovo* and *in vivo*.

In ovo evaluation was conducted in the virology laboratory, at the Faculty of Veterinary Medicine (FVM), SUA, Tanzania. Twenty 10-day-old embryonated chicken eggs were used, the eggs were randomly grouped into four groups (I, II, III, and IV) of 5 eggs each. Each egg in group I, II, and III was inoculated with 0.1 ml of 10^6 EID_{50}/0.1 ml of REV-free FWPV inoculum; REV-integrated FWPV inoculum; and PGPV inoculum; respectively, through CAMs. Group IV eggs served as control, each egg was injected 0.1 ml of PBS through CAMs. All eggs were incubated at 37ºC for 7 days, thereafter CAMs and chicken embryos were examined for gross pathological changes.

In vivo evaluation was carried out at the Animal Research Unit of the FVM, SUA, Tanzania. One hundred and forty chicks were used, at 26 days of age the chicks were randomly grouped into four groups (I, II, III, and IV) of 35 chicks each. Each chicken in group I, II, and III was inoculated with 0.1 ml of 10^6 EID_{50}/0.1 ml of

REV-free FWPV inoculum; REV-integrated FWPV inoculum; and PGPV inoculum; respectively, through the subcutaneous route at the ventral side of the neck. Chickens in group IV served as control, each chicken was injected 0.1 ml of PBS subcutaneously. Blood samples were collected from wing vein of each chicken before inoculation and at 4-day intervals post-inoculatio (pi) up to day 28 pi.

From day zero to day 28 pi, the chickens were examined for development of clinical signs and deaths; followed by necropsy of dead chickens using standard procedures described by Nyaga *et al*. (2014) and examination of samples of cutaneous nodular lesions from chickens inoculated with REV-free FWPV or REV-integrated FWPV for the presence of FWPV by using both virus isolation, according to procedures described by Wambura and Godfrey (2010), and PCR techniques. The findings from this work were published in paper IV presented in chapter 7 of this thesis.

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CHAPTER FOUR

PAPER I: Isolation and Molecular Biological Characterization of Fowlpox Virus from Specimen of Cutaneous Nodular Lesions from Chickens in Several Regions of Tanzania[a]

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ISOLATION AND MOLECULAR BIOLOGICAL CHARACTERIZATION OF FOWLPOX VIRUS FROM SPECIMEN OF CUTANEOUS NODULAR LESIONS FROM CHICKENS IN SEVERAL REGIONS OF TANZANIA

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ABSTRACT

The aim of this study was to investigate molecular and evolutionary characteristics of genes of fowlpox virus (FWPV) isolates from chickens in Tanzania. Samples of cutaneous nodular lesions were collected from featherless parts of chickens (n = 154) suspected to have fowl pox in 14 regions of Tanzania followed by virus isolation, DNA extraction, polymerase chain reaction (PCR) amplification of the P4b gene, gel electrophoresis of PCR products, purification of PCR products, sequencing of purified PCR products and finally analysis of sequence data using standard procedures. The disease was confirmed in 12 regions, out of 154 investigated samples 66 (42.86%) were found to contain FWPV, indicating that the 66 chickens from which the samples were collected had fowl pox as a result FWPV infection. Sequence analysis revealed that the Tanzanian FWPV isolates were 99.65 – 100% identical to each other and 99 – 100% identical to several published sequences of FWPV isolates from various countries in different continents of the world, including Europe and Asia. Phylogenetic analysis revealed that all Tanzanian isolates belong to clade A, subclade A1. Based on the findings of this study it is concluded that currently fowl pox is prevalent in several regions of Tanzania, caused by FWPVs which are genetically and phylogenetically closely related. However,

these findings do not rule out the possibility of existence of genetic divergence among FWPVs currently prevalent in Tanzania. In order to rule out or detect genetic divergence (if any) among FWPVs currently prevalent in the country, other studies aimed at investigating molecular and evolutionary characteristics of genes in other genomic regions are highly recommended.

Keywords: Fowlpox virus, fowl pox, cutaneous nodular lesions, virus isolation, PCR, sequencing, Tanzania.

1. INTRODUCTION

Fowlpox virus (FWPV) is a DNA virus that belongs to the family *Poxviridae*, subfamily *Chordopoxvirinae* and genus *Avipoxvirus* (Lüschow *et al*., 2004). The virus causes fowl pox in chickens. Clinically, the disease presents itself in three forms; the cutaneous, diphtheritic and systemic form (Kulich *et al*., 2008; Atkinson *et al*., 2010). The cutaneous form is characterized by formation of proliferative lesions ranging from papules to nodules in the unfeathered parts of the body, which eventually hardens to form scabs. The diphtheritic form is characterized by formation of fibrous necrotic proliferative lesions in the mucous membrane of the digestive and upper respiratory tracts (Thiel *et al*., 2005; Weli and Tryland, 2011). In the systemic form various body systems and tissues of an infected bird are involved (Atkinson *et al*., 2010).

Fowl pox is an important disease because it causes great economic losses to farmers due to mortality and decreased productivity of birds. The mortality rate, which is higher in birds with the diphtheritic form, may approach 50% in severe outbreaks, especially when accompanied by secondary bacterial and/or fungal infection(s).

Economic losses are largely due to transient decrease in egg production in laying birds and decreased growth in young birds (Quinn *et al.*, 2002).

Provisionally, fowl pox is suspected if papules or nodular lesions are evident on chickens during clinical or postmortem examination (Weli and Tryland, 2011). The definitive diagnosis is attained if the presence of FWPV in infected tissue samples is confirmed by using one or a combination of two or more of the following techniques: isolation of FWPV in cell culture of avian origin, or the chorioallantoic membrane (CAM) of embryonated chicken eggs (ECEs) (Tripathy, 2005; Carulei *et al*., 2009; Farias *et al*., 2010); demonstration of the characteristic viral inclusion bodies by examination of affected tissue specimen stained with Haematoxylin and Eosin under a light microscope, or by fluorescent antibody and immunohistochemical techniques (Tripathy, 2005); demonstration of viral particles with typical morphology of FWPV by negative staining electron microscopy (Weli *et al*., 2004; Tripathy, 2005; Kulich *et al*., 2008; Weli and Tryland, 2011) or by other techniques of transmission electron microscopy (Weli *et al*., 2004; Prukner-Radovčić *et al*., 2006; Kulich *et al*., 2008); demonstration of the presence of DNA of FWPV in the infected tissue specimen by using polymerase chain reaction (PCR) and identification of FWPV based on the sequence blast results (Palade *et al*., 2008). Fowl pox can also be confirmed by serological assays (Weli and Tryland, 2011).

Prior to this work no study on fowl pox and FWPV had been conducted in Tanzania, consequently no data on fowl pox and strain(s) of FWPV circulating in the country were available. The aim of this study was to investigate molecular and evolutionary characteristics of genes of FWPV isolates from chickens in Tanzania.

2. MATERIALS AND METHODS

2.1 Study Location

Field work (collection of samples of cutaneous nodular lesions from chickens suspected to have fowl pox) was conducted in various geographical locations in 14 regions of Tanzania (Fig. 1, Table 1). Laboratory work was conducted at the Faculty of Veterinary Medicine (FVM), Sokoine University of Agriculture (SUA), Morogoro, Tanzania. The country is located in Eastern Africa between latitudes 1°-12° South and longitudes 29°-41° East (Kireri, 2012; Adimola *et al.*, 2013).

2.2 Samples Collection and Storage

Between November 2011 and May 2013 samples of proliferative cutaneous nodular lesions were collected from chickens (n = 154) suspected to have fowl pox. Pieces of cutaneous nodular lesions collected from the same cadaver or live bird were put in one plastic vial and were considered as one sample. Each sample was labeled and stored in a deep freezer at -20°C at a nearby Veterinary Investigation Centre (VIC), Local Government Authority or Regional Secretariat office. Thereafter all samples were transported (in cool boxes) to the FVM, SUA, Morogoro; where they were also stored at -20°C until required.

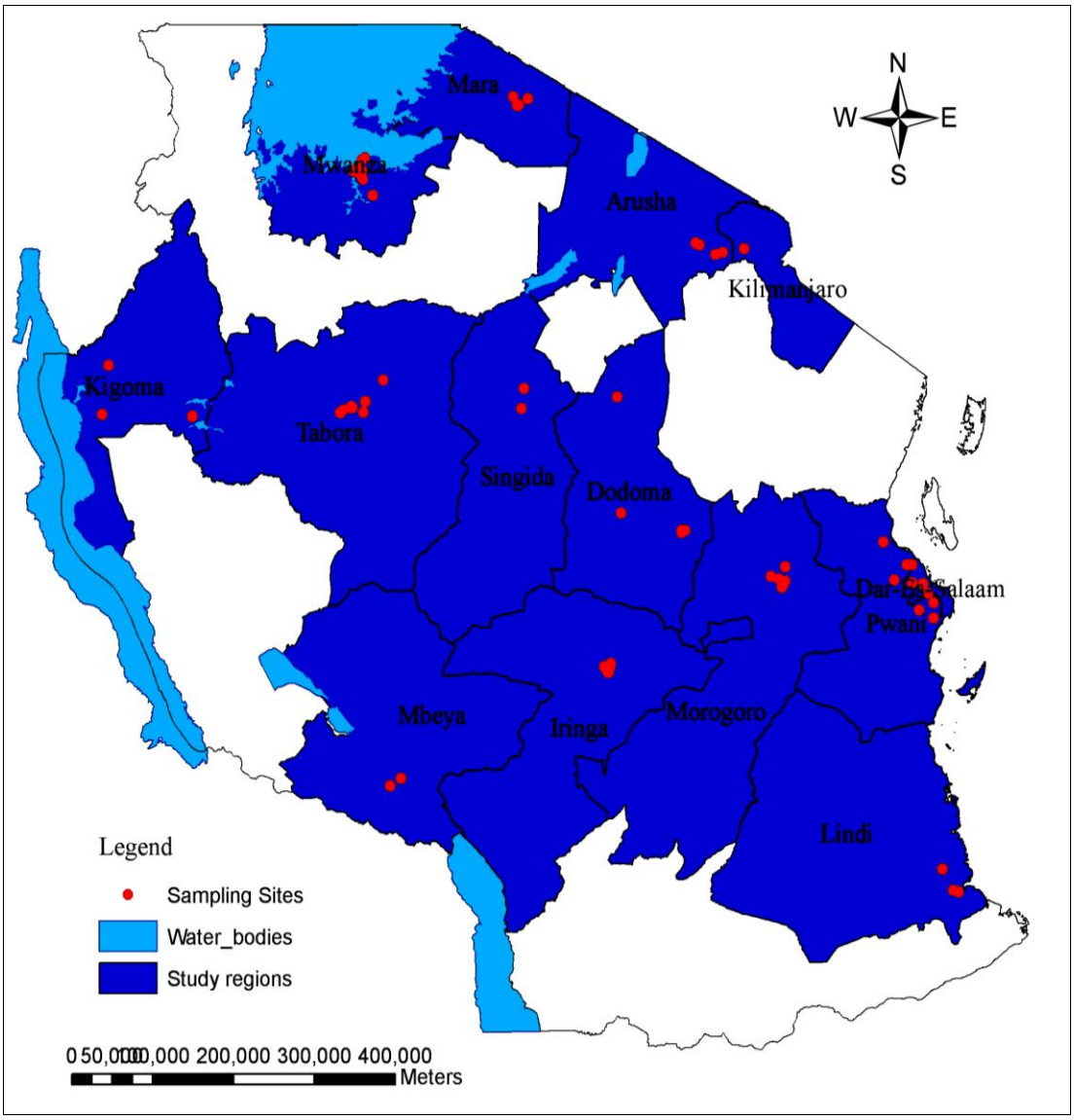

Figure 1. Map of Tanzania showing study regions and sampling sites

2.3 Commercial Fowl Pox Vaccines

Two samples of imported lyophilized fowl pox vaccines commercially available in Tanzania were purchased from some commercial sources in Morogoro Municipality, and stored at SUA in a refrigerator at 4°C until required.

Table 1. Details of samples of cutaneous nodular lesions used in this study

Source of samples		Number of samples collected
Geographical location	**Region**	
Eastern Tanzania	Dar es Salaam	11
Eastern Tanzania	Morogoro	20
Eastern Tanzania	Pwani	2
Central Tanzania	Singida	13
Central Tanzania	Dodoma	21
Western Tanzania	Kigoma	7
Western Tanzania	Tabora	13
North-western Tanzania	Mwanza	20
Northern Tanzania	Mara	5
Northern Tanzania	Arusha	9
North-eastern Tanzania	Kilimanjaro	1
Southern Tanzania	Mbeya	15
Southern Tanzania	Iringa	12
South-eastern Tanzania	Lindi	5
Total		**154**

2.4 Virus Isolation

Inoculums for CAM were prepared from 154 samples of cutaneous nodular lesions, followed by inoculation of each inoculum in 10 day-old ECEs through CAMs as described previously (Wambura and Godfrey, 2010). Briefly, each sample from a suspected chicken was finely ground in a pestle and mortar with sterile sand and suspended in phosphate-buffered saline (PBS). Thereafter the suspension was centrifuged (500g for 10 minutes at room temperature) and the supernatant was collected. The supernatant was filtered using a 0.45 µm membrane filter in order to prepare inoculum for CAM. Then 0.1 ml of inoculums was inoculated in 10 day-old

ECEs through CAMs. Similarly, 0.1 ml of each sample of the FWPV vaccine was inoculated in 10 day-old ECEs through CAMs (these served as positive control). Nothing was inoculated in the negative control ECEs. The eggs were incubated at 37℃ for 5-7 days, thereafter examined for the presence of nodular lesions on CAMs or generalized thickening and haemorrhage of the CAMs. The inoculums were passaged in the CAMs four times.

2.5 DNA Extraction

DNA samples were extracted from samples of CAM containing virus cultures after the 4th passage by using ZR Tissue and Insect DNA MiniPrep^{TM} Kit Catalog Number D6016 (Zymo Research Corp., USA) according to the manufacturer's instructions.

2.6 PCR for Amplification of the P4b Gene

Conventional PCR was conducted in Takara PCR Thermal Cycler (Takara Bio Inc., Japan) using a set of primers indicated below (Table 2). Each amplification reaction consisted of 12.5 µl of master mix, 1 µl of each primer, 5.5 µl of nuclease-free water, and 5 µl of template DNA; making a total volume of 25 µl . After an initial heat denaturation at 94℃ for 2 minutes; each mixture was subjected to 40 cycles of heat denaturation at 94℃ for 1 minute, primer annealing at 60℃ for 1 minute, and DNA extension at 72℃ for 1 minute. After the 40th cycle a final extension step was performed at 72℃ for 2 minutes.

Table 2. Primers used in this study

Primer	Sequence	Expected fragment size	References
Forward primer (P1)	5'-CAGCAGGTGCTAAACAACAA-3'	578 bp	Lüschow *et al*. (2004); Jarmin *et al*. (2006); Lierz *et al*. (2007); Rampin *et al*. (2007); Palade *et al*. (2008); Manarolla *et al*. 2010); Terasaki *et al*. (2010) and Biswas *et al*. (2011).
Reverse primer (P2)	5'-CGGTAGCTTAACGCCGAATA-3'		

2.7 Gel Electrophoresis

Five microlitres (5 µl) of each PCR product was loaded in a 1.5% agarose gel with ethidium bromide (10 mg/ml). Electrophoresis was conducted in 1X Tris-Borate-EDTA (TBE) buffer for 45 minutes at 100V. A DNA ladder with 100-bp increments was used as a molecular weight marker. Thereafter, gels were visualized under UV light using UVI tec transilluminator and photographed using a digital camera.

2.8 Purification of PCR Products, Sequencing and Analysis of Sequence Data

Samples of PCR products were purified using EXOSAP Amplicon Purification Kit (Affymetrix, USA) according to the manufacturer's instructions. The sequencing reaction was cleaned using ZR-96 DNA Sequencing Clean-up Kit™ Catalog Numbers D4052 and D4053 (Zymo Research Corp., USA) according to the manufacturer's instructions. Thereafter purified samples were injected in the ABI 3500XL with P0P7 and a 50 cm array (Applied Biosystems, USA). The primers used for PCR were used for sequencing. The samples were sequenced using ABI V3.1 Big Dye Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Thereafter the sequences of each PCR product were assembled using CLC Main Workbench version 6.7.1 software to get a consensus sequence.

After manual editing the sequence homology was investigated using BLAST algorithm (Palade *et al*., 2008; Biswas *et al*., 2011). Similarities among the Tanzanian FWPV isolates were investigated using BLAST two sequences programme which gives alignment of two sequences of interest (Biswas *et al*.,

2011). Prior to phylogenetic analysis the nucleotide sequences reported in this study (Table 3) were aligned with reference avipoxvirus (APV) sequences from the GenBank (Table 4) using Clustal Omega programme (Sievers *et al.*, 2011). Thereafter possible phylogenetic relationships and grouping of the APVs were investigated using procedures described previously (Manarolla *et al*., 2010) except that in the present study MEGA version 5.2.2 (Tamura *et al.*, 2011) was used instead of MEGA version 3.1, also no pairwise genetic and amino acid distances were calculated because none of the analyzed isolates displayed greater variability in the phylogenetic tree.

Table 3. Details of the Tanzanian FWPV isolates analyzed in this study

Source of isolates		Virus name	Acronym	Host	Nature	GenBank accession numbers
Geographical location	**Region**					
Central Tanzania	Dodoma	Fowlpox TZ 3/DOM	FWPV3DOM	Chicken	Clinical isolate	KF722863
Eastern Tanzania	Morogoro	Fowlpox TZ 6/MOR	FWPV6MOR	Chicken	Clinical isolate	KF722864
Western Tanzania	Tabora	Fowlpox TZ 19/TBR	FWPV19TBR	Chicken	Clinical isolate	KF722865
Southern Tanzania	Mbeya	Fowlpox TZ 28/MBY	FWPV28MBY	Chicken	Clinical isolate	KF722866
Southern Tanzania	Iringa	Fowlpox TZ 41IRG (TPV1)	FWPV41IRG (TPV1)	Chicken	Clinical isolate	KF032407
North-western Tanzania	Mwanza	Fowlpox TZ 47/MWZ	FWPV47MWZ	Chicken	Clinical isolate	KF722860
Northern Tanzania	Arusha	Fowlpox TZ 60/ARS	FWPV60ARS	Chicken	Clinical isolate	KF722861
Eastern Tanzania	Pwani	Fowlpox TZ 63/CST	FWPV63CST	Chicken	Clinical isolate	KF722859
Western Tanzania	Tabora	Fowlpox TZ 65/TBR	FWPV65TBR	Chicken	Clinical isolate	KF722858

Table 4. Details of reference APV sequences obtained from the GenBank

Virus name	Acronym	Host	P4b locus GenBank accession numbers
Fowlpox 174/4/04	FWPV174	Chicken	AM050377
Fowlpox Mild (Websters; Fort Dodge)	FWPVM	Chicken	AM050378
Turkeypox 2/11/66	TKPV66	Turkey	AM050387
Turkeypox 10/12/98	TKPV98	Turkey	AM050388
Albatrosspox 353/87	ABPV	Black-browed albatross	AM050392
Falconpox 1381/96	FLPV1381	Falcon	AM050376
Falconpox GB362-02	FLPV36202	Falcon	AY530306
Canarypox 1445/97/33	CNPV1445	Canary	AM050375
Canarypox (Duphar; Fort Dodge)	CNPVV	Canary	AM050384
Great titpox GTPV-A310	GTPVA310	Great tit	AY453173
Great titpox-A311	GTPVA311	Great tit	AY453174
Pigeonpox 950 24/3/77	PGPV950	Pigeon	AM050386
Pigeonpox B7	PGPVB7	Pigeon	AY453177
Starlingpox /27	SLPV	Starling	AM050391
Macawpox 1305/86	MCPV	Macaw	AM050382
Parrotpox 364/89	PRPV	Parrot	AM050383
Agapornispox APIII	AGPV	Agapornis	AY530311

Source: Manarolla *et al*. (2010).

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Virus isolation

Gross pathological changes on CAMs were first observed at the third passage, when two to three nodular lesions about 1 mm in diameter were observed on some CAMs without thickening of the CAMs. At the fourth passage marked proliferative nodular lesions were observed, the nodules had increased in number and size ranging from 1 to 2 mm in diameter, most of the nodules had coalesced to form large mass (Fig. 2). The lesions were demonstrated in CAMs inoculated with inoculums prepared from 66 samples of cutaneous nodular lesions from chickens in 12 regions (Table 5) and in all CAMs inoculated with inoculums prepared from samples of imported commercial fowl pox vaccines. None of these lesions were demonstrated in the CAMs of negative control ECEs, and CAMs of ECEs inoculated with inoculums prepared from the other 88 samples of cutaneous nodular lesions.

3.1.2 PCR and gel electrophoresis

DNA samples were isolated from samples of cutaneous nodular lesions collected from chickens suspected to have fowl pox, and from a sample of CAM with nodular lesions at the fourth passage after inoculation with inoculum prepared from imported commercial fowl pox vaccine currently used in Tanzania; followed by PCR for amplification of the P4b gene, and agarose gel electrophoresis. Positive results were indicated by migration of PCR products to approximately 578 bp, an expected size for the P4b gene amplicon for FWPV and other avipoxviruses (APVs) (Fig. 3). Out of 154 total samples tested 66 (42.86%) were positive (Table 5).

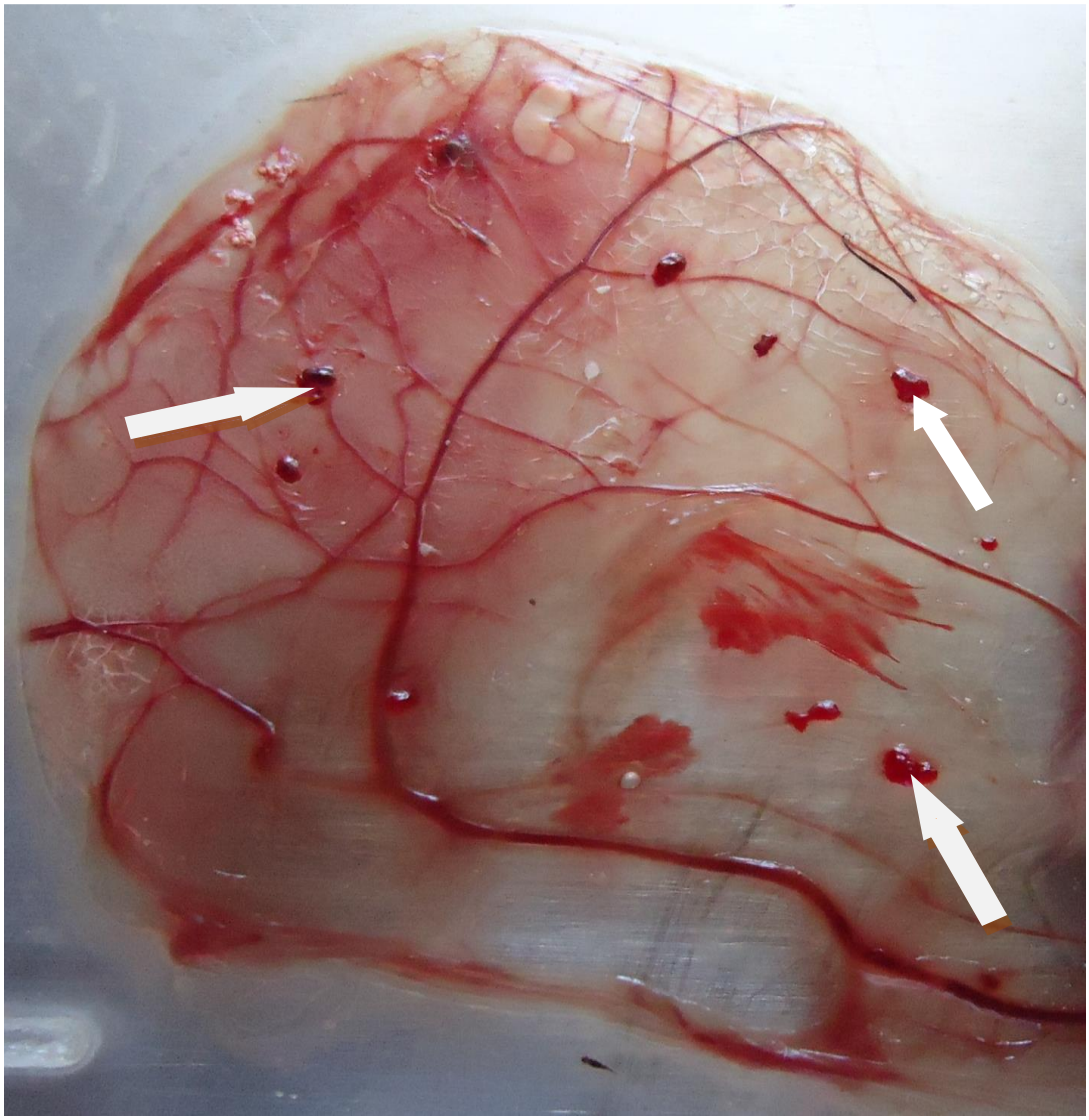

Figure 2. Chicken CAM during the fourth passage showing nodular lesions (arrows) characteristic of poxvirus infection.

Table 5. Results based on virus isolation and PCR

Source of samples		Number of samples of cutaneous nodular lesions analyzed	Number of positive samples (percentage positive)
Geographical location	**Region**		
Eastern Tanzania	Dar es Salaam	11	9 (81.82)
Eastern Tanzania	Morogoro	20	4 (20.00)
Eastern Tanzania	Pwani	2	1 (50.00)
Central Tanzania	Singida	13	4 (30.77)
Central Tanzania	Dodoma	21	10 (47.62)
Western Tanzania	Kigoma	7	3 (42.86)
Western Tanzania	Tabora	13	4 (30.77)
North-western Tanzania	Mwanza	20	8 (40.00)
Northern Tanzania	Mara	5	4 (80.00)
Northern Tanzania	Arusha	9	2 (22.22)
North-eastern Tanzania	Kilimanjaro	1	0 (0.00)
Southern Tanzania	Mbeya	15	14 (93.33)
Southern Tanzania	Iringa	12	3 (25.00)
South-eastern Tanzania	Lindi	5	0 (0.00)
Total		**154**	**66 (42.86)**

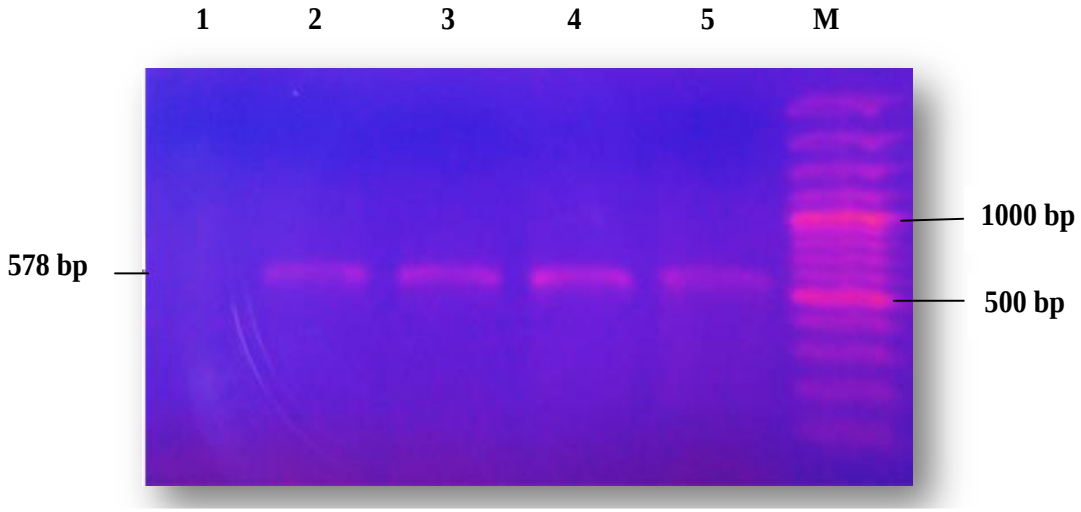

Figure 3. Agarose gel electrophoresis of PCR products of DNA extracted from samples of cutaneous nodular lesions collected from chickens suspected to have fowl pox (lanes 2, 3, and 4), and from a sample of CAM infected with inoculum prepared from imported commercial fowl pox vaccine currently used in Tanzania which served as a positive control (lane 5). The amplicons migrated to approximately 578 bp, which is an expected fragment size for the P4b gene of FWPV and other APVs. Lanes 1 and M are negative control and 100-bp molecular weight marker, respectively.

3.1.3 Sequencing and analysis of sequence data

In order to identify the virus, the obtained consensus nucleotide sequences were blasted in the GenBank. Each blasted sequence showed 99 – 100% identity to several published sequences of FWPV isolates (GenBank accession numbers AM050378, AM050379, AY453171, AY453172, AY530302, FR852586, GQ180201, GQ180212, GQ221269, GU108500, GU108501, GU108502, GU108503, GU108504, GU108505, GU108506, GU108507, GU108508,

GU108509, JQ665838, JX464819 and JX464820) from various countries in different continents of the world, including Europe and Asia.

Moreover, sequence analysis revealed that the Tanzanian FWPV isolates are 99.65 – 100% identical to each other. Phylogenetic analysis revealed that all Tanzanian isolates belong to clade A subclade A1 (Fig. 4). After analysis sequences of the Tanzanian FWPV isolates were deposited in the GenBank data base under accession numbers KF722858, KF722859, KF722860, KF722861, KF722862, KF722863, KF722864, KF722865 and KF722866.

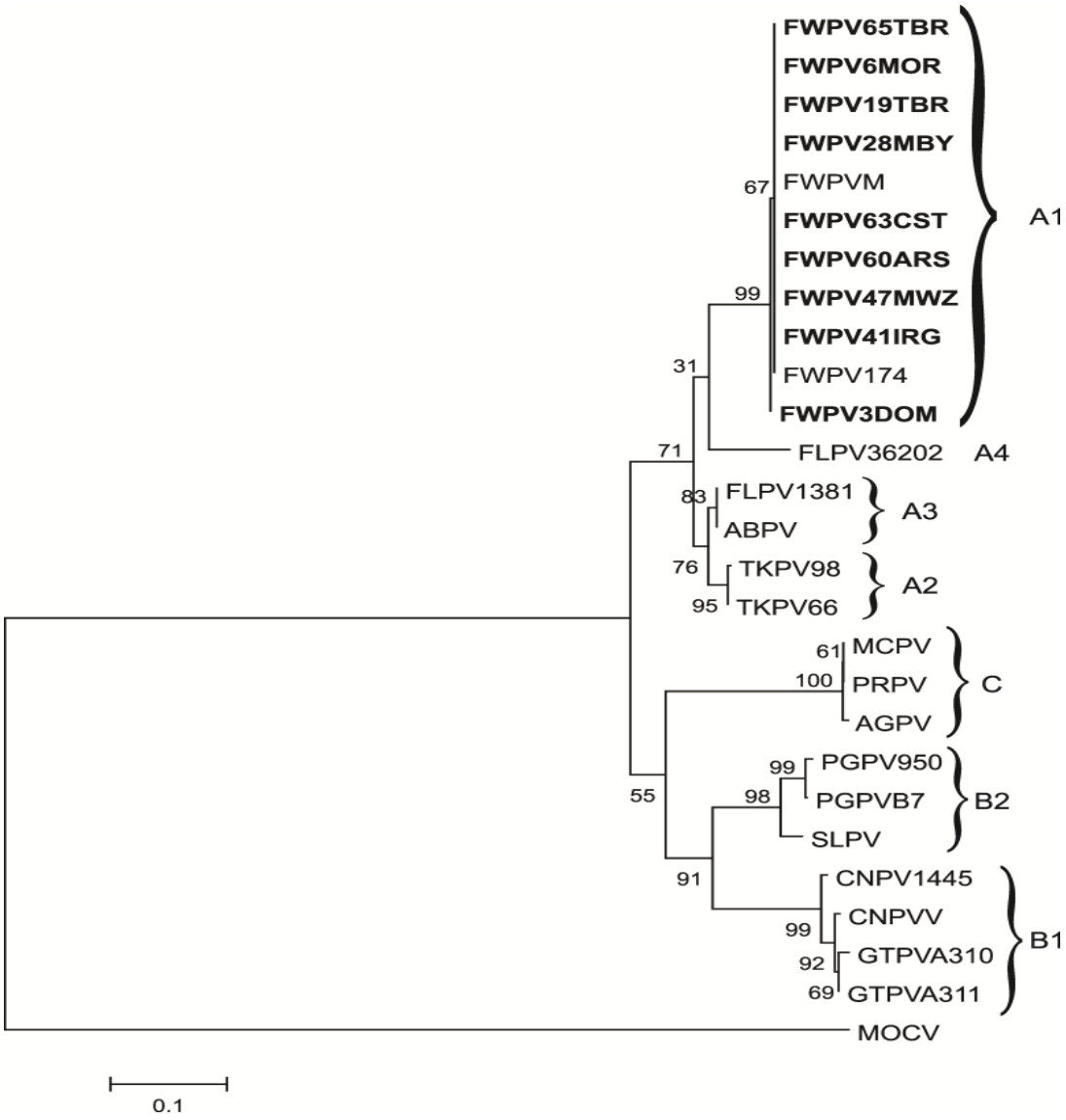

Figure 4. Phylogenetic tree of nucleotide sequences of the P4b gene of APVs and the Molluscum contagiosum virus (MOCV) orthologue sequence, rooted on MOCV, showing the phylogenetic relationship of FWPVs currently prevalent in Tanzania (bolded). The tree was obtained by the neighbour-joining method calculated with the Jukes and Cantor model. Bootstrap testing of phylogeny was performed with 1000 replications and values equal to or greater than 30 are indicated in the branches (as a percentage). The length of each bar indicates the amount of evolution along the horizontal branches as measured by substitution per site. APV subclades A1 to A4, B1, B2 and clade C are labeled.

3.2 Discussion

In this study 154 samples of cutaneous nodular lesions were analyzed to demonstrate the presence of FWPV in the samples. Out of 154 analyzed samples 66 (42.86%) were found to contain FWPV. This implies that the 66 chickens from which the samples were collected had fowl pox due to FWPV infection as revealed by virus isolation, PCR and sequence blast results. The findings of this study (Table 5) indicate that currently fowl pox is prevalent in various geographical locations and regions of Tanzania.

However, most of the analyzed samples [88 (53.14%)] were found to be FWPV-negative. This implies that the 88 chickens from which the samples were collected were not infected with FWPV. The proliferative cutaneous nodular lesions found on featherless parts of the chickens during samples collection could be attributed to other diseases such as papillomatosis (Literák *et al*., 2003) and/or mange (CFSPH, 2012; OIE, 2013) which have clinical signs similar to those of the cutaneous form of fowl pox.

Although results in Table 5 show that none of the analyzed samples from Kilimanjaro region in North-eastern Tanzania, and Lindi region in South-eastern Tanzania was confirmed to be FWPV-positive; this does not rule out fowl pox in these regions due to the fact that the analysis involved few samples of cutaneous nodular lesions from chicken(s) in Kilimanjaro (n = 1) and Lindi (n = 5) regions.

A close genetic relationship of the Tanzanian FWPVs to each other (99.65 – 100% identity) and to FWPV isolates of several other countries in the world (99 – 100% identity) demonstrated in this study shows how highly conserved the P4b gene is

(Jarmin *et al*., 2006; Manarolla *et al*., 2010). Phylogenetically all Tanzanian isolates belong to clade A subclade A1 (Fig. 4), this implies that based on sequences of P4b gene the FWPVs currently prevalent in Tanzania are phylogenetically closely related.

4. CONCLUSION

Based on the findings of this study it is concluded that currently fowl pox is prevalent in several regions and geographical locations of Tanzania, caused by FWPVs which are genetically and phylogenetically closely related. However, these findings do not rule out the possibility of existence of genetic divergence among FWPVs currently prevalent in Tanzania. In order to rule out or detect genetic divergence (if any) among FWPVs currently prevalent in the country, other studies aimed at investigating molecular and evolutionary characteristics of genes in other genomic regions are highly recommended.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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CHAPTER FIVE

PAPER II: Integration of Reticuloendotheliosis Virus in Most of Tanzanian Fowlpox Virus Isolates is not attributed to Imported Commercial Fowl Pox Vaccines[b]

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INTEGRATION OF RETICULOENDOTHELIOSIS VIRUS IN MOST OF TANZANIAN FOWLPOX VIRUS ISOLATES IS NOT ATTRIBUTED TO IMPORTED COMMERCIAL FOWLPOX VACCINES

S. N. Masola, A. Mzula, C. J. Kasanga and P. N. Wambura

ABSTRACT

The aim of this study was to investigate integration of reticuloendotheliosis virus (REV) in the Tanzanian fowlpox virus (FWPV) field isolates, and the imported commercial fowl pox vaccines currently used in the country. Fifty five samples of FWPV isolates from naturally infected chickens, and two isolates of FWPV from samples of the imported commercial fowl pox vaccines were analyzed for integration of REV envelope (*env*) gene and REV 5'long terminal repeat (5'LTR). The study involved polymerase chain reaction (PCR) amplification of FWPV P4b gene, REV *env* gene, and REV 5'LTR; agarose gel electrophoresis of PCR products, purification of PCR products, sequencing of the purified PCR products, and sequence analysis using standard procedures. Out of 55 analyzed field isolates 53 (96.36%) were found to have REV inserts. Most of them [38 (69.09%)] contained both REV *env* gene and REV 5'LTR inserts, 10(18.18%) contained inserts of REV *env* gene only, and 5 (9.09%) contained inserts of REV 5'LTR only. Two isolates (3.64%) were found to be integrated with neither REV *env* gene nor REV 5'LTR. None of the screened FWPV isolates from the imported commercial vaccines was found to have REV inserts. Sequence analysis revealed that genomic fragments of REV integrated in the Tanzanian FWPV isolate were closely related (99 – 100% identity) to REV sequences integrated in FWPV isolates from other countries. Based

on the findings of this study it is concluded that currently there is a heterogeneous population of FWPV in Tanzania comprising of REV-integrated FWPV strains and REV-free FWPV strains. Since strain(s) of REV-integrated FWPV are more virulent than strain(s) of REV-free FWPV, further studies on the REV-integrated Tanzania FWPV isolates aiming at obtaining the appropriate isolate for development of autogenous fowl pox vaccine are highly recommended.

Keywords: REV-integrated FWPV, REV-free FWPV, variant FWPV strains, Tanzania, PCR, sequencing, autogenous fowl pox vaccine.

1. INTRODUCTION

Reticuloendotheliosis virus (REV) comprises a group of avian RNA viruses that belong to the genus *Gammaretrovirus*, sub-family *Orthoretrovirinae* and family *Retroviridae* (Büchen-Osmond, 2004). Based on the replication ability in the host cells REV isolates are classified as either defective or non-defective. Defective REV refers to REV isolates which lack the ability to replicate in the cell cultures or naturally-infected host cells. Non-defective REV refers to those which can replicate in the cell cultures or naturally-infected host cells (Payne and Venugopal, 2000; Fadly, 2005). The group of defective REV comprises of strain T (REV-T) (Aly *et al*., 1993); a laboratory strain of REV with deletions within the genome and possesses a viral oncogene, *v-rel*, inserted at some site within the genome (Stephens *et al*., 1983; Payne and Venugopal, 2000). This strain transforms haematopoietic cells and fibroblasts to tumourigenic cells in vitro (Herzog *et al*., 1986). REV-T causes acute reticulum cell neoplasia if experimentally infected to susceptible birds (Payne and Venugopal, 2000).

Strains that comprise the non-defective group of REV include REV strain A (REV-A), spleen necrosis virus (SNV), chick syncytial virus (CSV), and duck infectious anaemia virus (DIAV) (Aly *et al*., 1993). Most field isolates of REV are non-defective (Fadly, 2005) and have been reported to cause runting disease syndrome in chickens and ducks characterized by runting, bursal and thymic atrophy, enlarged peripheral nerves, abnormal feather development, proventriculitis, enteritis, anaemia, liver and spleen necrosis, cellular and humoral immunosuppression (Payne and Venugopal, 2000). Apart from that non-defective REV cause chronic lymphoid neoplasms in chickens, ducks, geese, pheasants, quail, and turkeys (Payne and Venugopal, 2000).

Like other retroviruses non-defective REV have a simple genome that consists of four genes namely *gag*, which encodes for the synthesis of internal viral proteins which form the matrix, the capsid, and the nucleoprotein structure; *pol*, that encodes for an RNA-dependent DNA polymerase (also known as reverse transcriptase) and integrase enzymes; and *env*, which encodes for the viral envelope glycoproteins. In addition to these genes, a small gene known as *pro*, which encodes for viral protease is located within or adjacent to the *gag* gene (Weaver *et al*., 1990; Moore *et al*., 2000; Payne and Venugopal, 2000; Davidson *et al*., 2008). The structural genes (*env* and *gag*) are flanked by genomic sequences which regulate viral replication, which in the DNA provirus form the viral long terminal repeats (LTRs) that carry promoter and enhancer sequences (Payne and Venugopal, 2000).

Fowlpox virus (FWPV) is a DNA virus that belongs to the *Poxviridae* family, *Chordopoxvirinae* subfamily and genus *Avipoxvirus* (Lüschow *et al*., 2004). FWPV

and other avipoxviruses (APVs) cause pox in birds (Jarmin *et al*., 2006). Unlike REV, FWPV have a large genome that consists of 260 genes (Afonso *et al*., 2000).

Previous studies (Hertig *et al*., 1997; Diallo *et al*., 1998; Singh *et al*., 2000; García *et al*., 2003; Singh *et al*., 2003; Singh *et al*., 2005; Prukner-Radovčić *et al*., 2006; Davidson *et al*., 2008; Biswas *et al*., 2011) demonstrated the integration of various genomic fragments of REV in the genomes of field strains of FWPV isolated from chickens in various countries outside the African continent. Furthermore, Singh *et al*. (2005) demonstrated that integration of genomic fragments of REV into the FWPV genome leads to increased virulence of the later (REV-integrated FWPV). Apart from that Singh *et al*. (2005) demonstrated that in a country where fowl pox is endemic with a heterogeneous population of FWPV consisting of REV-integrated FWPV strains and REV-free FWPV strains, appropriate autogenous fowl pox vaccine can be developed from an appropriate REV-integrated FWPV isolate after attenuating it by removing the REV provirus provided that the removal of the REV provirus from the FWPV genome does not alter immunogenicity and antigenicity of the REV-less FWPV. According to Hertig *et al*. (1997) infection of susceptible chickens with REV-integrated FWPV may lead to dissemination of REV if a full length or near-full length REV provirus is integrated into the FWPV genome.

In the recent years incidences and prevalence of fowl pox in chickens have increased in Tanzania, characterized with high mortalities of chicks and growers (Oxfam, 2009). Increased virulence of field strains of FWPV currently prevalent in Tanzania due to integration of genomic fragments of REV in the FWPV genomes is one of the probable attributing factors implicated to be behind the increased incidences and prevalence of fowl pox in the country.

REV is known to be a potential contaminant of fowl pox vaccine (Awad *et al*., 2010). In 1966 to 1969 cases of a neoplastic disease were encountered in turkeys in Israel after vaccinating the birds against fowl pox using REV-contaminated fowl pox vaccine (Diallo *et al*., 1998). In the United States and Australia outbreaks of REV occurred after vaccination of chickens using REV-contaminated FWPV vaccine (Moore *et al*., 2000). Currently imported commercial fowl pox vaccines are used in Tanzania for control of fowl pox in chickens and turkeys. Although to date no cases of runting disease syndrome or chronic lymphoid neoplasms have been reported in domestic and/or wild birds in Tanzania, examination of the vaccines to determine their REV contamination status is important in order to make the Tanzanians (poultry keepers in particular) be certain with the safety of the vaccines. The objective of this study was to investigate integration of genomic fragments of REV in the Tanzanian FWPV field isolates, and the imported commercial fowl pox vaccines currently used in the country.

2. MATERIALS AND METHODS

2.1 Study Location

DNA samples analyzed in this study were extracted from samples of chorioallantoic membrane (CAM) containing virus cultures after inoculation of 10 day-old embryonated chicken eggs with inoculums prepared from samples of cutaneous nodular lesions collected from chickens suspected to have fowl pox in Eastern Tanzania (Dar es Salaam and Morogoro regions), Central Tanzania (Dodoma region), Western Tanzania (Kigoma and Tabora regions), North-western Tanzania (Mwanza region), Northern Tanzania (Mara region) and Southern Tanzania (Mbeya

and Iringa regions). Laboratory work was carried out at the Faculty of Veterinary Medicine (FVM), Sokoine University of Agriculture (SUA), Morogoro, Tanzania. The country is located in Eastern Africa between latitudes 1º - 12º South and longitudes 29º - 41º East (Kireri, 2012; Adimola *et al*., 2013).

2.2 DNA samples

Fifty seven samples of genomic DNA which were confirmed to contain FWPV-specific DNA in the previous work by Masola *et al*. (2014) were used in this study. Fifty five samples were extracted from samples of CAM containing virus cultures after inoculation of 10 day-old embryonated chicken eggs with inoculums prepared from samples of cutaneous nodular lesions collected from chickens suspected to have fowl pox. The other two samples were extracted from CAM containing virus cultures after inoculation of 10 day-old embryonated chicken eggs with inoculums prepared from two samples of imported commercial fowl pox vaccines (each sample from a different batch).

2.3 Amplification of FWPV P4b gene, REV *env* gene and REV 5'LTR

Three polymerase chain reaction (PCR) assays were conducted in Takara Thermal Cycler (Takara Bio Inc., Japan) using three sets of primers (Table 1). A PCR assay amplifying a P4b gene (578 bp) of FWPV was carried out using the first set of primers, P1 and P2. The second assay was conducted to amplify an 807 bp region of REV provirus *env* gene using primers P3 and P4. The third set of primers, P5 and P6, was used in the third assay for amplification of REV 5'LTR (370 bp), an REV integration site in the FWPV genome.

The components of each amplification reaction were as described previously by Masola *et al*. (2014). The cycling parameters described earlier by Masola *et al*. (2014) were used in the first PCR assay. For the second assay the cycling parameters included initial denaturation step at 94ºC for 1 minute, for 35 cycles; followed by heat denaturation at 94ºC for 1 minute, primer annealing at 57ºC for 2 minutes, DNA extension at 72ºC for 1 minute. The final extension cycle was performed at 72ºC for 6 minutes. The cycling parameters for the third assay were similar to those of the second assay except that the annealing temperature was 52ºC instead of 57ºC.

2.4 Agarose Gel Electrophoresis

Samples of PCR products were run in a 1.5% agarose gel as described previously by Masola *et al*. (2014).

2.5 Purification of PCR Products, Sequencing of Purified PCR Products and Sequence Analysis

Two samples of PCR products, one with fragment size of 370 bp and the other with fragment size of 807 bp, were selected. The selected samples were purified followed by sequencing and assembling of sequences (forward sequence and reverse sequence) of each sample to get a consensus sequence using procedures described by Masola *et al*. (2014). Thereafter each sequence was analyzed by blasting it in the GenBank using the BLAST algorithm (Palade *et al*., 2008; Biswas *et al*., 2011).

Table 1. Primers used in this study

Primer specific for	Primer sequence (5' →3')	Expected fragment size (bp)	Reference
APV P4b gene	Forward (P1): CAGCAGGTGCTAAACAACAA Reverse (P2): CGGTAGCTTAACGCCGAATA	578	Lüschow *et al.* (2004); Jarmin *et al.* (2006); Prukner-Radovčić *et al.* (2006); Lierz *et al*. (2007); Rampin *et al.* (2007); Palade *et al*. (2008); Manarolla *et al*. (2010); Terasaki *et al.* (2010); Biswas *et al*. (2011) and Masola *et al*. (2014).
.REV *env* gene	Forward (P3): TGACCAGGCGGGCAAAACC Reverse (P4): CGAAAGGGAGGCTAAGACT	807	García *et al*. (2003) and Biswas *et al*. (2011).
REV 5'LTR	Forward (P5): ACCTATGCCTCTTATTCCAC Reverse (P6): CTGATGCTTGCCTTCAAC	370	Biswas *et al*. (2011).

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Amplification of FWPV P4b gene, REV *env* gene and REV 5'LTR

Selected samples of genomic DNA known to contain FWPV-specific DNA were subjected to three conventional PCRs in order to amplify FWPV P4b gene, REV *env* gene and REV 5'LTR; followed by agarose gel electrophoresis. Integration of REV *env* gene and REV 5'LTR in the FWPV genome of the analyzed field isolates was indicated by migration of amplicons to approximately 807 bp and 370 bp, respectively (Fig. 1). Out of 55 field FWPV isolates screened for REV *env* gene and REV 5'LTR; 2 (3.64%) were found to be integrated with neither REV env gene nor REV 5'LTR, 10 (18.18%) were found to be integrated with REV *env* gene only, 5 (9.09%) were found to be integrated with REV 5'LTR only, and 38 (69.09%) were found to be integrated with both REV *env* gene and REV 5'LTR (Table 2). FWPV isolates from the two samples of fowl pox vaccine currently used in Tanzania were found to contain no inserts of REV *env* gene and/or REV 5'LTR.

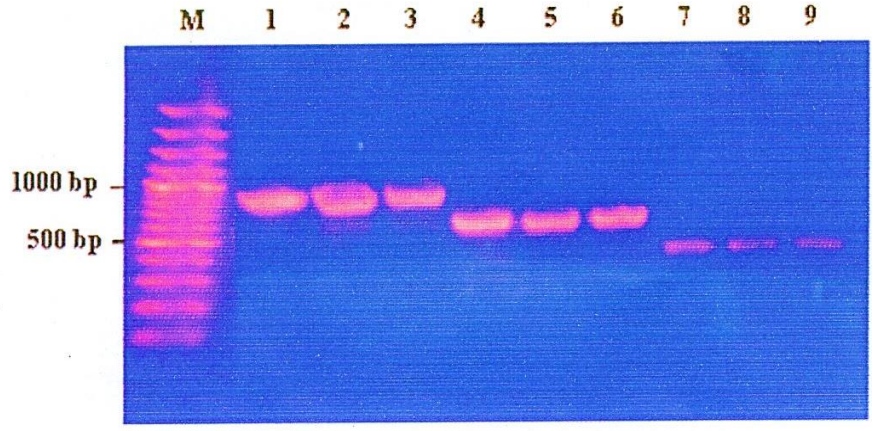

Figure 1. Agarose gel electrophoresis of FWPV-specific and REV-specific PCRs showing migration of amplicons of REV provirus *env* gene (807 bp) (lanes 1, 2, and 3), FWPV P4b gene (578 bp) (lanes 4, 5, and 6) and REV provirus 5'LTR (370 bp) (lanes 7, 8 and 9). Lane M is a DNA ladder with 100-bp increments.

Table 2. REV integration in the Tanzanian FWPV isolates in various geographical locations and regions

Geographical location	Region	Number of FWPV isolates screened for REV *env* gene and REV 5'LTR	Number of FWPV isolates integrated with[a]				Total percentage of FWPV isolates integrated with REV
			neither REV *env* gene nor REV 5'LTR	REV *env* gene only	REV 5'LTR only	Both REV *env* gene and REV 5'LTR	
Eastern Tanzania	Dar es Salaam	7	0 (0.00)	2 (28.57)	1 (14.29)	4 (57.14)	100
Eastern Tanzania	Morogoro	4	0 (0.00)	0 (0.00)	0 (0.00)	4 (100.00)	100
Central Tanzania	Dodoma	10	0 (0.00)	2 (20.00)	0 (0.00)	8 (80.00)	100
Western Tanzania	Kigoma	3	0 (0.00)	0 (0.00)	1 (33.33)	2 (66.67)	100
Western Tanzania	Tabora	2	0 (0.00)	2 (100.00)	0 (0.00)	0 (0.00)	100
North-western Tanzania	Mwanza	8	0 (0.00)	0 (0.00)	2 (25.00)	6 (75.00)	100
Northern Tanzania	Mara	4	0 (0.00)	0 (0.00)	0 (0.00)	4 (100.00)	100
SouthernTanzania	Mbeya	14	1 (7.14)	2 (14.29)	1 (7.14)	10 (71.43)	92.86
Souther Tanzania	Iringa	3	1 (33.33)	2 (66.67)	0 (0.00)	0 (0.00)	66.67
Total		**55**	**2 (3.64)**	**10 (18.18)**	**5 (9.09)**	**38 (69.09)**	**96.36**

[a]Numbers in brackets are the percentage of FWPV isolates integrated or not integrated with a particular genomic fragment(s) of REV.

3.1.2 Sequence analysis

In order to analyze the obtained sequences the later were blasted in the GenBank. Blast results indicated that the sequence of a PCR product with fragment size 807 bp showed 95 – 100% identity to sequences of several REV *env* gene obtained in the GenBank (accession numbers GU012641, GU012645, GU012646, GU012642, GU012639, GU012638, GU222416, KC884563, KC884554, GQ415646, GQ415647, GQ415645, GQ415644, GQ415643, GQ375848, FJ439120, FJ439119, AF246698, EU246946, DQ925492, KF305089, KC884562, GU222420, M22223, AY842951, DQ237903 and X01455); and 100% identity to sequence of *env* gene of REV provirus integrated in a FWPV isolate (GenBank accession number AF246698).

The sequence of a PCR product with fragment size 370 bp showed 88 – 99% identity to sequences of several REV LTR obtained in the GenBank (accession numbers KF305089, GQ415646, JQ804915, FJ439120, FJ439119, GQ375848, FJ496333, DQ387450, JX912710, M22224, M22223, S70398, GQ870289, GQ870290, AY842951, KC018475, KF709431 and DQ003591) (Fig. 2); and 99 – 100% identity to sequences of LTR of REV provirus integrated in several FWPV isolates from other countries (GenBank accession numbers AY255633, AY255632, AF246698, AJ581527, AF198100, AF006065, AF006064 and HQ111429) (Fig. 3). After analysis sequences of *env* gene and 5'LTR of REV provirus integrated in a Tanzanian FWPV isolate were deposited in the GenBank data base under accession numbers KF225480 and KF268024, respectively.

```
Tzfwpv  186  TGT-GGAGGGAGCTCCGGGGGGAATAGCGCTGGCTCGCTAACTGCCATATTAGCTTCTGT  244
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HA1101  3    TGTGGGAGGGAGCTCCGGGGGGAATAGCGCTGGCTCGCTAACTGCCATATTAGCTTCTGT  62

Tzfwpv  245  AATCATGCTTGCTTGCCTTAGCCGCCATTGTACTTGATATATTTCGCTGATATCATTTCT  304
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HA1101  63   AATCATGCTTGCTTGCCTTAGCCGCCATTGTACTTGATATATTTCGCTGATATCATTTCT  122

Tzfwpv  305  CGGAATCGGCATCAAGAGCAGGCTCATAAACCATAAAAGGAAATGTTTGTTGAAGGCAAG  364
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HA1101  123  CGGAATCGGCATCAAGAGCAGGCTCATAAACCATAAAAGGAAATGTTTGTTGAAGGCAAG  182

Tzfwpv  365  CATCAG  370
             ||||||
HA1101  183  CATCAG  188
```

Figure 2. Alignment of the sequence of LTR of REV provirus integrated in the genome of a Tanzanian FWPV isolate (designated as Tzfwpv) with sequence of LTR of REV "strain HA1101" obtained in the GenBank (accession number KF305089) (designated as HA1101) showing 99% identity.

```
Tz fwpv          1       ACCTATGCCTCTTATTCCACTATCGAAGTACGATTTTACATTTACTGATTTGAGTGTTAT  60
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HP-438[Munich]   221553  ACCTATGCCTCTTATTCCACTATCGAAGTACGATTTTACATTTACTGATTTGAGTGTTAT  221612

Tz fwpv          61      CAAATCAGAGAATGTTAAAACGGTACTCTCTAAAGTTCATACGAGTATGAAATCGTACTA  120
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HP-438[Munich]   221613  CAAATCAGAGAATGTTAAAACGGTACTCTCTAAAGTTCATACGAGTATGAAATCGTACTA  221672

Tz fwpv          121     CAACAATGATACGTCTCTTCCTGTCGCCGTTAAGGTGATTTACGGAACAGTAACAATATA  180
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HP-438[Munich]   221673  CAACAATGATACGTCTCTTCCTGTCGCCGTTAAGGTGATTTACGGAACAGTAACAATATA  221732

Tz fwpv          181     AAAAGTGTGGAGGGAGCTCCGGGGGGGAATAGCGCTGGCTCGCTAACTGCCATATTAGCTT  240
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HP-438[Munich]   221733  AAAAGTGTGGAGGGAGCTCCGGGGGGGAATAGCGCTGGCTCGCTAACTGCCATATTAGCTT  221792

Tz fwpv          241     CTGTAATCATGCTTGCTTGCCTTAGCCGCCATTGTACTTGATATATTTCGCTGATATCAT  300
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HP-438[Munich]   221793  CTGTAATCATGCTTGCTTGCCTTAGCCGCCATTGTACTTGATATATTTCGCTGATATCAT  221852

Tz fwpv          301     TTCTCGGAATCGGCATCAAGAGCAGGCTCATAAACCATAAAAGGAAATGTTTGTTGAAGG  360
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HP-438[Munich]   221853  TTCTCGGAATCGGCATCAAGAGCAGGCTCATAAACCATAAAAGGAAATGTTTGTTGAAGG  221912

Tz fwpv          361     CAAGCATCAG  370
                         ||||||||||
HP-438[Munich]   221913  CAAGCATCAG  221922
```

Figure 3. Alignment of the sequence of LTR of REV provirus integrated in the genome of a Tanzanian FWPV isolate (designated as Tz fwpv) with sequence of LTR of REV provirus integrated in a FWPV "isolate HP-438[Munich]" (GenBank accession number AJ581527) (designated as HP-438[Munich]) showing 100% identity.

3.2 Discussion

In the present study the Tanzanian FWPV isolates were analyzed between open reading frame (ORF) 201 and ORF 203 for REV integration (Singh *et al.*, 2003;

Davidson *et al.*, 2008). The analysis revealed integration of various genomic fragments of REV in the genome of most of the FWPV isolates. This implies that currently in Tanzania there is a heterogeneous population of FWPV circulating in the field due to integration of various genomic fragments of REV in their genome. This has led to emergence of variant strains of FWPV currently prevalent in the country, which according to Singh *et al.* (2005) are more virulent than REV-free FWPV strain(s).

Prior to this study integration of genomic fragments of REV in the genome of field isolates of FWPV had been reported in Australia (Hertig *et al.*, 1997; Diallo *et al.*, 1998), Croatia (Prukner-Radovčić *et al.*, 2006), India (Biswas *et al.*, 2011), Israel (Davidson *et al.*, 2008) and the United States (Singh *et al.*, 2000; García *et al.*, 2003; Singh *et al.*, 2003; Tadese and Reed, 2003; Singh *et al.*, 2005). The high proportion of near-full length REV integration in the genome of field isolates of FWPV observed in the present study (Table 2) is in agreement with a previous report by Biswas *et al.* (2011).

Fowl pox is mainly controlled by vaccination of susceptible chickens or turkeys using fowl pox vaccines of FWPV- or pigeonpox virus (PGPV)-origin (Fatunmbi and Reed, 1996). The findings of this study are a step toward development of autogenous fowl pox vaccine for control of fowl pox in Tanzania and other countries with populations of FWPV which are genetically and antigenically similar to FWPV strains currently prevalent in Tanzania.

Lack of genomic fragments of REV in the FWPV isolates from samples of the imported commercial fowl pox vaccines currently used in Tanzania, and

demonstration of the presence of REV *env* gene in most (90.57%) of REV-integrated Tanzanian FWPV isolates indicated that the vaccines are not a source of the variant strains of FWPV revealed in this study. The genetic diversity of the Tanzanian FWPV revealed in the present study could be a result of recombination between field strains of FWPV and field strains of REV (Davidson and Silva, 2008).

4. CONCLUSION

Based on the findings of this study the following conclusions are drawn:

i) Currently there is a heterogeneous population of FWPV in Tanzania comprising of REV-integrated FWPV strains and REV-free FWPV strains.

ii) The imported commercial fowl pox vaccine currently used in Tanzania is not contaminated with REV.

iii) The increased incidences and prevalence of fowl pox currently experienced in Tanzania, characterized with high mortalities of chicks and growers, is attributed to increased virulence of variant FWPV strains due to integration of genomic fragments of REV in their genome.

Further studies on REV-integrated Tanzanian FWPV isolates, aiming at obtaining the appropriate strain (isolate) for development of appropriate autogenous fowl pox vaccine for control of fowl pox in Tanzania, and other countries with populations of FWPV which are genetically and antigenically similar to FWPV currently prevalent in Tanzania are highly recommended. This recommendation is based on the fact that strains of REV-integrated FWPV are more virulent than strains of REV-free FWPV.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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CHAPTER SIX

PAPER III: Detection and Genetic Characterization of an Avipox Virus Isolate from Domestic Pigeon (*Columba livia domestica*) in Morogoro Region, Eastern Tanzania[c]

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DETECTION AND GENETIC CHARACTERIZATION OF AN AVIPOX VIRUS ISOLATE FROM DOMESTIC PIGEON (*COLUMBA LIVIA DOMESTICA*) IN MOROGORO REGION, EASTERN TANZANIA

S. N. Masola, A. Mzula, E. D. Mwega, C. J. Kasanga and P. N. Wambura

ABSTRACT

The aim of this study was to determine molecular and evolutionary characteristics of a newly isolated Tanzanian isolate of pigeonpox virus (PGPV). Samples of cutaneous nodular lesions were collected from 17 pigeons suspected to have pigeonpox in Morogoro (n = 11), Pwani (n = 2) and Dar es Salaam (n = 4) regions; followed by virus isolation, and confirmation by amplification and sequencing of P4b gene. Further the sequence was phylogenetically analyzed for its evolutionary relationship with other related viruses. PGPV was detected in two samples, both from Morogoro region. Sequence analysis revealed that the Tanzanian PGPV isolate derived in this study was 90 – 99% identical to several avipox virus isolates from birds belonging to different species from several countries; for instance the Tanzanian PGPV isolate was 91% identical to each of the Tanzanian fowlpox virus isolates derived in the previous study, and 99% identical to all three PGPV isolates whose sequences were obtained in the GenBank i.e PGPV isolates from India (accession number DQ873811), Egypt (accession number JQ665840) and a PGPV (accession number AY530303) whose country of origin is unknown. Phylogenetic analysis revealed that the Tanzanian PGPV isolate belongs to clade A in subclade A2, sharing a recent common ancestor with members of subclade A3. Based on the findings of this study it is concluded that currently pigeonpox virus is circulating in

Morogoro region. The present study warrants the further surveillance/ molecular epidemiology of PGPV in Tanzania in a large-scale

Keywords: Pigeonpox virus, cutaneous nodular lesions, virus isolation, Tanzania, amplification, sequencing.

1. INTRODUCTION

Avipoxviruses (APVs) are classified under the family *Poxviridae*, subfamily *Chordopoxvirinae* and genus *Avipoxvirus* (Manarolla *et al*., 2010; Parker *et al*., 2011; Gyuranecz *et al*., 2013). APVs are usually named according to the species of the birds from which they were originally isolated (Weli and Tryland, 2011). To date the International Committee on Taxonomy of Viruses (ICTV) considers the genus *Avipoxvirus* to be comprised of only ten species namely canarypox virus (CNPV), fowlpox virus (FWPV), juncopox virus (JNPV), mynahpox virus (MNPV), pigeonpox virus (PGPV), psittacinepox virus (PSPV), quailpox virus (QUPV), sparrowpox virus (SRPV), starlingpox virus (SLPV) and turkeypox virus (TKPV) (www.ictvonline.org/virusTaxonomy.asp). The other three species namely peacockpox virus, penguinpox virus and crowpox virus are considered as tentative members of the genus *Avipoxvirus* (Weli and Tryland, 2011).

APVs have a double-stranded DNA genome, ranging from 260 to 365 kb (Weli and Tryland, 2011). The avipoxvirus (APV) genomes which have been completely sequenced, showing considerable divergence between them are genomes of FWPV (Afonso *et al*., 2000) and CNPV (Tulman *et al*., 2004). APVs are worldwide distributed and cause pox in domestic, wild, and pet birds of several species (van

Riper and Forrester, 2007). They cause three forms of pox in birds; the cutaneous, diphtheritic, and systemic form (Kulich *et al*., 2008; Atkinson *et al*., 2010), characterized by formation of proliferative lesions ranging from papules to nodules in the unfeathered parts of the body, which eventually hardens to form scabs; formation of fibrous necrotic proliferative lesions in the mucous membrane of the digestive and upper respiratory tracts (Thiel *et al*., 2005; Weli and Tryland, 2011); and involvement of various body systems and tissues of an infected bird (Atkinson *et al*., 2010), respectively. Pigeonpox is a slowly developing disease affecting pigeons of all age groups and both sexes. The disease may be complicated with parasitism or poor body condition of birds leading to high mortality rates (Mohan and Fernandez, 2008).

APVs from different species of birds have been characterized in many countries based on their genetic, antigenic, biological or evolutionary properties (Shivaprasad *et al*., 2002; García *et al*., 2003; Lüschow *et al*., 2004; Weli *et al*., 2004; Smits *et al*., 2005; Thiel *et al*., 2005; Tripathy, 2005; Jarmin *et al*., 2006; Prukner-Radovčić *et al*., 2006; Lierz *et al*., 2007; Rampin *et al*., 2007; Kulich *et al*., 2008; Palade *et al*., 2008; Literak *et al*., 2010; Manarolla *et al*., 2010; Terasaki *et al*., 2010; Biswas *et al*., 2011; Chen *et al*., 2011; Lawson *et al*., 2012; Gyuranecz *et al*., 2013; Offerman *et al*., 2013; El-Mahdy *et al*., 2014; Masola *et al*., 2014a; Masola *et al*., 2014b). Avianpox is mainly controlled by vaccination of susceptible birds using appropriate vaccines. Each one of the currently available vaccines against fowlpox, canarypox, pigeonpox and quailpox is developed using virus strains isolated from the respective avian group (Gyuranecz *et al*., 2013). The understanding of genetic, antigenic, biological and evolutionary characteristics of field strains of APVs prevalent in a

particular region is of great value when planning for development of appropriate autogenous vaccines for control of avian pox. However, no information is available on the genetic, antigenic, biological and evolutionary characteristics of field strain(s) of PGPV isolates currently circulating in Tanzania. The objective of this study was to determine molecular and evolutionary characteristics of a newly isolated PGPV in the Eastern Tanzania.

2. MATERIAL AND METHODS

2.1 Study Location

Field work was conducted in Morogoro, Pwani and Dar es Salaam regions; Eastern Tanzania (Fig. 1). It involved collection of samples of cutaneous nodular lesions from live pigeons suspected to have pox, or pigeon cadavers suspected to have died of pox. Laboratory work was conducted at the Faculty of Veterinary Medicine (FVM), Sokoine University of Agriculture (SUA), Morogoro, Tanzania. Eastern Tanzania is located between latitudes 4º - 8º South, and longitudes 37º - 40º East (Adimola *et al*., 2013).

2.2 Samples Collection and Storage

Between October 2012 and June 2013 samples of proliferative cutaneous nodular lesions (n = 17) were collected from featherless or poorly feathered parts of pigeon cadavers suspected to have died of pox (Fig. 2), or live pigeons suspected to have pox; in Morogoro (n = 11), Pwani (n = 2) and Dar es Salaam (n = 4) regions of Eastern Tanzania. Pieces of cutaneous nodular lesions collected from the same bird were pooled as a single sample. Each sample was labeled and stored in a deep freezer at -20 ºC.

2.3 Virus Isolation

An initial polymerase chain reaction (PCR) assay on DNA isolated directly from the lesions produced negative results, so avipox virus DNA was isolated after virus growth on chorioallantoic membranes (CAMs). Inoculums for CAMs were prepared from 17 samples, followed by inoculation in 10 days-old embryonated chicken eggs (ECEs) through CAMs using procedures described by Wambura and Godfrey (2010). Inoculated eggs were incubated at 37 ºC for 5 – 7 days; thereafter they were examined for presence of pock lesions on the CAMs, or generalized thickening and haemorrhages of the CAMs. The inoculums were blind passaged four times.

2.4 DNA Extraction

DNA samples were extracted from samples of CAMs containing virus cultures after the 4th passage by using ZR Genomic DNA^{TM}-Tissue MiniPrep Kit (Zymo Research Corp., USA) according to the manufacturer's instructions.

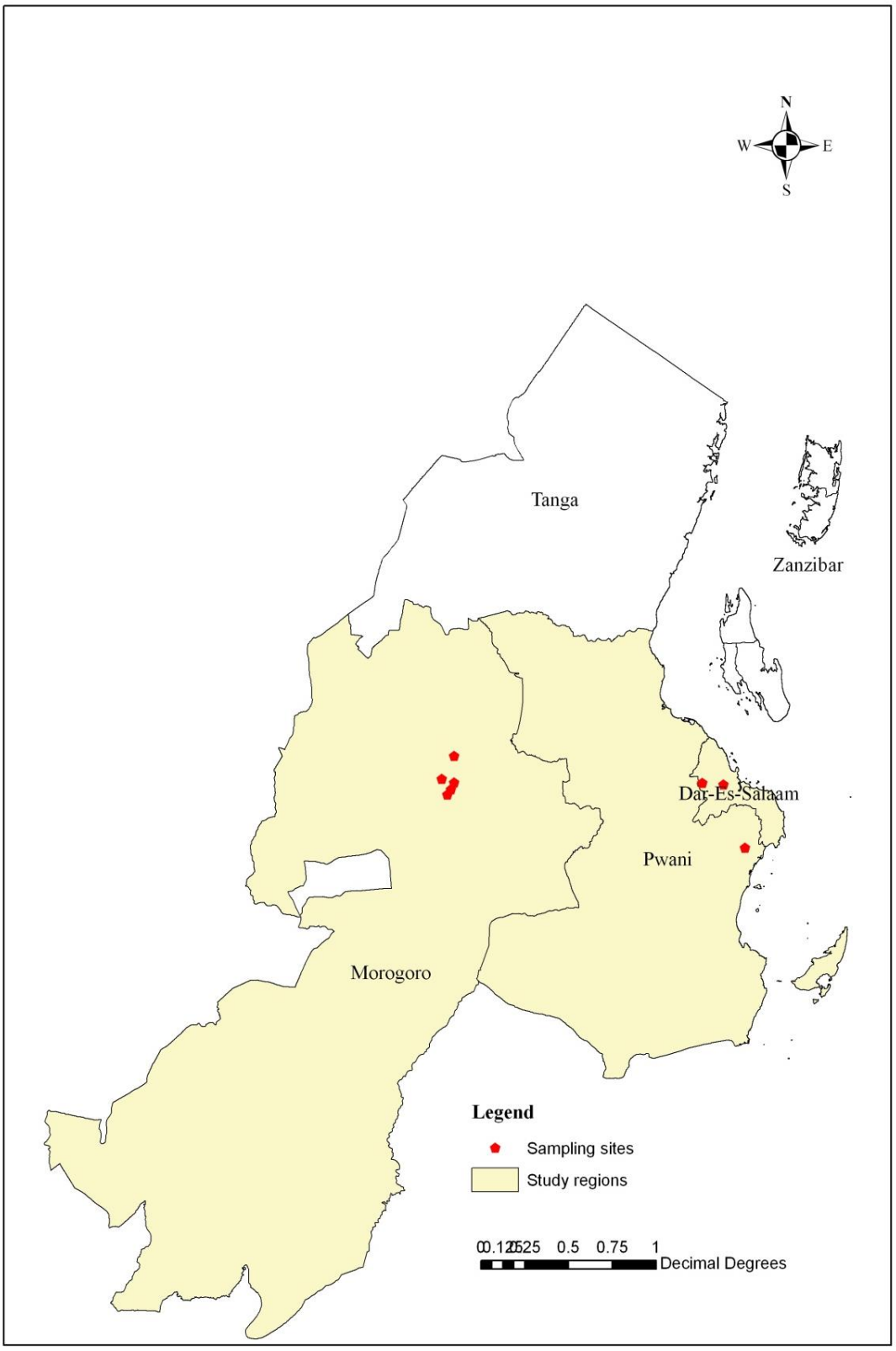

Figure 1. Map of Eastern Tanzania showing study regions and sampling sites

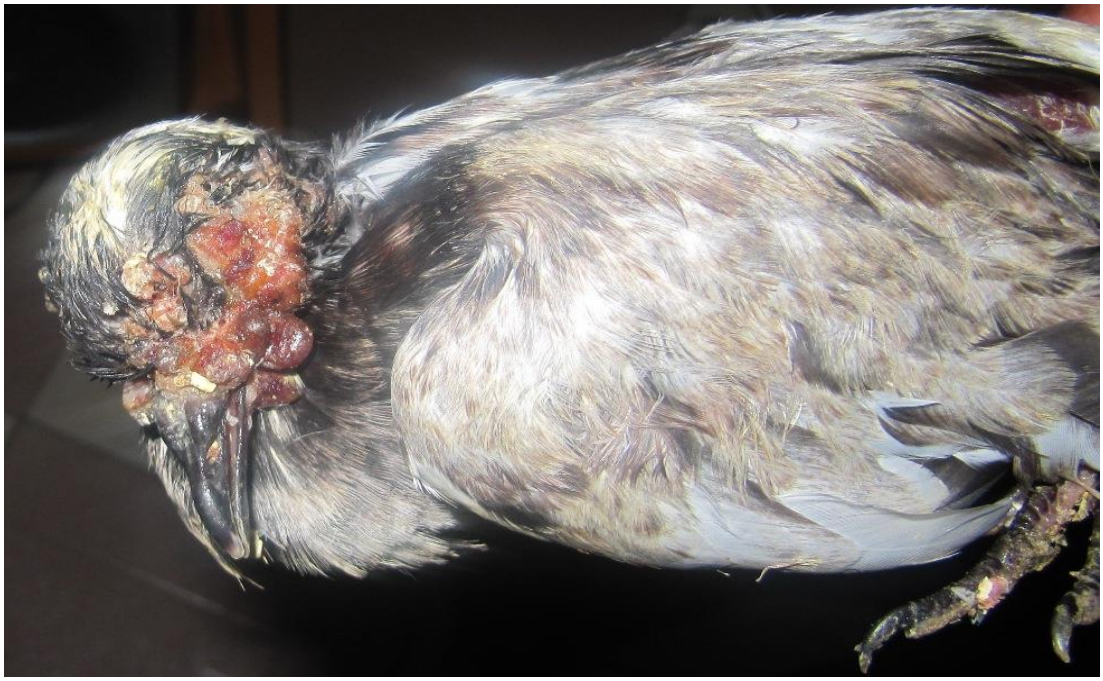

Figure 2. A pigeon cadaver found in one of the pigeon flocks in Morogoro Municipality, Tanzania, presenting proliferative cutaneous nodular lesions at the base of the beak and featherless parts of the head. Note obstruction of vision caused by a complicated eye lesion with caseous material.

2.5 PCR and Agarose Gel Electrophoresis

Conventional PCR was conducted in Takara PCR Thermal Cycler (Takara Bio Inc., Japan) using a set of primers designed to amplify the P4b gene (virus core protein) (Table 1). The components of each amplification reaction and the cycling parameters were as described by Masola *et al.* (2014a). PCR products were run in 1.5% agarose gel and visualized under UV light using UVI tec transilluminator and photographed using a digital camera.

Table 1. Primers used for PCR and sequencing

Primer	Sequence (5' → 3')	Expected fragment size	Reference
Forward primer	CAGCAGGTGCTAAACAACAA	578 bp	Huw Lee and Hwa Lee (1997)
Reverse primer	CGGTAGCTTAACGCCGAATA		

2.6 Purification of a Selected PCR Product and Sequencing of the Purified PCR Product

The PCR product was purified using ExoSAP-IT (Affymetrix, USA). The sequencing reaction was cleaned using ZR-96 DNA Sequencing Clean-up Kit™ (Zymo Research Corp., USA) and purified sample was injected in the ABI 3500XL with P0P7 and a 50 cm array (Applied Biosystems, USA). Thereafter the sample was sequenced using ABI V3.1 Big Dye Sequencing Kit (Applied Biosystems, USA) using primers used for PCR. The sequences (forward and reverse) were assembled using CLC Main Workbench version 6.7.1 to generate a consensus sequence.

2.7 Analysis of Sequence Data

Sequence homology between the P4b gene of an APV isolate derived in the present study and the P4b gene of APVs isolated from various bird species in several countries was investigated by blasting the sequence derived in this study in the GenBank using BLASTN (Altschul *et al*., 1990). Similarities between the Tanzanian APV isolate derived in the current study with other Tanzanian FWPV isolates derived in the previous study (Masola *et al*., 2014a) were investigated using BLAST two sequences programme which gives alignment of two sequences of interest (Biswas *et al*., 2011).

In order to establish evolutionary characteristics of the Tanzanian APV isolate, the nucleotide sequence derived in this study was aligned with reference sequences obtained from the GenBank (Table 2) using ClustalW programme (Thompson *et al*., 1994) incorporated in MEGA 6.06 software (Tamura *et al*., 2013). Thereafter possible phylogenetic relationship and grouping of the Tanzanian APV isolate were investigated using MEGA 6.06 software (Tamura *et al*., 2013); neighbour-joining method was used according to the maximum composite likelihood model. *Molluscum contagiosum virus* (MOCV) was used as an out group for the P4b gene.

3. RESULTS AND DISCUSSION

3.1 Results

Initial PCR assay on DNA isolated directly from samples of cutaneous nodular lesions indicated that all samples were negative for APV. This could be attributed to low concentration of APV DNA in the lesions. Therefore, inoculums prepared from the lesions were inoculated in the ECEs though CAMs so as to let the APV (if any) multiply and grow on CAMs in order to increase the concentration of APV DNA to levels which are detectable by PCR.

Table 2. Details of APVs used in this study

Virus name	Acronym	Host	P4b locus GenBank accession numbers
Pigeonpox virus PGPV-TZ	PGPV-TZ	Pigeon	KJ913659[a]
Fowlpox virus VR250	FWPVVR250	Chicken	AY453172[b]
Fowlpox virus HP-B	FWPVHPB	Chicken	AY530302[b]
Turkeypox virus GB 134/01	TKPV13401	Turkey	AY530304[b]
Pigeonpox virus TP-2	PGPVTP2	Pigeon	AY530303[b]
Pigeonpox virus Peekham	PGPVP	Pigeon	AM050385[b]
Ostrichpox virus GB 724/01-20	OSPV	Ostrich	AY530305[b]
Falconpox virus 1381/96	FLPV1381	Falcon	AM050376[b]
Albatrosspox virus 353/87	ABPV	Black-browed albatross	AM050392[b]
Falconpox virus GB362-02	FLPV36202	Falcon	AY530306[b]
Sparrowpox virus 9037 31/5/66/23	SRPV23	Sparrow	AM050390[b]
Great titpox virus GTPV-256	GTPV256	Great tit	AY453175[b]
Canarypox virus GB 724/01-22	CNPV72401	Canary	AY530309[b]
Pigeonpox virus 950 24/3/77	PGPV950	Pigeon	AM050386[b]
Pigeonpox virus B7	PGPVB7	Pigeon	AY453177[b]
Starlingpox virus /27	SLPV	Starling	AM050391[b]
Macawpox virus 1305/86	MCPV	Macaw	AM050382[b]
Parrotpox virus 364/89	PRPV	Parrot	AM050383[b]
Agapornis virus APIII	AGPV	Agapornis	AY530311[b]
Avipoxvirus P31		Trumpeter swan	KC017990[c]
Avipoxvirus P32		Mottled duck	KC017991[c]
Avipoxvirus P34		Redhead duck	KC017993[c]
Avipoxvirus P38		Mourning dove	KC017997[c]
Avipoxvirus P42		Rock dove	KC018001[c]
Avipoxvirus P43		Canada goose	KC018002[c]
Avipoxvirus P44		Bald eagle	KC018003[c]
Avipoxvirus P51		Red kite	KC018010[c]
Avipoxvirus P54		Mallard duck	KC018013[c]
Avipoxvirus P109		American robin	KC018068[c]
Avipoxvirus P29		Peregrine falcon	KC017988[c]
Avipoxvirus P30		Red-footed falcon	KC017989[c]
Avipoxvirus WCV34-03		American crow	DQ131893[c]
Avipoxvirus WCV52-04		Great blue heron	DQ131898[c]

[a]Sequence of a Tanzanian PGPV isolate derived in this study.

[b]Reference sequences - Source: Manarolla *et al.* (2010).

[c]Reference sequences - Source: Gyuranecz *et al.* (2013).

3.1.1 Virus isolation

3.1.1.1 Inoculation for CAMs

Grossly, signs of embryopathic effects characterized by focal proliferations started to be visible during the third passage. Two to three pocks about 1 mm in diameter were observed on the CAMs. Marked proliferative pock lesions were observed at the fourth passage and the pocks were increased in number and size ranging from 1 – 2 mm in diameter. Most of them had coalesced to form large mass. The number of samples examined, number of PGPV-positive samples and percentage of PGPV-positive samples for each region were as tabulated in Table 3.

3.1.2 PCR

Positive results were indicated by migration of PCR products to approximately 578 bp, an expected fragment size for the P4b gene amplicon for APVs. Out of 17 samples tested 2 (11.76%) were positive.

Table 3. Detection of PGPV in cutaneous nodular lesions from pigeons by virus isolation

Region	Number of samples examined for PGPV	Number of PGPV-positive samples	Percentage of PGPV-positive samples
Morogoro	11	2	18.18
Pwani	2	0	0.00
Dar es Salaam	4	0	0.00
Total	**17**	**2**	**11.76**

3.1.3 Analysis of sequence data

Sequence analysis revealed that the nucleotide sequence of a Tanzanian APV isolate derived in the present study was 90 – 99% identical to sequences of the P4b gene of several APV isolates from birds belonging to different avian species from several countries. For instance, the Tanzanian APV isolate derived in this study was 91% identical to the sequence of the P4b gene of each of the Tanzanian FWPV isolates (GenBank accession numbers KF032407, KF722858, KF722859, KF722860, KF722861, KF722862, KF722863, KF722864, KF722865 and KF722866) derived in the previous study (Masola *et al*., 2014a), and 99% identical to the P4b gene sequences of all three PGPV isolates obtained in the GenBank i.e an Indian PGPV isolate (accession number DQ873811); an Egyptian PGPV isolate, strain ELshargyia_PGPV (accession number JQ665840); and a PGPV strain TP-2 (accession number AY530303) whose country of origin is not available.

Phylogenetic analysis revealed that the Tanzanian PGPV isolate clustered together with members of clade A in subclade A2, sharing a recent common ancestor with members of subclade A3 (Fig. 3). After analysis the sequence of the Tanzanian isolate was deposited in the GenBank under accession number KJ913659.

3.2 Discussion

In the current study, PGPV was detected in samples of cutaneous nodular lesions from two domestic pigeons in Morogoro region using virus isolation and PCR assay. To the best of our knowledge this is the first report confirming pigeonpox in Tanzania. However, the virus could not be isolated from other 15 samples. This implies that in the pigeons from which the lesion samples were collected, the lesions

could be attributed to other diseases such as papillomatosis (Literák *et al*., 2003) and/or mange (CFSPH, 2012; OIE, 2013) which present clinical manifestations similar to those of the cutaneous form of pigeonpox. Although all samples from Pwani and Dar es Salaam regions were found to be PGPV-negative, this does not rule out pigeonpox in these regions because only few samples from Pwani (n = 2) and Dar es Salaam (n = 4) regions were examined.

High sequence identity (99%) of the Tanzanian PGPV isolate to sequences of all three PGPV isolates obtained in the GenBank demonstrates that the P4b gene is highly conserved (Jarmin *et al*., 2006; Offerman *et al*., 2013; Zhao *et al*., 2014). A relatively low sequence homology (91%) of the P4b gene of a PGPV isolate derived in the present study to the previously derived Tanzanian FWPV isolates (Masola *et al*., 2014a) indicates that the APV currently causing pigeonpox in Morogoro region is distinct from APVs currently causing fowlpox in chickens in Morogoro and other regions of Tanzania.

In the present study, the Tanzanian PGPV isolate clustered together with members of clade A in subclade A2 (Fig. 3). This implies that the isolate is phylogenetically closely related to APVs belonging to subclade A2, some of which include APV isolates from birds of the order *Columbiformes* such as rock doves (accession numbers KC017965, KC017966, KC017968, KC017969, and KC017971) from USA and Hungary, oriental turtle doves (accession numbers KC017972 and KC017973) from South Korea (Gyuranecz *et al*., 2013), a rock pigeon (accession number KC821559), racing pigeons (accession numbers KC821556, KC821552 and KC821557) and a feral pigeon (accession number KC821551) from South Africa,

pigeons (accession numbers JX464827, DQ873811 and AM050385) from Egypt, India and UK, and a common wood pigeon (accession number HM481409) from India (Offerman *et al*., 2013); birds of the order *Accipitriformes* such as booted eagles (accession numbers KC017976 and KC017979) from Spain, an eastern imperial eagle (accession number KC017967) from Hungary, and a red kite (accession number KC017978) from Spain; birds of the order *Gruiformes* such as great bustards (accession numbers KC017970 and KC017974) from Hungary and Spain; and birds of the order *Galliformes* such as an Indian peafowl (accession number KC017975) from Hungary, red-legged partridges (accession numbers KC017977 and KC017980) from Spain (Gyuranecz *et al*., 2013), and a grey partridge (accession number GQ180204) from Northern Italy (Manarolla *et al*., 2010). Others include APV isolates from a canary (accession number GQ180208), a gyrfalcon (accession number GQ180210) and a common buzzard (accession number EF016108) from Northern Italy (Manarolla *et al*., 2010).

Although the Tanzanian PGPV isolate (accession number KJ913659) clustered together with APV isolates belonging to subclade A2, some APV isolates from pigeons (accession numbers KC821550, KC821555, KC821557, KC821558 and KC821560) have been reported to cluster together with APV isolates belonging to subclade A3 (Offerman *et al*., 2013), and others (accession numbers AM050386 and AY453177) have been reported to cluster together with APV isolates belonging to subclade B2 (Jarmin *et al*., 2006; Rampin *et al*., 2007; Manarolla *et al*., 2010). Recently, an APV isolate from a rock dove (*Columba livia*) (accession number KC018001) has been reported to cluster in subclade A6 together with APV isolates

from mourning doves (*Zenaida macroura*) (accession numbers (KC017997, KC017998, KC017999 and KC018000) belonging to the order *Columbiformes*, and an APV isolate from a canada goose (*Branta canadensis*) (accession number KC018002) belonging to the order *Anseriformes* (Gyuranecz *et al*., 2013).

The grouping of the Tanzanian PGPV isolate in subclade A2 together with APV isolates from birds of the same species (*Columba livia*) and other APV isolates from birds not belonging to the species *C. livia*, while some APV isolates from birds belonging to species *C. livia* cluster in other subclades (subclades A3, A6 and B2) supports what was reported by Jarmin *et al*. (2006) that evolutionary taxonomy of the host doesn't appear to have a major role in driving evolution of APVs.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

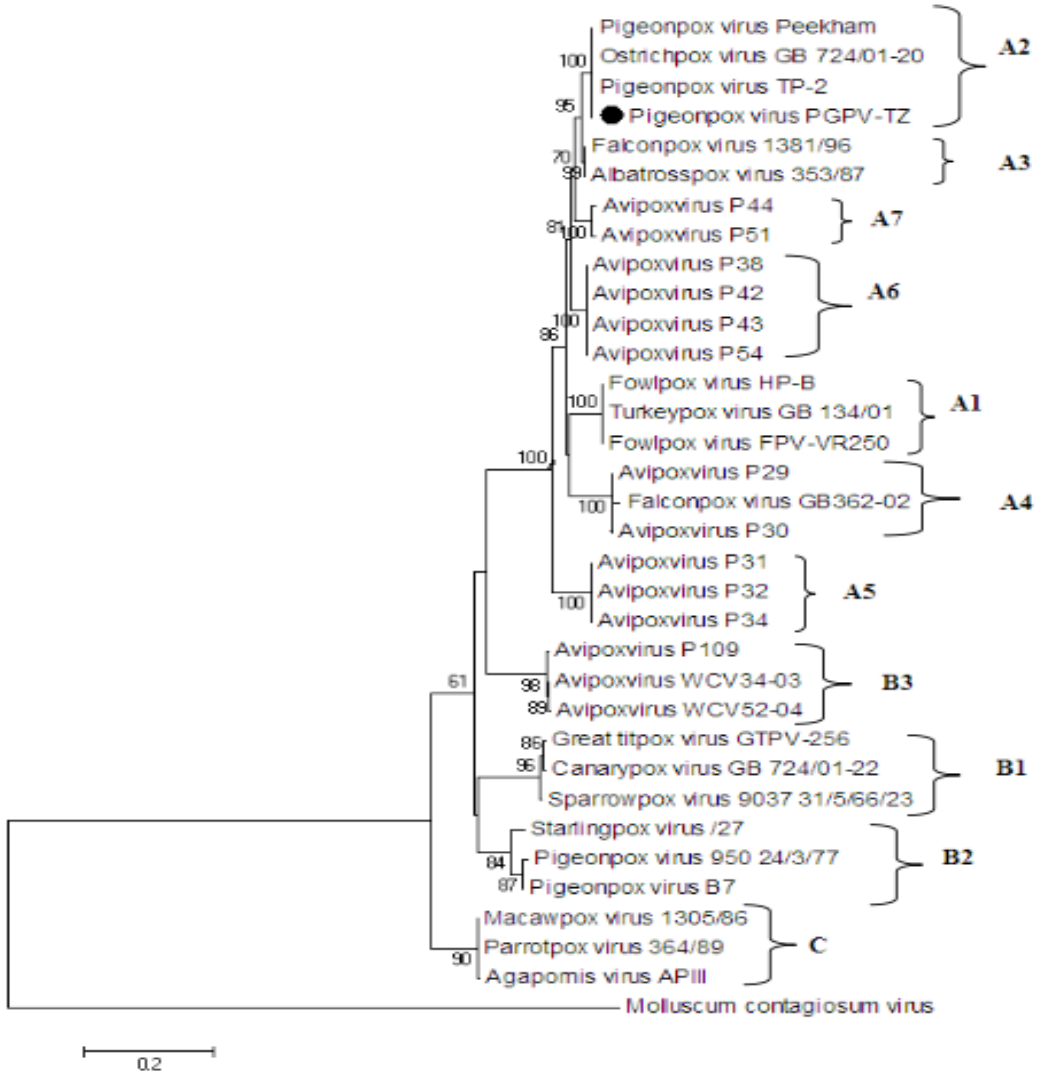

Figure 3. Phylogenetic tree of nucleotide sequences of the P4b gene of APVs and molluscum contagiosum virus (MOCV) orthologue sequence, rooted on MOCV, showing phylogenetic relationship of the Tanzanian PGPV isolate [marked with a black dot (●)] to APV isolates of other countries. The tree was obtained by the neighbour-joining method calculated with the maximum composite likelihood model. Bootstrap testing of phylogeny was performed with 1000 replications and values greater than 50 are indicated on the branches (as a percentage). The length of each bar indicates the amount of evolution along the horizontal branches as measured by substitution per site. APV subclades A1 to A7, B1 to B3, and clade C are labeled.

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CHAPTER SEVEN

PAPER IV: Evaluation of Virulence of Tanzanian Strains of Fowlpox and Pigeonpox Viruses in Chickens[d]

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EVALUATION OF VIRULENCE OF TANZANIAN STRAINS OF FOWLPOX AND PIGEONPOX VIRUSES IN CHICKENS

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ABSTRACT

The aim of this study was to evaluate the virulence characteristics of recently isolated Tanzanian strains of fowlpox virus (FWPV) and pigeonpox virus (PGPV) in chickens. Ten-day embryonated chicken eggs were used for *in ovo* evaluation. The eggs were randomly grouped into four groups (I, II, III, and IV) of 5 eggs each. Each egg in group I, II, and III was inoculated with 0.1 ml of 10^6 EID_{50}/0.1 ml of reticuloendotheliosis virus (REV)-free FWPV inoculum; REV-integrated FWPV inoculum; and PGPV inoculum; respectively, through chorioallantoic membranes (CAMs). Group IV eggs served as control. All eggs were incubated at 37ºC for 7 days, thereafter CAMs and chicken embryos were examined for gross pathological changes. One hundred and forty chicks were used for *in vivo* evaluation. At 26 days of age the chicks were randomly grouped into four groups (I, II, III, and IV) of 35 chicks each. Each chicken in group I, II, and III was inoculated with 0.1 ml of 10^6 EID_{50}/0.1 ml of REV-free FWPV inoculum; REV-integrated FWPV inoculum; and PGPV inoculum; respectively, subcutaneously. Chickens in group IV served as control. Thereafter from day zero to day 28 post-inoculation, the chickens were examined for development of clinical signs and deaths; followed by necropsy of dead chickens and examination of samples of cutaneous nodular lesions from chickens inoculated with REV-free FWPV or REV-integrated FWPV for the presence of FWPV by using standard procedures. Extensive pock lesions and severe

haemorrhages were evident on CAMs and embryos, respectively, of eggs inoculated with REV-integrated FWPV. Chickens inoculated with REV-integrated FWPV developed a severe disease, characterized by mortality rate of 57%. Based on the findings of this study it is concluded that REV-integrated FWPV strains are more virulent in susceptible chickens than REV-free FWPV strains.

Keywords: Tanzanian avipoxvirus strains, REV-free FWPV, virulence, PGPV, REV-integrated FWPV strains, chickens.

1. INTRODUCTION

The extent of virulence is usually correlated with the ability of the pathogen to multiply within the host. This ability, which is mediated by virulence factors, represents a genetic component of the pathogen; and the overt damage done to the host is an outcome of the host-pathogen interactions (Casadevall and Pirofski, 1999; Casadevall and Pirofski, 2001). Currently virulence is used to characterize the relative capacity of an infectious agent to cause disease in susceptible hosts and has traditionally been used to describe biological characteristics of infectious agents (Casadevall and Pirofski, 2001).

Fowlpox and pigeonpox viruses are DNA viruses belonging to the family *Poxviridae*, subfamily *Chordopoxvirinae* and genus *Avipoxvirus* (Hendrickson *et al*., 2010; Manarolla *et al*., 2010; Parker *et al*., 2011; Gyuranecz *et al*., 2013). Avipoxviruses (APVs) cause pox in birds, in chickens the disease is known as fowl pox (Tripathy, 2005). Clinically, there are three forms of avian pox; the cutaneous, diphtheritic and systemic forms (Kulich *et al*., 2008; Atkinson *et al*., 2010). The cutaneous form is characterized by formation of proliferative lesions ranging from

papules to nodules in the featherless or poorly feathered parts of the body, which eventually hardens to form scabs. The diphtheritic form is characterized by formation of fibrous necrotic proliferative lesions in the mucous membrane of the oral cavity and upper respiratory tracts (Tripathy, 2005). In the systemic form various body systems and tissues of an infected bird are involved (Atkinson *et al*., 2010). The cutaneous form is common and causes no or low mortality. Mortality rates are high in the diphtheritic and systemic forms. However, these forms of avian pox occur rarely (van Riper and Forrester, 2007; Alehegn *et al*., 2014).

Isolates of APVs from several avian species have been characterized in several countries based on their antigenic, genetic or biological properties. Some of the countries include Norway (Weli *et al*., 2004), Hawaii (Tripathy *et al*., 2000; Kim and Tripathy, 2006), Tanzania (Masola *et al*., 2014a; Masola *et al*., 2014b; Masola *et al*., 2015), Galápagos islands (Thiel *et al*., 2005), Czech Republic (Kulich *et al*., 2008), Croatia (Prukner-Radovčic *et al*., 2006), the United States (Shivaprasad *et al*., 2002; García *et al*., 2003), Italy (Rampin *et al*., 2007), Hungary (Palade *et al*., 2008), Japan (Terasaki *et al*., 2010), Great Britain (Lawson *et al*., 2012), Egypt (Abdallah and Hassanin, 2013), Australia (Hertig *et al*., 1997), Israel (Davidson *et al*., 2008) and India (Biswas *et al*., 2011). As far as biological characterization is concerned, reports (Holt and Krogsrud, 1973; Cox, 1980; Winterfield and Reed, 1985; Reed and Fatunmbi, 1993; Weli *et al*., 2004; Kim and Tripathy, 2006) indicate that several studies have been conducted to determine the pathogenicity or virulence of avipoxvirus (APV) isolates from birds belonging to different avian species.

In recent years fowl pox has been reported to cause high mortalities of chickens (particularly chicks and growers) in Tanzania (Oxfam, 2009; RLDC, 2012). It has been speculated that the increased mortality rate of chickens (chicks and growers in particular) due to fowl pox could be attributed to emergence of variant strains of APVs which are more virulent than FWPV strains which were circulating in the country in the past decades. This necessitated biological characterization of Tanzanian APV strains currently circulating in the country. The objective of this study was to investigate the virulence characteristics of recently isolated Tanzanian strains of FWPV and PGPV in chickens.

2. MATERIALS AND METHODS

2.1 Study Location

The study involved both *in ovo* and *in vivo* evaluation. *In ovo* evaluation was carried out in the virology laboratory, at the Faculty of Veterinary Medicine (FVM), Sokoine University of Agriculture (SUA), Tanzania. *In vivo* evaluation was conducted at the Animal Research Unit of the FVM, SUA, Tanzania.

2.2 Source of Chickens and Chicken Eggs

One hundred and forty (140) day-old layer chicks and 130 specific-pathogen free (SPF) eggs were purchased from Interchick Company Limited, Dar es Salaam, Tanzania; and a commercial farm in Morogoro, Tanzania, respectively.

2.3 Incubation of Eggs

The eggs were incubated at 37ºC for 10 days so as to obtain embryonated chicken eggs (ECEs) for *in ovo* evaluation and determination of mean (50%) embryo-infectious dose (EID_{50}).

2.4 Preparation and Storage of Virus Inocula

Samples of chorioallantoic membranes (CAMs) with pock lesions due to Tanzanian strains of fowlpox virus (FWPV) and pigeonpox virus (PGPV), previously isolated and genetically characterized (Masola *et al*., 2014a; Masola *et al*., 2014b; Masola *et al*., 2015), were homogenized. Thereafter, each homogenate was suspended in phosphate-buffered saline (PBS) along with antibiotics [i.e Gentamycin (10% w/v) and Penistrept®, consisting of Procaine Penicillin (200 mg/ml) and Dihydrostreptomycin Sulphate (200 mg/ml)]. The homogenates were centrifuged at 500g for 10 minutes at room temperature (25 - 28ºC); thereafter the supernatants were collected and filtered using 0.22 µm membrane filters to get the inocula. Each inoculum was stored at -20ºC in plastic vials containing 10^6 EID_{50}/0.1 ml of either reticuloendotheliosis virus (REV)-free FWPV inoculum, REV-integrated FWPV inoculum, or PGPV inoculum.

2.5 Management of Chickens

The chicks were reared at the Animal Research Unit of the FVM, SUA; in a well-ventilated concrete floor house, littered by rice husks. The chicks were given standard feed (chick starter) and water *ad libitum* and held in isolation until they were 21 days of age for maternally derived antibodies to wane. At this age all birds were screened for fowl pox and reticuloendotheliosis antibodies and were found to be seronegative.

2.6 Study Design

This was an experimental study design and it involved both *in ovo* and *in vivo* evaluation as described below:

2.6.1 *In ovo* evaluation

Twenty 10-day-old SPF ECEs were used for *in ovo* evaluation. The eggs were randomly grouped into four groups (I, II, III, and IV) of 5 eggs each. Each egg in group I, II, and III was inoculated 0.1 ml containing 10^6 EID_{50}/0.1 ml of REV-free FWPV inoculum; REV-integrated inoculum; and PGPV inoculum; respectively, through CAMs. Group IV eggs served as control, they were injected 0.1 ml of PBS through CAMs. All eggs were incubated at 37ºC for 7 days, thereafter CAMs were examined for the presence of pock lesions or generalized thickening and haemorrhages. Chicken embryos were also examined for gross pathological changes. The experiment was replicated one time.

2.6.2 *In vivo* evaluation

This involved inoculation of chickens with Tanzanian strains of REV-free FWPV, REV-integrated FWPV (previously demonstrated to be integrated with a near-full length REV provirus i.e 807 bp of REV envelope gene and 370 bp of 5' REV long terminal repeat (Masola *et al*., 2014b) or PGPV (Masola *et al*., 2015), clinical examination of both inoculated and control chickens, necropsy of dead chickens, examination of cutaneous nodular lesions from chickens inoculated with Tanzanian strains of FWPV for the presence of FWPV, antigen preparation and measurement of humoral immune responses as described below:

2.6.2.1 Inoculation of chickens with FWPV or PGPV

At 26 days of age the chicks were randomly grouped into four groups (I, II, III, and IV) of 35 chicks each. Each group was kept in a separate room. Each chicken in group I, II, and III was inoculated 0.1 ml containing 10^6 EID_{50}/0.1 ml of REV-free FWPV inoculum; REV-integrated FWPV inoculum; and PGPV inoculum,

respectively, through the subcutaneous route at the ventral side of the neck. Chickens in group IV served as control, they were injected 0.1 ml of PBS subcutaneously. Blood samples were collected from wing vein of each chicken before inoculation and at 4-day intervals post-inoculation (pi) up to day 28 pi.

2.6.2.2 Clinical examination

From day zero to day 28 pi all chickens were clinically examined for development of clinical signs and deaths.

2.6.2.3 Necropsy

Post-mortem examination was carried out to all dead chickens using routine procedures described previously (Nyaga *et al*., 2014), so as to establish gross pathological changes in internal organs.

2.6.2.4 Examination of cutaneous nodular lesions for the presence of FWPV

Samples of lesions were examined by using both virus isolation and polymerase chain reaction (PCR) techniques. The samples were collected from chickens inoculated with REV-free FWPV or REV-integrated FWPV strains and stored at -20ºC. Thereafter virus inocula were prepared as described earlier (Masola *et al*., 2014a). The inocula were inoculated on SPF CAMs of 10-day-old chicken embryos. The infected embryos were incubated at 37ºC for 7 days. Thereafter they were cooled for 2 hours and the CAMs were removed. The CAMs thickness and the presence of characteristic pock lesions were evaluated grossly. Thereafter samples of genomic DNA were extracted from samples of CAMs containing virus cultures as described earlier (Masola *et al*., 2014a), followed by examination for the presence of

FWPV-specific DNA by using PCR as described previously (Masola *et al.*, 2014a) using gene specific oligonucleotide primers indicated in Table 1. After PCR the amplicons were run in a 1.5% agarose gel as described earlier (Masola *et al*., 2014a) to determine the positive samples.

2.6.2.5 Antigens preparation and measurement of humoral immune responses

The antigens were prepared by using procedures described previously (Weli *et al*., 2004). Humoral immune responses of chickens after inoculation with REV-free FWPV, REV-integrated FWPV or PGPV were measured by using enzyme-linked immunosorbent assay as described earlier (Weli *et al.*, 2004).

Table 1. Primers used in this study

Primer specific for amplification of	Primer sequence (5' → 3')	Expected fragment size	Reference
P4b gene of APVs	CAGCAGGTGCTAAACAACAA[a] CGGTAGCTTAACGCCGAATA[b]	578 bp	Huw Lee and Hwa Lee (1997)

[a]Forward primer, [b]reverse primer

2.7 Biosecurity Measures

In order to ensure that no environmental contamination occurred during *in vivo* experiment, the following biosecurity measures were taken:

i. A disinfectant footbath was available at the entry point of the experiment facility, each personnel involved during the experiment had to disinfect his/her feet before getting in or out of the experiment facility.

ii. Each personnel involved during the experiment had to put on protective clothing (i.e coveralls), gum boots and gloves that were devoted solely to the experiment facility.

iii. All other equipments and supplies such as feeders and drinkers used during the experiment were solely devoted to the experiment facility.

iv. Each personnel involved during the experiment observed personal hygiene which included frequent hand washing with warm water and soap, cleaning and disinfection of gum boots, coveralls and equipments used for post-mortem examination.

v. All dead chickens were burnt and ashes were buried. All chickens that were alive at the end of the experiment were euthanized. The euthanized birds were burnt and ashes were buried.

vi. After completion of data collection the experiment facility was disinfected.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 *In ovo* evaluation

3.1.1.1 Pathological findings

Gross examination of CAMs and chicken embryos revealed development of pock lesions on CAMs of ECEs inoculated with PGPV, REV-free FWPV and REV-integrated FWPV strains. Apart from development of pock lesions the CAMs were thickened and haemorrhagic (Fig. 1). Embryos of ECEs inoculated with PGPV, REV-free FWPV and REV-integrated FWPV strains were haemorrhagic (Fig. 2). Table 2 shows virulence scores of PGPV and FWPV strains based on damage induced by the viruses on the embryos as indicated by the extent of haemorrhages on the embryos.

3.1.2 *In vivo* evaluation

3.1.2.1 Clinical findings

Cutaneous nodular lesions started to be evident on featherless parts of chickens such as eyelids and nostrils on day 10 pi, the lesions were evident in chickens inoculated with REV-free FWPV or REV-integrated FWPV. In addition to cutaneous nodules, on day 26 pi abnormal feathering started to be evident in five of 35 (14%) chickens inoculated with REV-integrated FWPV. Chicken deaths occurred on days 23, 25, 26, and 28 pi; where 3, 7, 6, and 4 chickens died, respectively. Chicken mortalities related to inoculation of each virus inoculum by day 28 pi were as shown in Table 3. No clinical signs were observed in chickens which served as control and those which were inoculated with PGPV. Virulence scores based on severity of disease as indicated by the number of cutaneous nodular lesions on chickens that developed fowl pox were as indicated in Table 4.

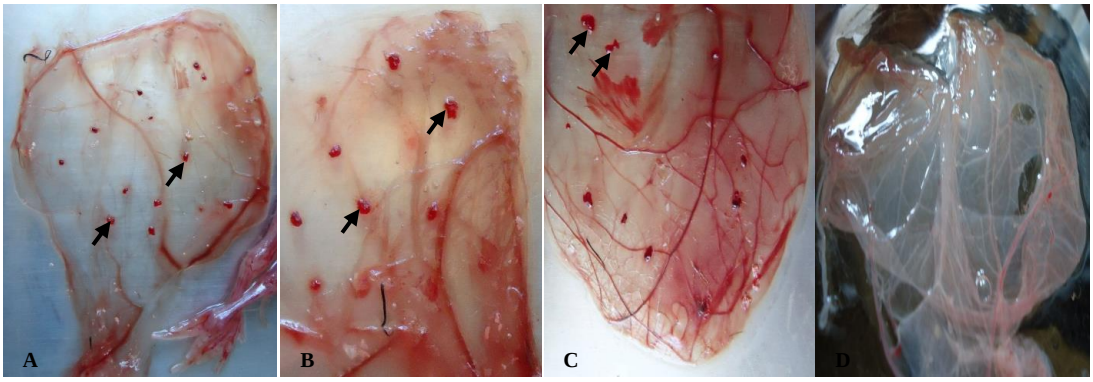

Figure 1A, B, C and **D**. Photographs showing gross pathological changes on CAMs of ECEs inoculated with Tanzanian strains of FWPV and PGPV. (A) Pock lesions (arrows) on CAM of ECE inoculated with PGPV. (B) Extensive pock lesions (arrows) and haemorrhages on CAM of ECE inoculated with REV-integrated FWPV. (C) Pock lesions (arrows) and haemorrhages on CAM of ECE inoculated with REV-free FWPV. (D) CAM of ECE not inoculated with any APV strain (control).

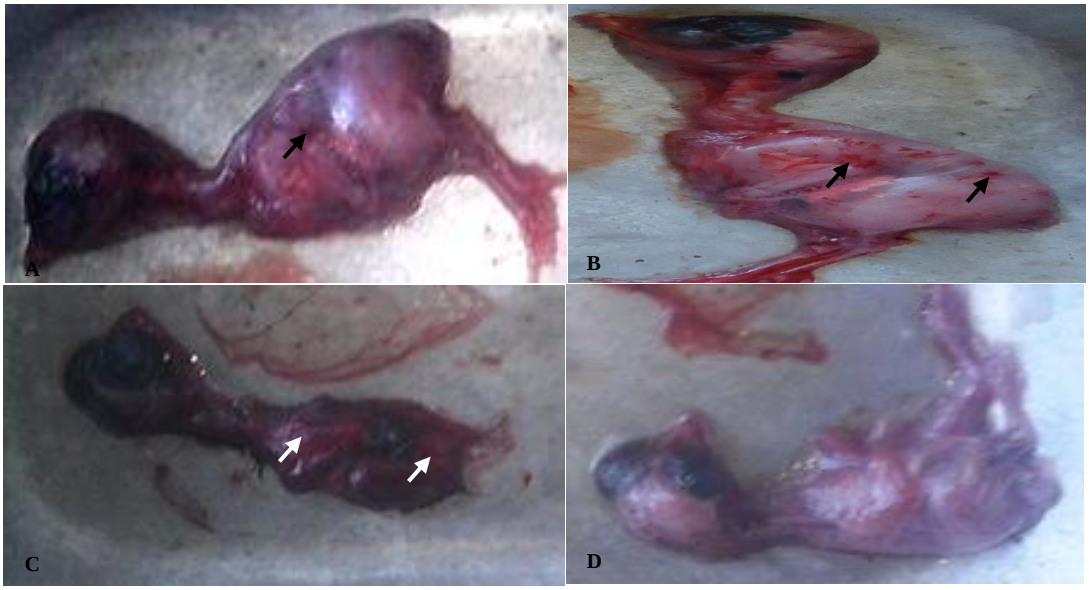

Figure 2A, B, C and **D**. Photographs showing haemorrhages on chicken embryos of ECEs inoculated with PGPV and FWPV strains. (A) Few haemorrhagic foci (arrow) on embryo of ECE inoculated with PGPV. (B) Embryos of ECE inoculated with REV-free FWPV demonstrating an increased number of haemorrhagic foci (arrows). (C) Embryo of ECE inoculated with REV-integrated FWPV demonstrating generalized haemorrhage (arrows). (D) Embryo of control ECE (not inoculated with any APV strain).

Table 2. Virulence scores based on the damage induced by PGPV and FWPV strains on chicken embryos as indicated by the extent of haemorrhage

Group	APV strains used for inoculation of ECEs	Virulence score	Reference
I	REV-free FWPV	2	Nguhiu-Mwangi
II	REV-integrated FWPV	3	and Mbithi (2007)
III	PGPV	1	
IV	Control[a]	0	

[a]ECEs were not inoculated with PGPV or FWPV strains. 0 = no haemorrhages, 1 = mild or slight haemorrhages, 2 = moderate haemorrhages, 3 = severe haemorrhages.

Table 3. Chicken mortalities related to inoculation of each virus inoculum by day 28 pi

Group	APV strains used for inoculation of chickens	Number of chickens			
		Inoculated	Infected	Contracted pox	Died
I	REV-free FWPV	35	35	35	0
II	REV-integrated FWPV	35	35	35	20
III	PGPV	35	35	0	0
IV	Control[a]	0	0	0	0

[a]Chickens were not inoculated with any APV strain.

Table 4. Virulence scores based on infectivity or damage induced by PGPV and FWPV strains on chickens as indicated by the number of cutaneous nodular lesions by day 28 pi

Group	APV strains used for inoculation of chickens	Virulence score
I	REV-free FWPV	1
II	REV-integrated FWPV	2
III	PGPV	0
IV	Control[a]	0

[a]Chickens were not inoculated with any APV strain. 0 = not affected, 1 = moderately affected (2 – 4 lesions), 2 = severely affected (> 4 lesions).

3.1.2.2 Necropsy findings

Gross examination of dead chickens revealed atrophy of bursa of Fabricius and thymus, spleen and liver necrosis, enlarged peripheral nerves, proventriculitis and enteritis.

3.1.2.3 Examination of cutaneous nodular lesions for the presence of FWPV

Successful isolation of FWPV from samples of cutaneous nodular lesions from chickens which developed fowl pox was indicated by the presence of characteristic pock lesions on CAMs. This was confirmed by detection of FWPV-specific DNA in samples of CAMs containing virus cultures, as indicated by migration of PCR products to approximately 578 bp which is an expected fragment size for the P4b gene amplicons for FWPV (Fig. 3).

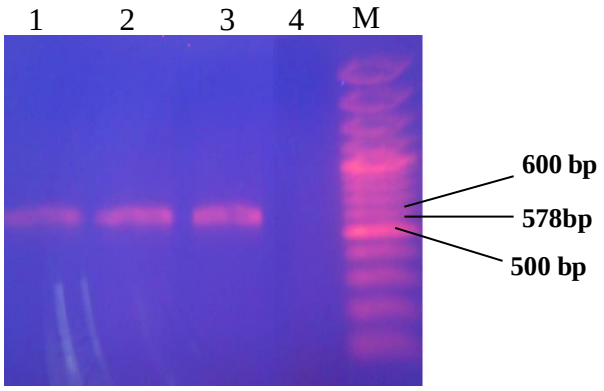

Figure 3. Agarose gel electrophoresis of PCR products of DNA extracted from samples of cutaneous nodular lesions collected from chickens inoculated with REV-free FWPV (lane 1) and REV-integrated FWPV (lane 2). Lanes 3 and 4 are positive and negative control, respectively. Lane M is a molecular weight marker with 100-bp increments. The amplicons migrated to approximately 578 bp, which is an expected fragment size for the P4b gene of FWPV.

3.1.2.4 Humoral responses

All chickens inoculated with the APVs showed antibody responses against the APVs antigens. The highest antibody titre was reached on day 20 pi. Chickens inoculated with PGPV had the highest antibody response, followed by chickens inoculated with

REV-free FWPV. Chickens inoculated with REV-integrated FWPV had relatively low antibody titres compared to chickens inoculated with PGPV or REV-free FWPV. Chickens in the control group did not show any humoral response (Fig. 4). No antibodies against the APVs antigens were detected in chickens before inoculation.

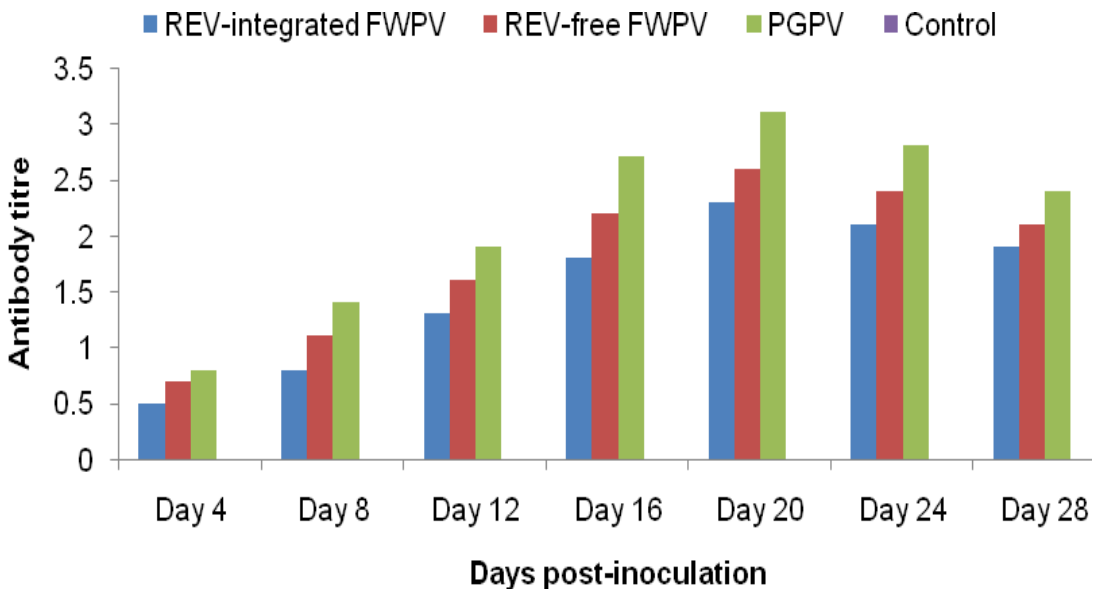

Figure 4. Humoral responses of chickens following inoculation with REV-integrated FWPV, REV-free FWPV or PGPV.

3.2 Discussion

In the present study, virulence characteristics of Tanzanian strains of FWPV and PGPV in chickens were evaluated both *in ovo* and *in vivo*. *In ovo* evaluation revealed that chicken embryos inoculated with REV-integrated FWPV were more affected, as indicated by the extent of haemorrhages, as compared to chicken embryos inoculated with REV-free FWPV or PGPV. This suggests that REV-integrated FWPV are more virulent in susceptible chickens than REV-free FWPV.

In *in vivo* evaluation; clinical examination revealed that chickens inoculated with REV-integrated FWPV developed severe fowl pox, as indicated by high number of cutaneous nodular lesions, as compared to chickens inoculated with REV-free FWPV. In addition to that, 20 of 35 (57%) chickens inoculated with REV-integrated FWPV died. Apart from chicken deaths; abnormal feathering, which is considered a pathognomonic clinical sign of reticuloendotheliosis caused by REV (Payne and Venugopal, 2000), was also observed in five of 35 (14%) chickens inoculated with REV-integrated FWPV. The relatively low antibody titres demonstrated in chickens inoculated with REV-integrated FWPV, as compared to antibody titres in chickens inoculated with PGPV or REV-free FWPV, could be attributed to the immunosuppressive effects of REV provirus integrated in the genome of FWPV to the chickens (Wang *et al*., 2012; Xue *et al*., 2013). It is likely that the integration of a near-full length REV provirus in the genome of a FWPV strain that was inoculated in the chickens gave rise to infectious REV which, in turn, caused immunosuppression and reticuloendotheliosis that led to deaths of 20 of 35 (57%) chickens inoculated with REV-integrated FWPV. Necropsy findings ruled out diphtheritic and systemic forms of fowl pox, which are usually characterized by high mortality rates (van Riper and Forrester, 2007; Alehegn *et al*., 2014). This suggests that gross lesions observed in internal organs during necropsy could be attributed to a near-full length REV provirus integrated in the genome of the FWPV strain inoculated to the chickens.

Isolation of FWPV from samples of cutaneous nodular lesions collected from chickens inoculated with REV-free FWPV or REV-integrated FWPV, which was demonstrated by presence of characteristic pock lesions on CAMs, and thereafter

confirmed by demonstration of FWPV-specific DNA in samples of CAMs containing virus cultures; indicates that the cutaneous nodular lesions on the chickens were attributed to inoculation of the chickens with the FWPV strains.

As opposed to a previous study by Weli *et al*. (2004) which demonstrated a Norwegian strain of PGPV isolated from a Norwegian wood pigeon (*Palumbus palumbus*) to be pathogenic in chickens, and more virulent in chickens than a vaccine strain of FWPV; the present study has demonstrated that the Tanzanian strain of PGPV isolated from domestic pigeons (*Columba livia domestica*) can infect but does not cause disease (pox) in chickens, thus ruling out the possibility of a PGPV strain currently circulating in Tanzania being one of the attributing factors to the increased incidences and prevalence of fowl pox currently experienced in the country.

With regard to humoral responses, antibody titres in chickens inoculated with REV-integrated FWPV were low as compared to antibody titres in chickens inoculated with PGPV or REV-free FWPV. This could be attributed to immunosuppressive effect of REV provirus integrated in the genome of FWPV to chickens inoculated with REV-integrated FWPV.

4. CONCLUSION

Based on the findings of this study it is concluded that integration of REV provirus in the genome of FWPV renders REV-integrated FWPV strains more virulent in susceptible chickens than REV-free FWPV strains; and the Tanzanian strain of PGPV isolated from domestic pigeons is not pathogenic in chickens. This implies

that the increased incidences and prevalence of fowl pox currently experienced in chickens in Tanzania are attributed to emergence of variant strains of FWPV which are REV-integrated. It also implies that, the Tanzanian strain of PGPV is not one of the attributing factors to the increased incidences and prevalence of fowl pox currently experienced in the country.

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ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (National Institutes of Health [NIH] publication No. 85-23, revised 1985) were followed, and the Tanzania Animal Welfare Act of 2008 was complied. All experiments were approved by the Research, Publication and Ethics Committee of the FVM, SUA, Tanzania. The reference number for the ethical approval is SUA/VET/012/03.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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CHAPTER EIGHT

8.0 GENERAL DISCUSSION

8.1 Significance and Implications of the Findings from this Study

This study was set to investigate genetic and biological characteristics of FWPV and PGPV currently circulating in chickens and domestic pigeons in Tanzania, respectively, focusing on development of appropriate fowl pox vaccine. The study has demonstrated high level of significance because of two reasons. First, the findings of the present study have provided the answers to the research questions; also have achieved the specific objectives presented in chapter 1. In other words this study has significant contribution to knowledge, because it has added the following information to the existing knowledge:

i. Ten (10) novel nucleotide sequences (GenBank accession numbers KF722858, KF722859, KF722860, KF722861, KF722862, KF722863, KF722864, KF722865, KF722866 and KF032407) of the P4b gene of 10 Tanzanian isolates of FWPV, and one novel nucleotide sequence (GenBank accession number KJ913659) of the P4b gene of a Tanzanian isolates of PGPV derived from this study have been deposited in the GenBank. These sequence data will be used as baseline data in future studies.

ii. Genetic characteristics of FWPV strains currently prevalent in various regions and geographical locations of Tanzania have been determined. Based on sequences of the P4b gene (ORF 167) the Tanzania isolates of FWPV

showed 99 – 100% identity to several published sequences of FWPV isolates (GenBank accession numbers AM050378, AM050379, AY453171, AY453172, AY530302, FR852586, GQ180201, GQ180212, GQ221269, GU108500, GU108501, GU108502, GU108503, GU108504, GU108505, GU108506, GU108507, GU108508, GU108509, JQ665838, JX464819 and JX464820) from various countries in different continents of the world, including Europe and Asia. Moreover, sequence analysis revealed that the Tanzanian FWPV isolates are 99.65 – 100% identical to each other. This close genetic relationship of the Tanzanian FWPV isolates to each other and to FWPV isolates from several other countries in the world demonstrates how highly conserved the P4b gene is, as previously reported (Jarmin *et al*., 2006; Manarolla *et al*., 2010). Phylogenetic analysis revealed that all analyzed Tanzanian isolates belong to clade A in subclade A1. These findings imply that based on sequences of the P4b gene the FWPV strains currently prevalent in Tanzania are genetically and phylogenetically closely related.

However, examination of the isolates for REV integration revealed that various genomic fragments of REV provirus are integrated in the genome of field strains of FWPV currently circulating in the country. This is a key finding because an understanding of the genetic diversity of FWPV currently prevalent in Tanzania is of great value for development of appropriate fowlpox virus vaccines for control of fowl pox in the country. Recent reports indicated that a DNA vaccine expressing *env* and *gag* offers partial

protection against REV infection in prairie chicken (*Tympanicus cupido*) (Drechsler *et al*., 2013), and a DNA prime-protein boost vaccination strategy could enhance both humoral and cellular immune responses in chickens against REV infection (Li *et al*., 2013). According to Hertig *et al*. (1997) infection of susceptible chickens with FWPV whose genome contains a near-full length REV may lead to dissemination of REV. This study (paper II) has revealed that most of field strains of FWPV currently circulating in Tanzania are integrated with near-full length REV provirus. This finding implies that, since REV has immunosuppressive effects to chickens (Liang *et al*., 2013; Xue *et al*., 2013), a recombinant vaccine against both FWPV and REV infections could be appropriate for a successful control of fowl pox in Tanzania and other countries with populations of FWPV which are genetically and antigenically similar to the FWPV strains currently circulating in Tanzania.

iii. REV contamination status of the imported commercial FWPV vaccines currently used in Tanzania for control of fowl pox has been determined. This study has revealed that the imported commercial vaccines currently used in Tanzania are REV-free.

This implies that the integration of various genomic fragments of REV in the genome of field strains of FWPV currently circulating in Tanzania could not be attributed to the imported FWPV vaccines currently used in the country for control of fowl pox.

iv. Genetic characteristics of a PGPV strain currently circulating in Morogoro region, Eastern Tanzania, have been determined. This study has revealed that based on nucleotide sequences of the P4b gene, the PGPV strain is 91% identical to FWPV strains that are currently prevalent in various regions and geographical locations of Tanzania.

This relatively low sequence homology (91% identity) implies that the APV strain that causes pox in pigeons in Morogoro region is genetically distinct from APV strains that cause pox in chickens in Morogoro and other regions of Tanzania.

v. Virulence (biological) characteristics of the Tanzanian strains of FWPV in susceptible chickens have been established. The present study has revealed that Tanzanian strains of FWPV which are integrated with genomic fragments of REV are more virulent in susceptible chickens than REV-free FWPV strains.

This finding is in agreement with a previous report by Singh *et al*. (2005) who demonstrated that REV-integrated FWPV strains, isolated from outbreaks of fowlpox in vaccinated chicken flocks in Minnesota and Nebraska, were more virulent to susceptible chickens than REV-less FWPV strains.

The increased virulence of REV-integrated FWPV strains could be attributed to immunosuppressive effect of REV provirus integrated in the genome of the FWPV to the chickens (He *et al*., 2004; Liang *et al*., 2013; Xue *et al*., 2013); which could explain the increased occurrence of fowl pox in Tanzania, characterized with high mortality rates of chicks and growers.

vi. Virulence (biological) characteristics of a Tanzanian strain of PGPV in susceptible chickens have been established. As opposed to a PGPV isolate from a Norwegian wild pigeon, a wood pigeon (*Palumbus palumbus*), that could infect and cause pox in chickens (Weli *et al*., 2004); this study has revealed that the Tanzanian PGPV strain currently circulating in Morogoro region can infect but does not cause pox in chickens. This finding implies that the Tanzanian PGPV strain does not pose a threat to chickens in the country

In addition to that, the present study revealed that chickens infected with the Tanzanian PGPV isolate had higher antibody titre compared to those infected with REV-free FWPV isolates or REV-integrated FWPV isolates. This implies that the PGPV isolate could make a good candidate for development of fowl pox vaccine.

Secondly, 66 novel isolates of FWPV and two novel isolates of PGPV have been derived from this study. One of the REV-free FWPV isolates (an isolate from Iringa region) was used for development of a tailored thermostable fowl pox vaccine. This

implies that the present study has contributed to development of the vaccine. Currently, mass production of the vaccine for commercial purpose is in progress. Once the vaccine is commercially available and used by poultry keepers for control of fowl pox in Tanzania and other countries having populations of FWPV which are genetically and antigenically similar to strains of FWPV currently prevalent in Tanzania, the study will have contributed to improved health and productivity of chickens, as well as improved poultry keepers' income and livelihood. As far as Tanzania is concerned, the ultimate outcome of this study could contribute to the national level goal of reducing poverty for improved people's livelihood within the framework of the National Strategy for Growth and Reduction of Poverty-Phase II, the National Development Vision 2025, and the first Millenium Development Goal.

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CHAPTER NINE

9.0 GENERAL CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusions

Based on the key findings reported in chapters 4, 5, 6 and 7; also outlined in chapter 8, the following conclusions are drawn:

i. Currently there is a heterogeneous population of FWPV in Tanzania comprising of strains which are integrated with various genomic fragments of REV and strains which are REV-free. This is a key finding because an understanding of the genetic diversity of FWPV currently prevalent in Tanzania is of great value for development of appropriate fowlpox virus vaccines.

ii. The increased occurrence of fowl pox currently experienced in Tanzania, characterized with high mortality rates of chicks and growers, could be attributed to increased virulence of REV-integrated FWPV strains as a result of immunosuppressive effect of REV provirus integrated in the genome of the FWPV to the chickens.

iii. The integration of various genomic fragments of REV provirus in the genome of most strains of FWPV currently prevalent in Tanzania is not attributed to imported commercial fowl pox vaccines currently used in the country for control of fowl pox, and therefore the vaccines can safely continue to be used in the country. The integration could be attributed to recombination between field strains of FWPV and field strains of REV.

iv. Based on nucleotide sequences of the P4b gene, the APV strain currently causing pox in pigeons in Morogoro region is genetically and phylogenetically distinct from APV strains currently causing fowl pox in chickens in Morogoro and other regions of Tanzania.

v. Unlike the PGPV isolate from a Norwegian wild pigeon, a wood pigeon (*Palumbus palumbus*) which infected and caused pox in chickens, the Tanzanian strain of PGPV currently circulating in Morogoro region is not pathogenic in chickens; therefore it does not pose a threat to chickens in the country.

9.2 Recommended future studies

Based on the findings reported in chapters 4, 5, 6 and 7; also outlined in chapter 8, the following studies are recommended:

i. More studies aiming at detection and characterization of PGPV isolates from other regions and geographical locations of Tanzania should be conducted in order to establish genetic, antigenic, virulence and immunogenic characteristics of PGPV currently circulating in the country. This recommendation is based on the fact that in the present study only one strain of PGPV currently circulating in Morogoro region, Eastern Tanzania, was obtained and characterized.

ii. Studies to determine pathogenicity and lethality of PGPV and FWPV in different host systems are required.

iii. Epidemiological features and risk factors for FWPV and PGPV transmission ability and spread should be investigated.

iv. Evolutionary characteristics of FWPV, PGPV and REV should be systematically studied to unravel possible factors that could be linked with their genetic and antigenic diversity.