MAKERERE  UNIVERSITY

SEROTYPES OF FOOT AND MOUTH DISEASE VIRUS IN AFRICAN BUFFALOES AND CATTLE IN QUEEN ELIZABETH NATIONAL PARK

BY

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DEDICATION

I dedicate this work to my wife Susan Ndibugaya and to my daughters Emmanuella Kogere and Estherine Kasiime.

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LIST OF ABBREVIATIONS

BHK	Baby Hamster Kidney
cDNA	Complementary Deoxyribonucleic Acid
cre	*cis*-acting replicative element
DANIDA	Danish International Development Agency
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agricultural Organization
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
GDP	Gross Domestic Productivity
H_2O	Water
IRES	Internal Ribosomal Entry Sites
kDa	Kilodaltons
M	Molar
MAAIF	Ministry of Agriculture Animal Industry and fisheries
ml	Militres
MW	Molecular Weight
NADDEC	National Animal Disease Diagnostic Epidemiology Center
NCBI	National Center for Biotechnology Information
NSP	Non structural Protein
OD	Optical Density
ODP	Optical Density Percentage
OIE	International Office of Epizootics
ORF	Open Reading Frame

PBS	Phosphate Buffered Saline
PI	Percentage Inhibition
PCR	Polymerase Chain Reaction
QENP	Queen Elizabeth National Park
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT-LAMP	Reverse Transcriptase Loop mediated isothermal Amplification
RT-PCR	Reverse Transcriptase Chain Reaction
SAT	South African Serotypes
SPBE	Solid Phase Blocking ELISA
SVD	Swine Vesicular Disease
TADEA	Transboundary Animal Diseases in East Africa
TMB	Tetramethyl benzidine
UBOS	Uganda Bureau of Statistics
UTR	Untranslated Region
UWA	Uganda Wildlife Authority
VNT	Virus Neutralization Test
VPg	Virion Protein Genome
µl	microliter

ABSTRACT

A cross-section study was done in Queen Elizabeth National Park (QENP) to determine the FMDV serotypes circulating in buffaloes and cattle. Serum and oro-pharygeal fluids (probang samples) were collected from African buffaloes (n=36) and cattle (n=114) from August 2011 to June 2012. Serum was screened using Priocheck FMDV NS ELISA and oro-pharyngeal probang samples were screened using Real-Time PCR. Serotype specific antibodies were determined by solid phase blocking ELISA (SPBE) and Virus Neutralization Test (VNT). The VP1 gene of positive PCR probang samples was amplified and genetic relatedness determined by construction of a phylogenetic tree. On Priocheck NS ELISA; 20/114(18%) cattle and 26/36 (72%) buffalo sera were positive. On SPBE; antibodies against serotypes; O (5/7; 71.4%), SAT 2 (1/6; 16.7%) and SAT 3 (2/8; 25%) in cattle and serotype; O 1/22(4.5%), A 1/8 (12.5%), SAT 1 (4/20; 20%), SAT 2 (5/29; 17.2%) and SAT 3 (3/23; 13%) in buffalo were detected. By virus neutralization test only antibodies against serotype; O 3/23 (13%) in cattle were detected, while in buffaloes it was only antibodies against SAT 2 (10/29, 35.4%) and SAT 3 (2/23, 8.6%) were detected. Real-Time PCR detected positive 3/20(15%) in cattle and (16/26) 61.5% in African buffalo. The VP1 gene, responsible for coding the major antigenic determinant of FMD virus, was used to characterize the SAT 2 sequence (UGA 11/13) that was got from buffalo. The phylogenetic analysis showed that it belonged to the East African buffalo lineage and was closely related to the previously isolated SAT 2 FMD sequences Buffalo 6 QE with pair wise identity of 83%, and Buffalo 10 QE with pair wise identity of 82%. The findings confirmed that SAT serotypes are found among African buffaloes in QENP. This study also showed that the circulating FMDV serotype antibodies in buffaloes and cattle were not the same since only antibodies against serotype; O were found in cattle sera while serotype; SAT 1 and SAT 2 in buffaloes by VNT.

CHAPTER ONE

1.0. INTRODUCTION

1.1. Background of the study

Foot-and-mouth disease (FMD) is a highly contagious and an economically important disease (Alexandersen and Mowat, 2005). It affects all cloven hoofed animals (Bronsvoort *et al*., 2004). The disease is believed to be endemic to most of the sub-Saharan African countries (Vosloo *et al*., 2002a). Foot and Mouth Disease is caused by foot-and-mouth disease virus (FMDV). This virus belongs to genus *Aphthovirus* and family *Picornaviridae* (Bastos *et al*., 2003). The FMD virus is non enveloped single stranded, positive sense RNA virus (approx. 8.3 kb) (Belsham, 2005; Bronsvoort *et al*., 2004). The RNA is surrounded by four structural proteins to form icosahedral capsid (Rueckert *et al*., 1996). The viral RNA is translated into a single polypeptide (L-P1-P2-P3) that is cleaved into the structural and non-structural proteins (Sorensen *et al.*, 1998b).

There are seven distinct FMDV serotypes and these include; O, A, C, Asia 1 and the Southern African Territories (SATs). Each of these serotypes has subtypes or topotypes (Samuel and Knowles, 2001a). Six of the seven serotypes have been found within Africa with exception of Asia 1 (Ansell *et al.*, 1994; Rweyemamu *et al*., 2008a; Vosloo *et al*., 2002b). The SAT 1, SAT 2 and SAT 3 are mainly endemic to sub-Saharan Africa, with SAT 3 mainly occurring in Cape buffaloes in Southern Africa (Thomson, 1994), SAT 2 is widely distributed all over sub-Saharan Africa (Brooksby, 1972; Ferris *et al*., 1992) and serotype; O is widely spread across the world (Knowles *et al.*, 2001; Reid *et al*., 2002).

Disease spread is mainly by inhalation of aerosolized droplets either by direct or indirect contact between animals (Grubman and Baxt, 2004). All tissues and discharges from infected animals are highly infective and will cause disease on contact with susceptible animals. The FMD virus Infection may occur indirectly through viral particles in milk, on dry material and tissues of slaughtered animals (NARO, 2001). The disease may also be airborne and can spread over considerable distances of up to 100 Km, if the relative humidity is above 60-70 % (Sutmoller and

Casas Olascoaga, 2003). The exhaled air from an infected animal acts as an important source of spread in temperate airs however this may not apply to dry areas such as East African region (Alexandersen *et al*., 2002a). The FMD spread by wildlife has not been well studied however it appears that infections in wildlife especially African buffalos and Impala (*Aepyceros melampus*) are subclinical (Thomson *et al*., 2003; Vosloo *et al*., 2009).

In Uganda FMD was first diagnosed in 1953 according to Ministry of Agriculture Animal Industry and Fisheries reports (NARO, 2001). Serotypes A and O were the first to be isolated and confirmed in eastern Uganda in 1953. The SAT 1 was first confirmed in Ankole in 1956 and in west Buganda in 1959 and C in the Aswa valley, Acholi in 1970. The SAT 3 has not been reported in cattle but was isolated from buffaloes in 1970 (Kalema-Zikusoka *et al*., 2005). West Nile, Kigezi and Bunyoro did not report FMD up to the early 1970's. After 1976, FMD was reported in several districts of Uganda. Initially only serotype O and A were involved in the outbreaks but later SAT 2 has also become significant in FMD outbreaks (NARO, 2001).

The FMD affects livestock and wildlife health globally (Hedger, 1981). From 1958 to 2000 there are an estimated 73 FMD serotyped outbreaks that have been published in cattle and few samples from healthy African buffaloes. About 31.5% of the out-breaks were attributed to serotype O, 26% to A, 24.7% to SAT 2, 13.7% to SAT 1, 2.7% to C and 1.4% to SAT 3 in livestock ((Vosloo *et al*., 2002a). However seroprevalence of FMDV serotypes in African buffalos in East Africa seems to be high for serotype; SAT 2 than the other SAT serotypes (SAT 1 and SAT 3) (Bronsvoort *et al*., 2008). The World organization for animal health (OIE: Office International des Epizooties), prohibits trade in livestock and livestock products from FMD affected countries or zones. This has led to exports of livestock products from Africa being limited to specific FMD-free zones in South Africa, Namibia and Botswana (OIE, 2009). Thus this is one of the reasons for low revenues from livestock products since FMD is endemic in Uganda.

In East Africa especially in Uganda further insight into understanding the molecular epidemiology of FMD is needed as well as the characterization of the disease. The molecular epidemiology of FMDV has been studied greatly through the understanding of the most variable capsid protein, the VP1 coding region of the virus genome (Knowles and Samuel, 2003). This

region has the major immunogenic sites that have been used to genotype the seven serotypes into topotypes. By comparing the sequences of VP1 coding region from different strains of FMDV, the circulating serotypes in cattle and African buffaloes can be determined (Knowles and Samuel, 2003). It is against the above background that this study was done to determine genetic relationship of FMD virus strains circulating in QENP.

1.2. Problem statement

Foot and Mouth Disease (FMD) is endemic in Uganda and ranked first among the notifiable infectious animal diseases (OIE, 2000). The FMD has debilitating effects including weight loss, low milk production, loss in drought power and it leads to restrictions on market access. These effects on livestock production and marketing lead to massive losses in revenue. The economy of Uganda is characterized by heavy dependence on agriculture and on foreign trade in agricultural products. Livestock is one of the major factors in providing valuable services and products for rural and urban households (LID, 1999), in 2008 about 71% of all households in Uganda owned livestock. And livestock production contributed 1.6 percent to total GDP in 2008, (UBOS/MAAIF, 2009). Uganda has an estimated cattle population of 11.4 million (UBOS/MAAIF, 2009). The FMD has been responsible causing severe economic losses in livestock keeping communities in Uganda (Rutagwenda, 2003). Currently in Uganda there have been frequent FMD outbreaks throughout the country for example between the period of 2001 and 2008, 311 FMD outbreaks in 56 district out of the 80 districts (Ayebazibwe *et al*., 2010b) have occurred, and also between January 2009 and May 2011, about 32 FMD outbreaks have occurred in different districts of the country (MAAIF reports) and serotypes O, SAT 1 and SAT 2 have been implicated in most of the out breaks (Vosloo *et al*., 2002b). African buffaloes (*Syncerus caffer*) are thought to play an important role as reservoirs of the SAT serotypes (Ayebazibwe *et al*., 2010b; Thomson *et al*., 2003) and it is believed that the disease is sometimes transmitted between livestock and buffaloes (Dawe *et al*., 1994; Sutmoller *et al*., 2000). Little is known about the relationship between the circulating FMD virus serotypes in African buffaloes and cattle in Uganda. It has been speculated that it is possible for the persistence of FMD in livestock to be linked to infections in buffaloes (Ayebazibwe *et al*., 2010b; Vosloo *et al*., 2002b). However, though Queen Elizabeth National Park (QENP) is a wildlife reservoir of FMDV and is located in Kasese district and the district is often involved in the FMD outbreaks, there have

been no reported FMD outbreaks since 2006 (Mwiine *et al.*, 2010). Therefore this study will help in understanding the genetic relatedness of the circulating FMDV serotypes in buffaloes and cattle and also elucidate on the prevalence of FMDV in African buffaloes and cattle.

1.3. Objectives of the research

1.3.1. General objective

To determine FMDV serotypes circulating in cattle and buffaloes within QENP.

1.3.2. Specific objectives

a) To determine the prevalence of FMDV antibodies in cattle and buffaloes in QENP.
b) To determine the FMDV serotypes circulating in cattle and buffaloes in QENP and their genetic relationship.

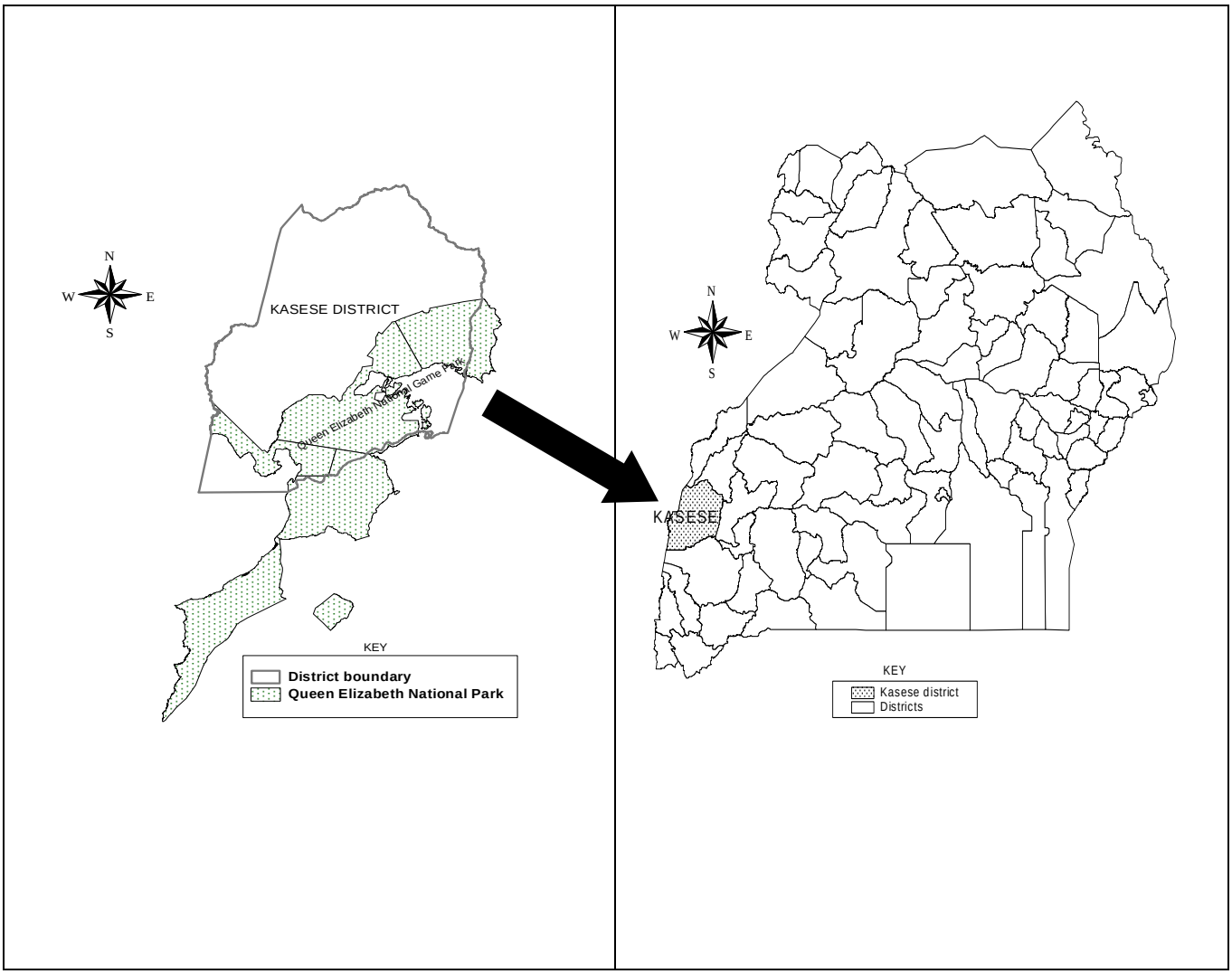
1.4. Research questions

Is the prevalence of FMDV higher in African buffaloes than in cattle in Queen Elizabeth National Park (QENP)?

Do African buffaloes and cattle in QENP have the same FMDV serotypes?

1.5. Significance of the study

The Queen Elizabeth National Park (QENP) is found in Kasese district in western Uganda (Figure: 1). QENP is occupied by cattle keepers whose cattle mingle at water points and graze with the freely roaming African buffaloes. This study aimed at understanding the prevalence of FMDV in both cattle and African buffaloes and also to know the relationship that occurs between the circulating FMDV serotypes in African buffaloes and cattle in QENP. This study will perhaps demonstrate the importance of African buffaloes as reservoirs in contributing to the spread and maintenance of FMD, an aspect that is unknown.



(a) (b)

Figure 1: (a) Map of QENP, (b) A map showing Kasese district in western Uganda

1.6. Justification of the Study

The FMD is endemic in Uganda, and has led to massive losses in revenue both at house hold and national level. This study was conducted in QENP because it contains settlement areas occupied by cattle keepers. Most times buffaloes and cattle from within and around the settlement areas end up mixing as they graze and drink water thus this interaction could lead to cross infections of FMDV between buffaloes and cattle. In East Africa the epidemiology of FMD is complex, due to poor monitoring animal systems, wild and domestic animal reservoirs (Ayebazibwe *et al*., 2010b). Currently little is known about the FMD virus populations circulating in these animals and about the serotypes involved or shared between livestock and wildlife buffaloes. The FMDV

so far serotyped in QENP indicates that SAT 1, SAT 2 and SAT 3 viruses exist in buffaloes in QENP. However, no SAT 3 has ever been confirmed in livestock in Uganda and it would be interesting to know if it certainly exists in livestock that graze close to buffaloes in QENP. Characterization of FMDV in QENP will promote serotype matching and improve the efficiency of vaccination programmes. This study will also promote conservation programmes, once the farmers or wildlife managers appreciate that both livestock and wildlife species may co-exist without significant transmission of FMDV. This is most likely the case since it is not a fact that in Uganda, most outbreaks occur around national parks (Ayebazibwe *et al.*, 2010c). It is also relevant to study the strains responsible for out breaks in the country.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Foot and Mouth Disease background

The FMD mainly affects cloven hoofed animals (Alexandersen and Mowat, 2005; Bastos *et al.*, 2003) and is endemic in most areas of Africa, Asia and South America. It is economically important to countries that internationally trade in animals and animal products (Mwiine *et al*., 2010; Rweyemamu *et al*., 2008b). It causes trade barriers that are being imposed on the affected countries thus affecting the livestock production and marketing (Sorensen *et al.*, 1998b).

The disease is caused by FMDV that has a remarkable ability to spread over long distances and to cause epidemics in previously free areas, as seen in the 2001 epidemic in the UK, France and the Netherlands and the outbreaks in South Korea and Japan in the year 2000 (Knowles *et al*., 2001). Seven major serotypes of the virus exist: the European serotype; O, A C Asia 1 and the Southern African territories (SATs) SAT 1, SAT 2, and SAT 3 (NARO, 2001). However there are a number of immunologically and serologically distinct subtypes for each individual serotype with different levels of virulence, especially within the A and O types. There are also some biotypes which get adapted to certain animals species and only affect others with difficulty.

The FMD manifests by fever, appearance of lesions on the teats, snout and feet, and lameness. The affected animals may also develop anorexia, shivering, excessive salivation and subsequent low milk production and weight loss (Alexandersen *et al*., 2003). The FMD viruses may occur in the oro-nasal secretions up to 3 days before and 7 to 14 after development of lesions (Sellers, 1971; Thomson, 1994). The virus sometimes persists in the osophago- pharyngeal region after infection of cattle for 2.5-3.5years and of African buffaloes for up to 5 years (Alexandersen *et al.*, 2003; Condy *et al.*, 1985b). The carriers are considered to be a potential risk for the spread of infection (Thomson *et al*., 2003). The virus is sometimes shade before the affected animal shows any clinical signs (Mansley *et al*., 2003).

The incubation period is about 2-3 days however it could go upto 14 days depending on the routes of the infection (Donaldson, 1994). Mortality may be low in adult animals however

morbidity can be very high and the disease can cause severe production loses (Vosloo *et al*., 2002b). Many susceptible wild species become persistently infected, the carrier status appears to occur only in ruminants, and however the virus may persist in absence of any obvious sign of the disease. These animals rarely transmit the infection to other species with which they are in close contact however African buffaloes are able to transmit the disease in the carrier state and thus play a big role in maintaining the virus (Dawe *et al.*, 1994; Thomson, 1996).

Two FMD cycles are said to occur in sub-Saharan Africa in which the virus circulates in the domestic animals and wild animals, in one cycle both the wildlife and domestic animals are involved. The other cycle only domestic animals are involved (Thomson and Bastos, 2004). This has been demonstrated in Southern Africa and in East Africa, where FMD viruses circulate between wildlife and domestic animals while in West Africa it is only the domestic animals that are involved (Vosloo *et al*., 2006).

Foot and Mouth Disease might have first occurred in the 15th century in 1514, when Hieronymi Fraeastorii described it as un usual disease affecting cattle (Wright, 1930). In the 18th century it was discovered that the disease agent causing FMD was ultrafiterable (Loeffler and Frosch, 1897). The virus is believed to have probably originated from Africa this is because of the great genetic variation in SAT types (Bastos *et al*., 2000; Bastos *et al*., 2003; Vosloo *et al*., 1995), and because of the sub-clinical cycle that occurs in African buffaloes (*Syncerus caffer*) in which the virus can persist in a single animal for up to five years and in an isolated herd for at least 24 years (Condy *et al.*, 1985b). The animals generate numerous antigenic and genetic variants as a result of the persistence of the virus (Vosloo *et al.*, 1996). The FMDV serotypes; O, A and C are widely distributed across the world and they have been isolated from many different parts of the globe (Grubman and Baxt, 2004).

The SAT serotypes are mainly distributed in the Southern African regions while Asia 1 is only limited to Asia (Ayelet *et al.*, 2009; Knowles and Samuel, 2003). In Uganda and other East African countries, FMD outbreaks have been mainly found to be due serotypes; O, A, SAT 1 and SAT 2 (Balinda *et al*., 2010; Balinda *et al*., 2009; Mwiine *et al*., 2010).

Serotype C was last detected in early 1990s and SAT 3 was last isolated in Uganda from buffaloes in 1971 (Vosloo *et al*., 2002b).

In Africa, the epidemiology of FMD in wild species has not been fully documented or understood and this has been due to wide range of host reservoirs involving the wild life and the domestic animal reservoirs, the wide spread movement of animals and presence of many serotypes and topotypes in Africa (Ayebazibwe *et al*., 2010b). However African buffaloes (*Syncerus caffer*) have been recognised as major reservoirs of the SAT-type viruses (SAT 1, SAT 2 and SAT 3); they may be carriers of the virus for several years (Condy *et al.*, 1985b; Thomson *et al.*, 2003). Other cloven hoofed wild animal species like the impala and kudu may be naturally involved in epidemiology of FMDV and may develop antibodies against FMD infection but their roles in excretion and transmission of the FMDV to other susceptible species has been less studied and also shown to be of less importance as compared to the African buffaloes (Ayebazibwe *et al*., 2010b; Bronsvoort *et al.*, 2008). For example the impalas (*Aepyceros melampus*) in South Africa have been shown to play an important role in the propagation of FMD outbreaks between livestock and wildlife (Vosloo *et al*., 2009).

In East Africa Serotypes O, A, C and SAT 1 and 2 have been many times isolated mainly from livestock and SAT 3 isolated a few times from African buffaloes. The SAT serotypes have been also found in many other sub-Saharan countries but it's only that those from East Africa belong to particular lineages (Bastos *et al*., 2003). The role played by buffaloes in the epidemiology of FMD serotypes other than the SAT serotypes is still unclear (Ayebazibwe *et al*., 2010b).

2.2. Foot and Mouth Disease virus genome

The FMDV genome is similarly organized like other members of the *Picornaviridae* family. The FMDV is a positive sense, single stranded RNA virus (Belsham, 1993). Its genome is approximately 8500 bases. The genome is surrounded by four structural proteins forming an icosahedral capsid (Rueckert *et al*., 1996). The virion (140S particles) consists of single-stranded RNA genome and 60 copies each of the four structural proteins (VP1 [1D], VP2 [1B], VP3 [1C], and VP4 [1A]). The virion also may contain one or two units of the structural proteins VP0 (1AB), the precursors of VP2 and VP4 (Acharya *et al*., 1989; Domingo *et al*., 1992), a copy of a

23 to 24 amino-acid genome linked protein, 3B VPg (Grubman, 1980). The virus is covalently linked to this small protein, VPg, at the 5'- terminus. The genome also contains the untranslated RNA, in the untranslated region upstream of 5'end (5'UTR) and downstream of the 3'end of the RNA open reading frame (ORF) (Robertson *et al*., 1985). The UTR has about 1300 bases and is divided into five segments which play an important role in the replication and translation of the RNA (Newton *et al*., 1985). These include the S-segment, the poly C tract, then just at the 3' end of the poly C tract is the pseudoknot, downstream of this, is the *cis*-acting replicative element (*cre*). Then between the cre and the ORF are the highly conserved stem loop structures which make up the internal ribosomal entry sites (IRES) (Costa Giomi *et al.*, 1984; Grubman and Baxt, 2004). Following ORF termination codon at the 3' end is the folded RNA stem loop structure and the poly A tract (Pilipenko *et al*., 1992). Translation of RNA yields a single polypeptide (L-P1-P2-P3). Cleavage of the polypeptide results into the structural from the P1 region and non-structural proteins (NSP). There are four structural proteins VP1, VP2, VP3, and VP4, encoded by genes 1D, 1B, 1C, and 1A respectively. The VP1, VP2, VP3 (MW≈24 kDa) contain surface components in the capsid, VP4 (MW≈8.5 kDa) is internal (Figure 2).

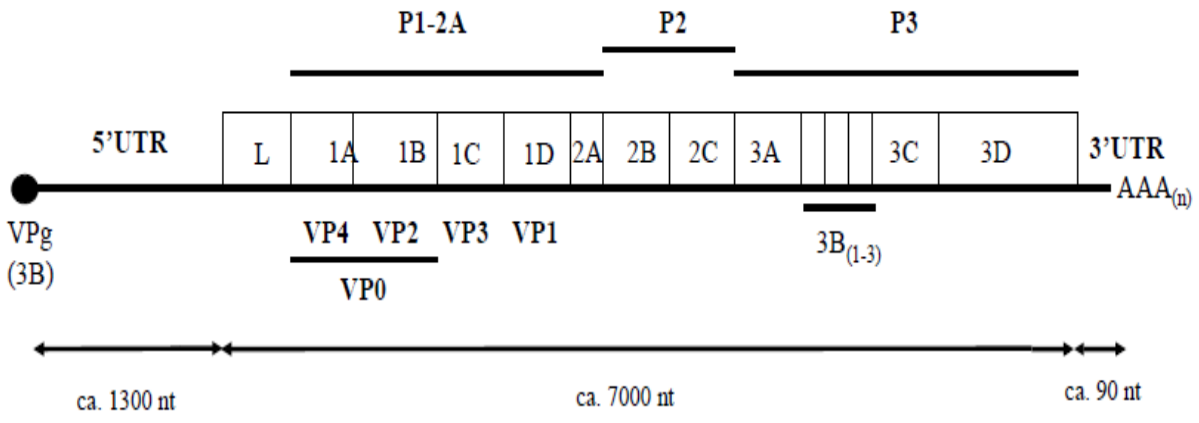


Figure 2: The FMDV genome structure

The genome structure includes the 5'untranslated region covalently linked to the small protein VPg, the polyprotein region (P1-2A, P2 and P3) and the 3'untranslated region. (Belsham, 2005)

2.3. Diagnosis of Foot and Mouth Disease

The FMD is usually diagnosed on farms through clinical symptoms however various laboratory techniques have been invented, for example; virus isolation by cell culture, immunologically through screening of antibodies against FMD non-structural proteins to detect FMDV infections through the detection of serotype specific FMDV antibodies by ELISA (Ferris *et al.*, 2009; Roeder and Le Blanc Smith, 1987). And the rapid detection of the FMDV nucleic acids by reverse transcriptase loop mediated isothermal amplification (RT-LAMP) (Notomi *et al.*, 2000), detection by real time polymerase chain reaction (Callahan *et al.*, 2002), and reverse transcriptase polymerase chain reactions and serotyping by use of serotype specific primers (OIE, 2009).

2.3.1. Clinical diagnosis/ differential diagnosis of Foot and Mouth Disease

Infection with FMDV can lead to development of vesicles on the feet, in and around the oral cavity and on the mammary gland of females. These vesicles rapture and then heal whilst coronary band lesions may give rise to growth arrest lines that grow down the side of the hoof. These changes can be used as an indicator as to when the infection occurred (MAAIF, 1980). The Vesicles can also occur inside the nostrils and at pressure points of the limbs especially in pigs. Severity of clinical signs varies with the strain of the virus, the breed of the animal and age, the exposure doze, the host species and the immunity of the animal. The FMD signs can range from mild to severe infection (Alexandersen *et al*., 2003) and death of the animals may occur in some cases.

2.3.2. Virus isolation of Foot and Mouth Disease viruses

This is performed by isolation of viruses through cell cultures or by inoculation of the viruses into unweaned mice (OIE, 2009). Mice of 2-7 days old and of selected inbreed are used however some field viruses require several passages in order to get adapted to the mice (Skinner, 1960). Then cultures are used, epithelium samples are taken from PBS/glycerol blotted on absorbent paper to reduce on the glycerol content since its toxic to cell cultures, and weighed. The sample is prepared by grinding in sterile sand in sterile pestle and mortar with a small volume of tissue culture medium and antibiotics. Medium is added until the final volume is 9x that of the epithelium sample. This is centrifuged at 2000Xg for 10 minutes. Suspensions with samples

screened both serologically and virologically to have FMD viruses are inoculated into uni-cell cultures or unweaned mice. The sensitive cell culture systems include bovine serum (calf) thyroid (BTY) cells and primary pig, calf of lamb kidney cells. Established cell lines like the BHK-21(baby hamster kidney) and IB-RS-2 cells can also be used but they are generally less sensitive than primary cells for the detection of low amounts of infectivity. The IB-RS-2 cells also can help in differentiation of swine vesicular disease virus (SVDV) from FMDV since SVDV only grow in cell of porcine origin. The sensitivity of any cell is tested with the standard preparation of FMD. The cells are then examined for cytopathic effect (CPE) after 48hrs and when no cytopathic effect is detected, the cells are frozen and thawed and (passaged again) used to inoculate fresh cultures. Again the CPE is detected after another 48hrs.

2.3.3. Serological tests

Serological tests used include Enzyme Linked Immunosorbent Assays (ELISAs) and these are the most preferred techniques for the viral detection and serotype identification of FMDV (Ferris *et al.*, 1992; Roeder and Le Blanc Smith, 1987). These tests have helped to detect antibodies against structural proteins in non vaccinated animals, infection in mild condition or in situations where epithelial tissues are not easy to collect. Antibodies against non-structural proteins have helped in detecting previous or current infections irrespective of the vaccination status. For example the priorcheck NS ELISA (used in this study) (Sorensen *et al.*, 1998b) that targets the 3ABC non structural proteins of FMDV, this helps to screen infected from non infected animals regardless of the serotype causing the infection and independent of the vaccination status. The ELISA used is indirect sandwich test. Multi-well micro-titer plates are coated with rabbit anti-sera to each of the seven serotypes of FMDV; these are the 'capture' sera. Then samples are added to each of the rows and appropriate controls also added. Then guinea pig anti-sera to each of the seven serotypes are added next followed by rabbit guinea pig anti-serum conjugated to an enzyme. Between each step there is extensive washing to remove unbound reagents. A color change following the addition of an enzyme substrate and the chromogen, a color change indicates positive reaction. Optical densities are determined using a spectrophotometer at an appropriate wavelength. The varied serotypes of FMDV can hence be identified. In these assays an absorbance greater than 1.0 above background indicates positive reaction and values close to one are confirmed by retesting. Other protocols are available with slightly different formats and

interpretation criteria (Alonso *et al*., 1992). However there serological limitations that involve cross reactivity as result of detecting antibodies of more than one serotype.

2.3.4. Nucleic acid recognition techniques

These methods include reverse transcriptase polymerase chain reactions (RT-PCR), real time polymerase chain reactions and rapid detection of FMDV using reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) (Notomi *et al.*, 2000; Reid *et al.*, 2002; Reid *et al.*, 2003). These are used to amplify the fragments of FMDV genome in diagnostic materials like milk, epithelial tissues and serum (Amarel-Doel *et al*., 1993). These techniques basically involve extraction of template viral RNA, and cDNA synthesis from the extracted RNA through reverse transcription then PCR amplification of the cDNA and finally detection of the PCR products. These techniques are available to only specialized laboratories however simplified systems for field use are under development (Callahan *et al*., 2002). These techniques are very important to the battle against animal diseases such as FMD. In this study, one step real-Time RT- PCR was used to screen for samples with viral RNA. This technique was targeting the 3D IRES region of the FMDV RNA strand which is a highly conserved region. Then these were subjected to conventional PCR targeting the 5' UTR region (multi-ii PCR) (Balinda *et al.*, 2010) and then VP1 region. The VP1 region was considered because it is the structural protein coding region and has been shown to vary significantly between strains and serotypes. Therefore it helped to determine the FMDV serotypes present before sequencing.

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study area description

A cross-sectional study was carried out on cattle and African buffaloes (*Syncerus caffer*) in Queen Elizabeth National Park (QENP) in Kasese district. The QENP is located in western Uganda across the districts of Kamwenge, Bushenyi, Rukungiri and Kasese. The park constitutes gazetted human settlement areas that include Katwe kabatooro and legally accepted fishing villages; Kahendero, Hamukungu, Kasenyi, Kisenyi, Katunguru K and B, and Katwe. The park also borders other settlements of Muhokya and Nyakatonzi (Figure: 3). The fishing and settlement areas within the park in Kasese district are mainly occupied by the Basongora tribe, a cattle keeping community. Queen Elizabeth National Park has a population of approximately 6,807 African buffaloes (UWA research and monitoring Unit, 2006) and Kasese district has an approximate of 17,000 to 140,000 heads of cattle (UBOS/MAAIF, 2009).

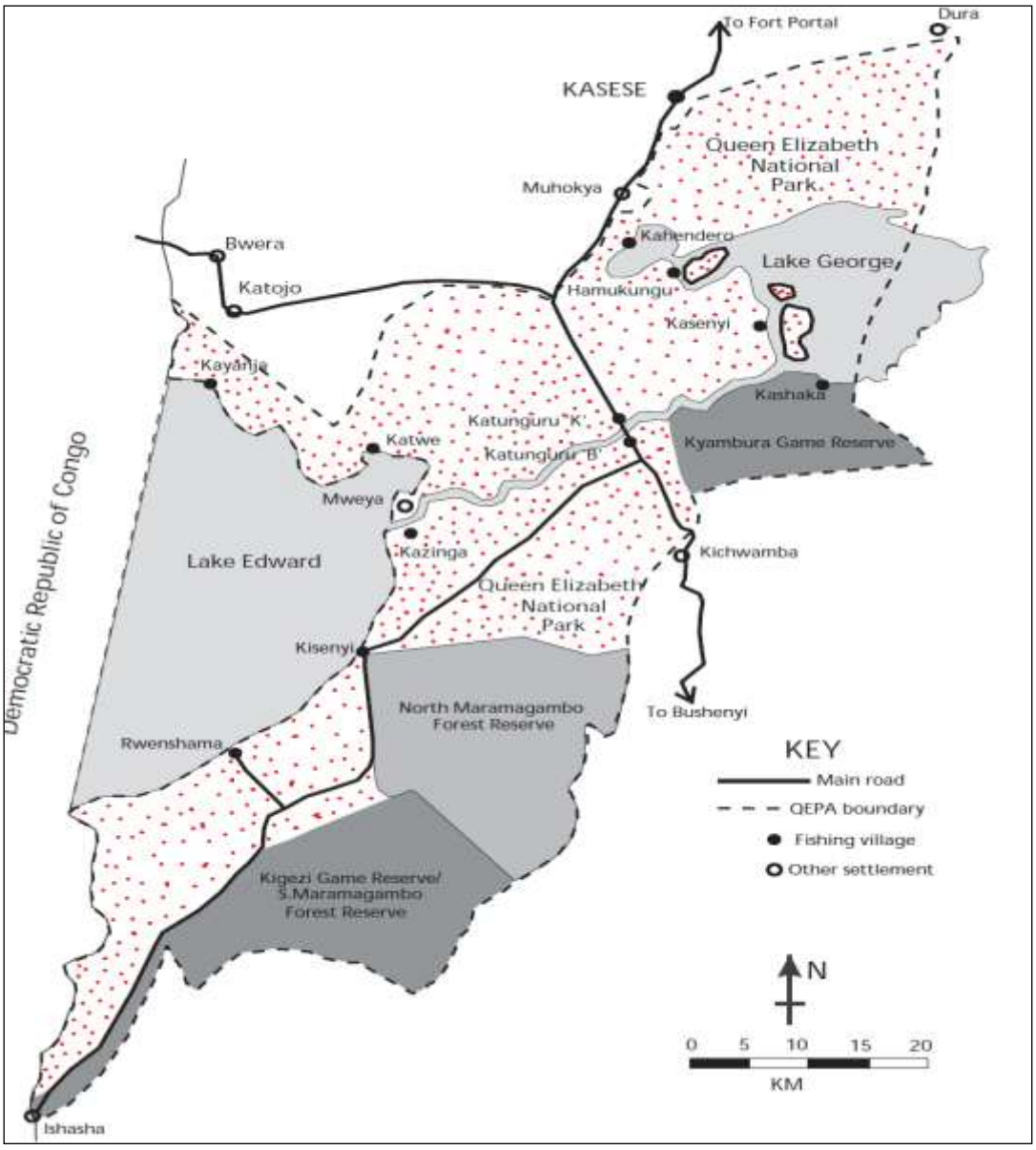


Figure 3: Map of QENP showing human settlement Areas, (Blomley, 2000).

3.2. Sample size

Buffalo and cattle sample sizes were determined by the Leslie Kish formula (Kish, 1965) below;

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

n- Sample size,

p - Prevalence of the disease, q=1-p, e - Desired level of precision.

Therefore for buffaloes sample size;

$$n = \frac{1.96^2(0.85)\,(0.15)}{0.05^2}$$

$$= 196 \text{ buffalo}$$

P=85% prevalence of FMDV antibodies in African buffaloes in QENP (Ayebazibwe *et al*., 2010a) and e =0.05

Cattle sample size;

$$n = \frac{1.96^2(0.37)\,(0.63)}{0.05^2}$$

$$= 358 \text{ cattle}$$

P = 63%, prevalence of FMDV antibodies in cattle in QENP (Mwiine *et al.*, 2010), E = 0.05.

3.3. Sampling method

Samples were collected from both buffaloes and cattle that graze within Queen Elizabeth National Park mainly from areas of Kasese district. The samples collected included serum and probang (oro-pharygeal fluids) samples.

3.3.1. Buffalo sampling

Sampling of African buffaloes was generally purposively done different herds of buffaloes from different localities, as guided by experienced rangers. Buffaloes were immobilized by chemical capture method (Ayebazibwe *et al.*, 2010b; Paling *et al*., 1979), this method, it involves immobilization of the identified buffalo for sampling (Harthoorn, 1976). Two cars were used, one for identifying the animal for darting and another for tracking the darted animal. Thus this made it possible to dart only one animal at a time.

Buffalo herds were located and one animal identified according to sex and size. The identified buffalo would then be darted by the veterinary doctor. Animals were darted with a Dan-Inject dart gun. (Age was estimated from the teeth from darted animals and all buffaloes fell within the age group used for rinderpest serosurveillance 1.8-20 years). The immobilization drugs or anesthetic combination were prepared in dosages according to age, weight and sex. These drugs included 8-10 mg Etorphine (Kyron, South Africa) and 70-90 mg Xylazine (Kyron,South Africa). The darted animal would be monitored and after 5-10 min it would be cautiously located

and approached. After making sure that it was fully sedated it was held by the horns and the head rose, then blind folded, the mouth opened and the tongue pulled out and examined for lesions and probang samples collected (appendix III). Meanwhile one of the team members would be monitoring the respiration of the animal. Samples would be taken and after sampling, the sedative was reversed by combination of drugs that included14-18 mg Diprenorphine and 60-70 mg Yohimbine (Kyron, South Africa) by intravenous infusion through the ear. Only thirty seven buffaloes were sampled, this low number of sampled buffaloes was due to some of the located buffalo herds dispersing before sedating any and sometimes running to inaccessible areas upon darting one of them. The use of two cars was also another limitation. At times it was hard to locate herds in some areas of the park as it is wide. Thirty six probang (oro-pharygeal fluids) and serum samples were collected from buffaloes in different areas of the park as indicated in the Table 1: below.

Table 1: Buffalo samples collected from different areas in QENP

	AGE GROUP AND SEX						
LOCATION	Adult (age ≥ 5 years)		Adult Total	Juvenile (1.8-5 years)		Juvenile Total	Total
	F	M		F	M		
QENP/Hakabale	0	0	0	1	1	2	2
QENP/Katunguru	2	2	4	2	0	2	6
QENP/Nyamugasani	3	1	4	2	1	3	7
QENP/Nyamunuka	0	2	2	0	0	0	2
QENP/Track3	2	1	3	2	1	3	6
Katwe	0	0	0	2	1	3	3
Kasenyi	4	2	6	2	2	4	10
Total	9	7	19	7	3	17	36

Key: **F**-Female, **M**- Male

3.3.2. Cattle sampling

Animals from herds of a few selected farmers were randomly sampled. In each herd, old animals mostly from 10 month and above were selected so as to avoid maternal antibodies (Mwiine *et al*., 2010). The age of the animals were gauged by examining at teeth (Mwiine *et al*., 2010) and consulting the farmers. Farmers were also interviewed about management practice, herd size and previous exposure to FMD and FMDV vaccination. Bias was avoided as much as possible by randomly selecting the animals with the right age for sampling. Cattle (114) probangs and serum samples were collected.

3.3.3. Serum samples

Blood samples were collected in sterile 10ml red top vacutainer tubes without anticoagulants, and 4.5ml serum extracted by centrifugation (1000rpm for 15min) method and aliquoted into cryo-vials. The serum aliquots were then kept at -20°C and transported to NADDEC (National Disease Diagnostics and Epidemiology Center) laboratory in Entebbe where they were stored at -20^{o}C until tested. The collected serum was then subjected to prior check NS ELISA to screen out the non-infected and then to Solid Phase Blocking ELISA (SPBE) to assess the antibodies against different FMDV serotypes.

3.3.4. Oro-pharygeal fluids (Probang samples)

Probang samples were collected from each of the restrained cattle and darted buffalo, from the oro-pharygeal cavity using a technique described by Kitching and Donaldson, (1987). Using this technique, prior to sampling 1ml of 0.04M phosphate buffer (appendix I) was added to cryo-vials and each was identified with a water proof label. After collection of the oro-pharyngeal fluid/probang samples, the oro-pharyngeal fluids were poured from the probang cup into 50ml falcon tubes and examined for the quality. About 1ml with some cellular contents was poured into the previously prepared vials containing 1ml of buffer and thoroughly mixed by gentle shaking. Samples heavily contaminated with ruminal contents were discarded and if possible sampling was repeated. Between sample collections from each animal, the probang cups were disinfected in a bucket containing 0.2% citric acid. After disinfection, the probangs were thoroughly rinsed in three separate buckets of clean water placed in series. After collection, samples in PBS were transported under liquid nitrogen to the laboratory for analysis. At the

laboratory the samples were stored at -70^{o}C or lower until they are worked on. Probang samples corresponding to the serum positive samples on NSP ELISA were subjected to molecular analysis tests which included Real-Time PCR to determine the prevalence of FMD viruses and Reverse Transcriptase PCR to serotype the FMD viruses.

3.4. Determination of the prevalence of FMDV antibodies in cattle and buffaloes in QENP

The prevalence of FMDV antibodies was determined by screening all the serum samples against non-structural FMDV proteins using the Priocheck® NS ELISA. This ELISA targets the 3ABC non-structural proteins of the FMDV. It detects animals infected with foot and mouth disease independent of the serotype that caused the infection and independent of the fact that animals were vaccinated or not. Serotype specific antibodies were determined by Solid Phase Blocking ELISA. This helped to determine which FMDV serotype was responsible for the infection in a particular sample.

3.4.1. Screening for antibodies against non-structural FMDV proteins by ELISA

Serum samples were screened using Priocheck® NS ELISA according to manufactures instructions (Sorensen *et al.*, 1998b). Briefly in this technique the 20µl of test samples and 80µl of ELISA buffer (appendix I) was added in the ratio of 1:5 respectively to all the wells on the micro-titer plates (the test samples were added to the micro-titer plates in duplicates), the plates were then sealed using enclosed plate sealers and incubated at room temperature with gentle shaking for 16-18hrs. After incubation, the plates were emptied and washed six times using 300µl of the washing solution for each wash (appendix I). Then 100µl of diluted conjugate (diluted 1/30 with ELISA buffer) were dispensed to all wells and the plates were sealed, and incubated for 60min at room temperature. After the 60min the plates were emptied and washed six times with 300µl of washing solution per wash. The plates were then tapped firmly after the last washing step on blotting paper. And 100µl of chromogen (TMB) substrate was dispersed to all wells, and incubated for 20min at room temperature. The reaction was stopped by adding 100µl of stop solution (1M sulfuric acid) to all wells.

Reading the plate and calculation of the results

The optical density (OD) was measured using MULTISKAN EX ELISA reader (Thermo Labsystems Oy, Helsinki, Finland) at 450nm within 15min after stopping color development. The mean OD_{450} value for the negative control wells was calculated (OD_{450} max).

The percentage inhibition (PI) of controls and test sera was calculated according to the formula below;

$$PI = 100 - \left(\frac{OD_{450}\ \text{test sample}}{OD_{450}\ \text{max}}\right) \times 100$$

Interpretation of results

The OD_{450} max of the negative control must to be >1.000, the mean percentage inhibition of the weak positive control must be >50%, and the mean percentage inhibition of positive control must be >70%. If any of these was not met then the results were discarded. PI =<50% negative (no antibodies against NS protein of FMDV), PI =>50%, positive against antibodies of NS protein of FMDV.

3.4.2. Serotype-specific ELISA (Solid Phase Blocking ELISA)

The serum samples which were positive on Priocheck NS ELISA were then subjected to Solid Phase Blocking ELISA (SPBE) (Ayebazibwe *et al.*, 2010a; Balinda *et al.*, 2009; Have and Holm Jensen, 1983; Mwiine *et al.*, 2010; Sorensen *et al.*, 1998a). Serum was screened at a dilution of 1:10 in SPBEs for antibodies against all the seven FMDV serotypes O, A, C, Asia1, SAT 1, SAT 2 and SAT 3. The SPBE ELISA was performed according to manufacturer's instruction. All seven FMDV strains as well as guinea pig and rabbit immune sera against the SAT-serotypes were provided by Lindholm, Denmark. Optimal dilutions of antigens, guinea pig sera and rabbit sera were predetermined for each of the seven serotype-specific antibody ELISAs, and the tests were run on separate micro-titer plates (Nunc-Maxisorp F96).

The plates were coated with 100µl of serotype-specific guinea pig immune sera, optimally diluted in carbonate buffer at a pH of 9.6. For guinea pig immune sera O-BFS was diluted at 1:1000, A-Iraque 1:4000, C-Turup 1:1000, Asia 1 Shamir 1:1000, and 1:1000 for SAT 1, SAT 2

and SAT 3 and incubated for one hour. The plates were then washed 3 times with 100µl of ELISA buffer (0.5M NaCl, 14.5mM KH_2PO_4, Tween 20 and distilled water) per wash.

Inactivated FMDV antigens (100µl) pre-diluted in ELISA buffer (O-Manisa diluted 1:5, A-Iraque 1:20, C-Noville 1:20, Asia 1 Shamir 1:10, SAT 1 1:10, SAT 2 1:2 and SAT 3 1:10) homologous to the guinea pig antiserum was then added and incubated for one hour. The plates were then washed 3 times with 100µl of ELISA buffer per wash.

After washing the plates, then 10µl of test sera and control sera were added in dilution of 1:5 for FMDV serotypes O, A, C and Asia 1 and 1:10 for the SAT serotypes in sample dilution buffer (ELISA buffer, 10% NCS, 0.05% sodium azide), then followed by addition of 90µl of sample dilution buffer to make 100µl per well. (Each plate contained duplicates of strong and weak positive control sera and four wells with negative control serum (Normal calf serum). The plates were incubated on an orbital shaker at room temperature for overnight.

The next day the plates were washed 3 times using 100µl of ELISA buffer per wash. This was followed by addition of 100µl of serotype-specific rabbit anti-sera diluted in ELISA buffer (containing 10% normal calf serum), for rabbit O-manisa 1:1000, rabbit A-Iraq 1:4000, rabbit C-Noville 1:2000, rabbit Asia 1 shamir 1:1000, and 1:1000 for SAT1, SAT 2 and SA3. This was followed by incubation at room temperature for one hour and then the plates were washed 3 times using 100µl of ELISA buffer per wash. After which 100µl of peroxidase conjugated swine anti-rabbit IgG (Dakopatts P0217) diluted 1:1000 in ELISA buffer (containing 10% NCS and 1% normal guinea pig serum) was added and then plates were incubated at room temperature for 30 min. Tetramethylbenzidine substrate (TMB) (1 part TMB mixed with 9 parts buffered hydrogen peroxidase) was added and plates left at room temperature. The reaction was stopped after 15min of color development by adding 1 M sulphuric acid. Reading of the plates was performed with a micro plate Multiskan Ascent spectrophotometer (Thermo Labsystems Oy, Helsinki, Finland) at a wavelength of 450 nm and with a reference wavelength of 620 nm and Ascent software 2.6. The results were expressed as optical density percentages (ODP).

Calculation of the OD percentage and interpretation of results

$$ODP = \frac{(OD_{sample})}{\text{mean } OD_{negative\ control}} \times 100$$

For each plate the mean OD value of the negative controls was calculated and divided into each individual sample OD value. This figure was then multiplied with 100 to give the ODP. The cut off values varied between serotypes; for ODP< 50% for serotypes O, SAT 1, SAT 2, SAT 3, <45% for type A and <35% for serotypes C and Asia 1, sera was considered positive. All sera with positive reaction for each of the seven serotype-specific ELISAs were titrated from 1:10 to 1:640. A serum sample was considered positive for antibodies against a particular serotype, if log10 (titer) was ≥80.

Accepted criteria for plates:

For each plate the variations between the four negative control wells must to exceed 20%. The OD of the negative control wells has to be in the range 0,500-1,000 (the 450 nm measurement after deducting the 620 nm measurement). However plates with negative control well OD-values in the range 0,400-1,500 were accepted.

3.4.3. Virus Neutralization Test (VNT)

This test was carried out in Lindholm, Denmark. It was carried out to check the cross reactions that have been implicated in serotype-specific ELISAs. The principle is that any antibodies present in the serum samples against a specific FMDV serotype will interfere with the adsorption of the virus to cell receptors, and thereby prevent the virus from infecting cells, where there are no antibodies; the virus will attack the cells suspended in wells of the micro-titer plates. By titrating the samples, the antibodies strength is determined.

3.5. Determination of prevalence of Foot and Mouth Disease viruses in QENP

To determine the prevalence of foot and mouth disease viruses in buffaloes and cattle in QENP, RNA was extracted from oro-pharyngeal fluids or probang samples. Then a one step Real-Time reverse transcriptase-PCR (rRT-PCR) for FMDV identification was done. The PCR used primers that targeted the 3D IRES region of the FMDV genome which is the most conserved region.

3.5.1. The RNA extraction

Ribonucleic acid (RNA) was extracted from oro pharyngeal fluid (from all samples for both buffaloes and cattle) using QIAamp RNA mini kit for purification of viral RNA from plasma, serum, cell-free body fluids and cell-culture supernatants (Qiagen Inc). The following steps were undertaken according to manufacturer's instructions. About 560µl of prepared AVL buffer containing carrier RNA (see appendix II) were pipetted into 1.5ml micro centrifuge tubes, and then 140µl of oro-pharygeal fluids added. These were pulse vortexed for 15s and then incubated at room temperature for 10min. The tubes were then centrifuged briefly to remove drops from the inside of the lid and then 560µl of absolute ethanol added to the sample, pulse vortexed and centrifuged to remove drops from the inside of the lid. Then 630µl from the above was applied to the QIAamp mini column (in 2ml collection tube) without wetting the rim. These were then centrifuged at 8000 rpm for 1min. after the filtrate was discarded and the QIAamp mini columns placed in new collection tubes. The columns were opened and 500µl of buffer AW1 added. Then centrifuged at 8000rpm for 1min and the filtrate discarded, the QIAamp mini columns were then placed in new collection tubes. The columns were carefully opened and 500ul of AW2 buffer added, the caps closed, the columns centrifuged at full speed (14000 rpm) for 1min. This process was repeated to eliminate possible chances of carryover of AW2. The QIAamp mini columns were placed in clean 1.5ml micro-centrifuge tube and the filtrate discarded. Then 60ul of buffer AVE kept at room temperature was added, the caps closed and incubated at room temperature for 1 min and centrifuged at 8000rpm for 1min. The QIAamp mini columns were discarded and the filtrate was stored at -20^{o}C till used. After RNA extraction one step real time reverse transcriptase-PCR was performed to both reverse transcribe the RNA in the sample to cDNA and also for amplification

3.5.2. The 3D Real-Time Reverse Transcriptase (rRT)-PCR

The 26 buffalo and 20 cattle samples were subjected to Taqman real-time RT PCR for the FMDV identification. This PCR was run using the master mix one step RT-PCR. The master mix consisted of 12.5µl of 2X reaction mix, 2.0µl of forward primer targeting the IRES 3D (5'-ACT GGG TTT TAC AAA CCT GTG A-3') and 2.0µl reverse primer (5'- GCG AGT CCT GCC ACG GA- 3') targeting the 3D region of FMDV genome.

The master mix also consisted of 1.5µl of the probe (FAM-TAMRA), 5'-TCC TTT GCA CGC CGT GGG AC-3' (Callahan *et al.*, 2002; Polichronova *et al.*, 2010; Reid *et al.*, 2002) 1.5µl RNAse free water and 0.5µl platinum Taq mix. Then 5µl of the extracted RNA (using QIAamp Viral RNA Mini kit) was added to the master mix and amplification was performed under the following conditions: Cycle 1:48 ^{o}C for 30min. (RT step), Cycle 2:95 ^{o}C for 10min and Cycle 3:40 ^{o}C cycles at 95 ^{o}C for 15 sec and 60 ^{o}C for 1min, the last cycle was repeated for 40 times. This process was performed by real time PCR machine spectrum 48. To confirm the serotypes, samples with expected threshold cut-off values were subjected to additional PCR targeting the cDNA corresponding to the VP1 region.

3.5.3. Genetic relatedness of FMDV circulating in cattle and buffaloes in QENP

To determine the genetic relatedness of FMD viruses circulating in buffaloes and cattle in QENP, sample which were positive by Rea-Time PCR were subjected to multi-ii PCR (+ve/-ve PCR). This PCR was used to screen against SAT serotypes and the other serotypes O, A, C and Asia 1. The VP1 coding regions of samples positive on multi-ii PCR were then amplified. These VP1 products were then sent to FMD reference laboratory in Lindholm for sequencing.

3.5.3.1. The cDNA synthesis

All the RNA from samples that were positive (16 buffalo and 3 cattle samples) on real time reverse transcriptase PCR was extracted above was reverse transcribed to cDNA. This PCR had reaction mix consisting of 3.0µl of 10x TaqMan RT-buffer, 6.6µl M $MgCl_2$ (25mM), 6.0µl dNTP (2.5mM each, with dTTP), 1.0µl Random hexamers, 0.6µl RNase inhibitor (20U/ul) and 0.75µ Multiscribe reverse transcriptase (100 U/µl Moloney RT). The total reaction was 30µl. And the reaction was run using THERMO Px2 machine under the following conditions: {(48^{o}C; 45 min) (95^{o}C; 5min) (5^{o}C; holding temp).

3.5.3.2. Confirmation of FMDV cDNA

To confirm the presence of cDNA a standard diagnostic PCR (+ or – PCR) targeting the 5' UTR of the FMDV RNA was carried out using two forward primers, Multi-II (F) and Multi-II SAT (F) and a reverse primer Multi-II (R-1) (Reid, 2002) Multi-II (F): 5'-CAC(T/C)T(T/C)AAG(G/A)TGACA(T/C)TG(G/A)TACTGGTAC-3', Multi-II SAT (F): 5'CAC(T/C)T(T/C)AAG(G/A)TAACA(T/C)TG(G/A)GACTGGTAC3', Multi-II(R-1):

CAGAT(C/T) CC (G/A) AGTG (T/A) C (I) TGTT-3' (Balinda *et al.*, 2010). The reaction consisted of 10pmol/µl forward primer and Reverse primer; 1.0µl of RNase free water, 12.5µl of 2x TaqMan Universal master mix in total per reaction 18µl. 7µl sample cDNA was added. The reaction was run using the THERMO PX2 M PCR machine (TECHNE) under the following conditions: 50^{o}C; 2min (UNG digestion) 95^{o}C; 10min. (40x (95oC; 15s-60^{o}C; 60s) (5^{o}C holding temperature). To determine the presence of cDNA in the amplicons, the amplified samples were electrophorised on 2% agarose gel stained in ethidium bromide, using a ϕx 174 marker and visualized under UV light. At the end of this analysis it was determined whether samples had the SAT serotypes or serotype O, A, C, and Asia 1.

3.5.3.3. Serotype specific PCR and sequencing

Samples positive from the +ve or –ve diagnostic PCR (6 buffalo and 2 cattle samples) were subjected to an additional PCR to determine the serotypes. This was through amplification of cDNA corresponding to the VP1 coding region using TaqMan Gold RT-PCR kit (Applied Biosystems, USA). For FMDV SAT1 and SAT 2, two sets of SAT specific primers were used: FMD SAT1-F 5'-ATGGGACACAGGTCTGAACTCGA-3', FMD SAT1-R 5'-GACATGTCCTCCTGCATCTG-3' and SAT 2-F 5´-TTAACTACCACTTCATGTACAC(CG)G-3´, SAT 2-R 5´-GAAGGGCCCAGGGTTGGACTC -3´ (Sangula *et al*., 2010). The PCR master mix constituted of 0.5µl of forward and reverse primers, 2.5µl of Tag buffer, 0.5µl of dNTPs (GACT mix), 1.5µl of $MgCl_2$ 16.75µl water, 0.25µl of amplitaqGold polymerase and 2.5µl of cDNA template. The total volume per reaction was 25µl. The amplification was performed under following conditions: 95°C for 5min for TaqGold activation, 95°C for 15s, 56°C for 1min for primer anealing, 72°C for 2min for strand extension. The three steps were repeated for 40 cycles followed by a final extension temperature of 72°C for 5min and a subsequent hold temperature of 5°C using THERMO PX2 M PCR machine (TECHNE). To determine the presence of cDNA, samples were loaded on 2% agarose gel stained in ethidium bromide, using a ϕx 174 marker and visualization under UV light. The amplicons were purified using QIAquick PCR purification kit (QIAGEN) PCR purification kit following manufacturer's instructions. The purified PCR products then were kept at -20^{o}C until sent for sequencing. The PCR products 4 from buffalo samples and 2 from cattle samples were sent to Lindholm FMD Laboratory (Denmark) for sequencing.

3.6. Data analysis

Descriptive statistics were performed. Both the sequence chromatograms of the forward and reverse sequences were visually analyzed using BioEdit (Hall, 1999) computer programme, assembled into contigs that resulted into overlaps. The consensus nucleotide sequence was manually aligned using the same programme. The reference serotype sequences were obtained from the NCBI Genebank (www.ncbi.nlm.nih.gov). The Multiple sequence alignments were made using Muscle (Edgar and Robert, 2004) incorporated in Mega 5.0 computer software (Tamura *et al*., 2007). The genetic relatedness of FMDV circulating in buffaloes and cattle in QENP was determined by phylogenetic inference from VP1 sequences obtained from the genebank. The genetic relatedness between VP1 coding sequences was determined by maximum likelihood method incorporated within MEGA 5.05 (Saitou and Nei, 1987).

The optimal tree (Figure 6) was constructed, bootstrap values ≥ 50, based on 1,000 replicates are indicated next to the relevant node (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa *et al*., 1985). The tree with the highest log likelihood (-5120.8117) is shown (Figure 6). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.3080)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 648 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

CHAPTER FOUR

4.0. RESULTS

4.1. Sero-prevalence of FMD in buffaloes and cattle

A total of 36 buffaloes and 114 cattle serum samples were screened against non-structural proteins (NSP) antibodies. The prevalence of antibodies against NSP was 18% (20/114) in cattle and 72% (26/36) in buffaloes**.** The 114 cattle serum samples and 36 buffalo serum samples were further screened at dilution of 1:10 by SPBE for antibodies against all the seven FMDV serotypes. There was no reaction for antibodies against serotype; Asia 1 in both cattle and buffalo serum. And there was no antibodies against serotype; C in buffalo serum. Cattle serum had high ODPs for antibodies against serotype O; 7/114(6%), A; 2/114(2%), C 1/114(1%), SAT1; 1/114(1%), SAT2; 6/114(5%) and SAT3; 8/114(7%). The buffalo serum had high ODP titers for serotype O; 22/36(61%), A; 8/36(22%), SAT1; 20/36(56%), SAT2; 29/36(81%) and SAT3; 23/36(64%) (Table: 2).

Table 2: The percentage positivity of cattle and buffalo samples on SPBE 1:10

FMDV serotypes

Animal species	**O**	**A**	**C**	**ASIA 1**	**SAT 1**	**SAT 2**	**SAT 3**
Cattle	7/114 (6%)	2/114 (2%)	1/114 (1%)	0/114 (0%)	1/114 (1%)	6/114 (5%)	8/114 (7%)
Buffalos	22/36 (61%)	8/36 (22%)	0/36 (0%)	0/36 (0%)	20/36 (56%)	29/36 (81%)	23/36 (64%)

The samples which were positive on SPBE 1:10 were further titrated in relevant SPBEs. Sera titers ≥80 was used as cut-off , a number of sera with titers above this cut-off were identified in SPBEs for serotypes O (5/7; 71.4%), SAT 2 (1/6; 16.7%) and SAT 3 (2/8; 25%) in cattle, and serotypes SAT 2 (5/12; 42%) and; O 4/22(18.2%), A 1/8 (12.5%), SAT 1 (4/20; 20%), SAT 2 (5/29; 17.2%) and SAT 3 (3/23; 13.4%) in buffalo (Table: 3).

Table 3: Titration of serotype specific antibodies against FMD viruses

		Number of positive sera per serotype			
		O	**SAT 1**	**SAT 2**	**SAT 3**
	Titers				
Cattle	≥80	3/6	0/20	1/6	1/8
	≤640	2/6	0/20	0/6	1/8
		O	**SAT1**	**SAT 2**	**SAT3**
Buffaloes	≥80	4/22	1/20	1/29	3/23
	≤640	0/22	3/20	4/29	0/23

-Titers are expressed on log 10 and the cut off is 80

The Virus Neutralization Test (VNT) only confirmed antibodies against O in three of 3 (3/23, 13%) cattle samples, while in buffaloes antibodies against SAT 2 (10/29, 35.4%) and SAT 3 (2/23, 8.6%) were found.

4.2. Prevalence of FMD viruses

The prevalence of FMD viruses in oro-pharygeal samples was 15% (3/20) in cattle and 61.5% (16/26) in buffaloes; the prevalence of FMD viruses was determined by Real-Time PCR.

4.3. Serotypes of FMD viruses in QENP

All the 16 buffalo and 3 cattle probang samples which were positive on Real-Time PCR were screened using two sets of primers (multi-II primers) against FMD SAT serotypes and the other FMDV serotypes O, A, C and Asia 1. Only 6 buffalo (figure 4) and 2 cattle (figure 5) probang samples yielded PCR products of approximately 100bp (DNA bands) with the multi II SAT (F) and multi II R-1 primers. No bands were seen with the multi-II F and multi II R-1 primers with both cattle and buffalo samples. The results show that all the samples (6 buffaloes and 2 cattle) had SAT serotypes.

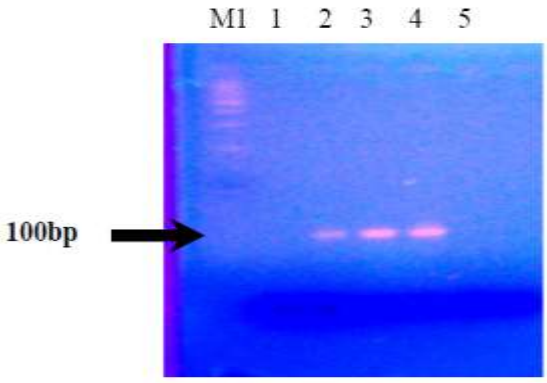


Figure 4: Agarose gel electrophoresis showing PCR products from buffalo probang samples.

The lanes 1-5 and are multi II PCR products (amplicons); lane 4 is the positive control, 5 is the negative control and no PCR product was seen. M1-is 100bp DNA ladder (Promega, madson WI, USA)

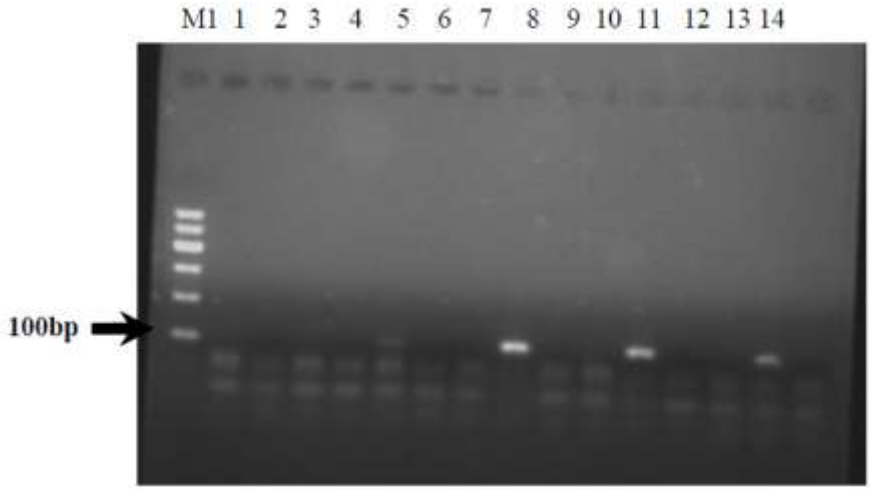


Figure 5: Agarose gel electrophoresis showing PCR products from cattle probang samples.

The lanes 1-14 are the multi II PCR products (amplicons) and lane 14 is positive control, 13 is negative control no PCR product was seen. M1-is 100bp DNA ladder (Promega, madson WI, USA)

4.4. Serotype identity of FMDV and sequencing results

The 6 buffalo and 2 cattle SAT positive probang samples were subjected to conventional PCR amplifying VP1 region. Primers targeting the VP1 region of both SAT 1 and SAT 2 were used.

Only 4 samples of the buffalo samples and 2 of cattle yielded PCR products. And only one sample Buffalo 11(UGA/BUFF11/13) was able to yield a DNA sequence. No sequence was obtained from any of the cattle samples from QENP as they had inadequate cycle threshold (CT)-values to be considered for sequencing. To determine which serotype the sequence represented, a nucleotide sequence comparisons were conducted using BLAST searches from National center for biotechnology information (NCBI). These showed that sequence obtained from buffalo sample (UGA/BUFF11/13) had the greatest sequence similarity to serotype SAT 2 FMDV isolates (Table 3). A comparison of the sample nucleotide sequence with selected FMDV sequences from the genebank shown in Table: 4 indicated that it had highest similarity with the Ugandan isolate from QENP BUFFALO 6 QE SAT 2 (HM067704) and BUFFALO 10 QE (HM067705) with 83% and 82% nucleotide identity respectively. The Genebank sequences from other countries had lower sequence similarity ranging from 71-75% (Table: 3).

Table 4: The FMDV sequences used in the phylogenetic analysis in this study

Accession number	Virus name	FMDV serotype	Host species	Year identified	Country of origin	% nucleotide identity
Ruhweza et al., (study sequence)	UGA/BUFF11/13	SAT 2	Buffalo	2013	Uganda	Reference sequence
AF367124	BOT/29/98	SAT 2	Buffalo	1998	Botswana	73
AF367136	ZIM/1/00	SAT 2	Buffalo	2000	Zimbabwe	73
HM623685	K52/84	SAT 2	Cattle	1984	Kenya	74
HM623708	K/59/07	SAT 2	Cattle	2007	Kenya	74
JX570628	KEN/11/2009	SAT 2	Cattle	2009	Kenya	73
AF137016	KNP/18/95	SAT 2	Buffalo	1995	S.Africa	71
AF137017	KNP/31/95	SAT 2	Buffalo	1995	S. Africa	72
AF367114	KNP/1/92	SAT 2	Buffalo	1995	S. Africa	71
AF367110	KNP/19/89	SAT 2	Buffalo	1989	S. Africa	72
AF367136	ZIM/1/00	SAT 2	Cattle	1996	Zimbabwe	73
Sabenzia *et al*., (unpublished)	KEN/MMB14/12	SAT 2	Buffalo	2012	Kenya	*

This study	UGA/BUFF11/13	SAT 2	Buffalo	2013	Uganda	*
HM067705	Buffalo 10QE	SAT 2	Buffalo	2010	Uganda	82
HM067704	Buffalo 6QE	SAT 2	Buffalo	2010	Uganda	83
Namatovu *et al*.,(unpublished)	U/30/12	SAT 2	Cattle	2012	Uganda	*
AF367134	RWA/1/00	SAT 2	Cattle	2000	Rwanda	75

Key: * there was no % nucleotide identity in the NCBI gene bank since the corresponding sequences in the table above are not yet published, KNP-Kruger National Park, RWA-Rwanda, K/KEN-Kenya, BOT-Botswana, ZIM-Zimbabwe, U/UGA-Uganda, QE/QENP-Queen Elizabeth National Park

4.5. Phylogenetic relatedness of sequence UGA/BUFF11/13 with those from the genebank

The complete VP1 coding sequences of FMDV serotypes SAT 2 (from the Genebank) and the test sample sequence (UGA BUFF 11/13 Ruhweza *et al*., (unpublished)) were used to construct phylogenetic tree (Figure: 6). The phylogenetic tree indicated two main lineages; one comprising East Africa Buffalo isolates and another lineage further subdivided into two sub-lineages of SAT serotype sequences from Kenya cattle and South African buffaloes. The test sequence (UGA 11/12) belonged to the East African lineage and was closely related to the previously isolated SAT 2 FMDV sequences Buffalo 6 QE (genebank accession number: HM067704) with pair wise identity of 83%, and buffalo 10 QE (accession number: HM067705) with pair wise identity of 82%. These two homologous sequences were obtained from the previous study conducted in 2010 (Ayebazibwe *et al*., 2010b). The buffalo sequence was however distinct from the SAT 2 cattle sequences from Kenya and Uganda with an average sequence divergence of 37.3%.

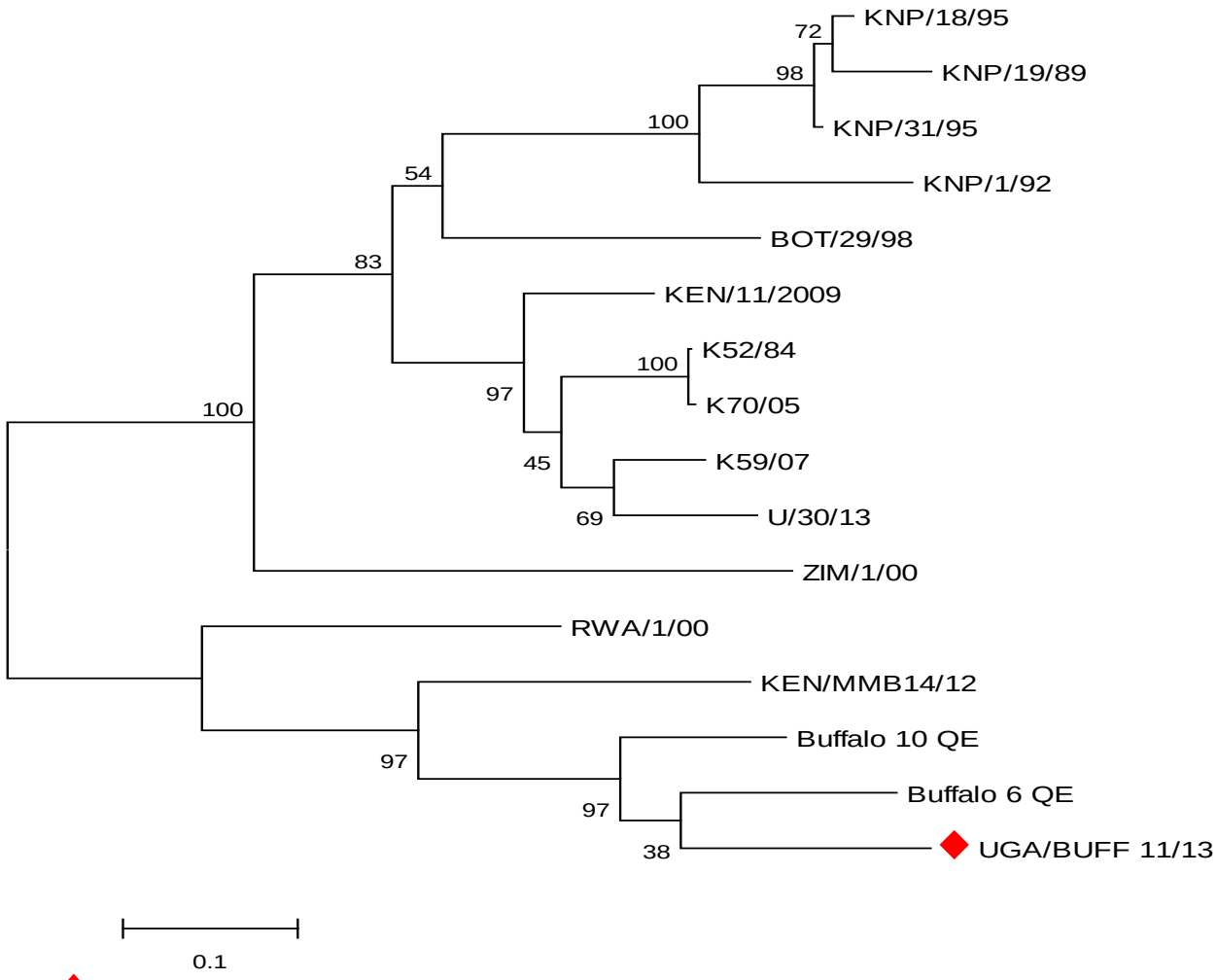


Figure 6: Maximum likelihood phylogenetic tree showing the relationship of SAT 2 VP1 coding sequences from East Africa and Southern Africa.

Key: Sequence from present study, KNP-Kruger National Park, QE- Queen Elizabeth National Park, K/KEN- Kenya, ZIM- Zimbabwe, U/UGA- Uganda, RWA- Rwanda, BOT- Botswana

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4.6. Amino acid alignment of SAT 2 VP1 coding sequences (216 amino acids) from East Africa and Southern Africa

The sequence was translated into an amino acid and compared with other sequences from the genebank. The amino acid alignment is shown in figure 7. Sequence K52/84 was used as a reference.

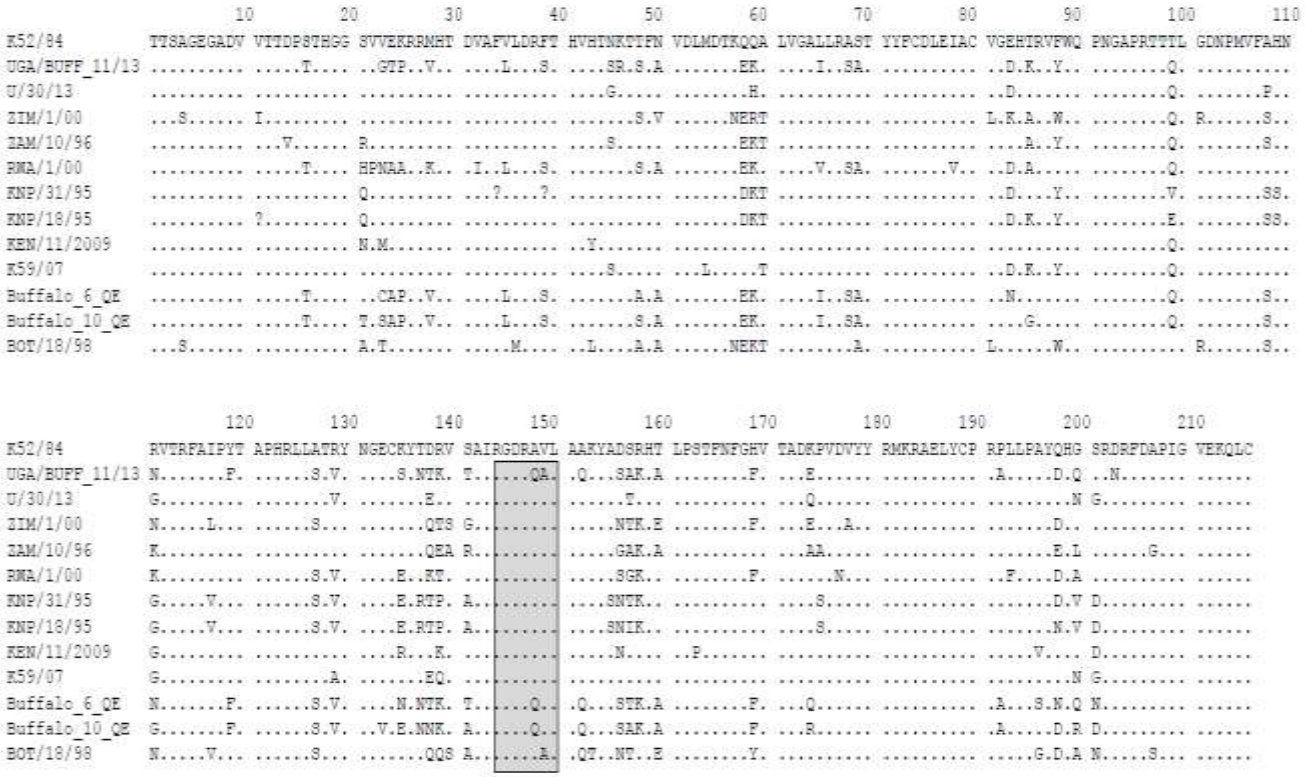

Figure 7: Amino acid alignment of 13 SAT 2 sequences of C-terminal region of VP1 from East Africa and South Africa.

Key: UGA/BUFF 11/13 sequence from present study, K52/84 reference sequence

Figure 7 shows the sequence alignment of the variable amino acids within the VP1 capsid protein coding region of approximately 216 amino acids. The alignment is comprised of 13 SAT 2 sequences (from buffaloes and Cattle) from the East and Southern African regions, these were compared to the vaccine strain, K52/84 (KEVEVAPI). A comparison between the test sequence (UGA/BUFF11/13) and reference sequence showed that 80.6% of amino acid were conserved.

The RGD motif which binds to the cellular integrin receptor located at residues 144–146 (in the highlighted box figure 7) was conserved. Residues flanking this motif were also conserved with the exception of position 148 and 149 (where an alanine was changed to glutamine; valine to alanine) respectively.

CHAPTER FIVE

5.0 DISCUSSION

This study was undertaken on healthy livestock and wildlife without any signs of FMD infection. No FMD outbreak had been reported in Kasese district since 2006. The last cattle vaccination was carried out in 2006. All serum was screened using Priocheck FMDV NS ELISA to detect potentially infected animals. Priocheck FMDV NS ELISA identified antibodies against FMDV in 18% of the tested cattle (20/114). In this study the prevalence of antibodies against non-structural proteins was low compared to the one reported by Mwiine *et al*. (2010) which indicated a prevalence of 63% in cattle in (or around QENP), however higher than 5%, the prevalence of non-structural proteins in cattle without clinical signs (Mwiine *et al*., 2010) within QENP (Katwe-Kabatoro) in Kasese district. The presence of non-structural protein antibodies against FMDV in cattle without clinical symptoms, and the fact that there has been no FMD outbreak since 2006 and last vaccination was carried out in 2006 in QENP could probably imply the possibility of FMDV infection in symptomless cattle.

This study revealed a prevalence of non structural antibodies of 72% of the African buffaloes (26/36) tested. This was slightly low compared to the study by Ayebazibwe *et al*. (2010) which showed a prevalence of 87% in QENP (Ayebazibwe *et al*., 2010a). Though these two studies were carried out at different times and they differ in the sample size. The high prevalence of non-structural protein antibodies realized in buffaloes was an indication that both buffaloes and cattle are exposed to FMDV.

The prevalence of FMDV antibodies was higher in buffaloes (72%) than in cattle (18%). The higher prevalence in buffaloes than in cattle might be an indicator of buffalo calves being naturally infected as early as 6 months when maternal antibodies wane out or due to the possibility of buffalo herds persistently being infected for up to 24 years with the all the FMDV SAT serotypes (Condy *et al.*, 1985a). The presence of non-structural proteins in cattle in an area which has not recently experienced out-breaks could probably be an indicator that carrier state in cattle could be prolonged for up to 3.5 years (Alexandersen *et al*., 2002b).

The antibodies were serotyped using a combination of serotype specific SPBE ELISAs and Virus Neutralization Test (VNT) techniques for both cattle and buffaloes serum samples. Sera of both cattle and buffaloes were serotyped at different SPBE titers. There were no antibodies detected for serotype; A, C and Asia 1 in both buffaloes and cattle. This agrees with the suspected absence of Asia 1 in Africa and the possible reports of disappearance of serotype C in East Africa (Vosloo *et al*., 2002a).

Some of the buffalo and cattle serum samples showed cross reactions against all the seven FMDV serotypes at low dilutions. This has been previously shown to be common especially in serotype specific SPBE ELISAs due to lack of specificity of this serological test (Mackay *et al*., 2001). The cross reactions could be due to repeated vaccinations with the multivalent vaccine (combining serotype; O, SAT 1 and SAT 3 strains) carried out to control FMDV infections from the previous outbreaks in cattle (Mwiine *et al*., 2010). In buffaloes this could be probably due to natural infections (Paling *et al*., 1979).

The serotype specific ELISAs indicated; serotypes O, SAT 2 and SAT 3 in cattle. This was in agreement with the previous study carried out in this area by Mwiine *et al*., (2010). Serotypes: O, SAT 1, SAT 2 and SAT 3 were found in buffaloes. This was the same with the previous studies done by Ayebazibwe *et al*., (2010) who found high antibody titers (≥ 160) of FMDV against FMDV serotypes; SAT1, SAT 2 and SAT3 in buffaloes and by Kalema-Zikusoka *et al*., (2005), who found SAT3 in buffaloes. In this study all SPBE titer values ≥ 80 were considered positive.

The serological finding of serotypes SAT 2 and SAT 3 after titration could have been due to cross reactions as SPBE ELISAs were prone to cross reactions *(*Ayebazibwe *et al., 2010b;* Mackay *et al*., 2001; Mwiine *et al.*, 2010). The serum samples were further tested using VNT which was conducted at National Veterinary Laboratory, Technical University of Denmark, Lindholm, 23 cattle and 26 buffalo samples were tested. This was done in order to confirm the serotypes circulating in both cattle and buffaloes in QENP. The VNT results were able to only confirm serotype O in three 3/23 (13%) cattle samples, this was in agreement with the findings of Balinda *et al*. (2009) who recovered serotype O and Mwiine *et al*. (2010) who found high titer values for antibodies against serotype O in Kasese district, therefore the circulating FMDV

serotype in QENP in cattle could probably be only serotype; O in cattle. The FMDV serotypes; SAT 2 and SAT 3 were found in buffalo samples. The VNT findings from this study still confirm the findings from previous work in buffaloes conducted by Ayebazibwe *et al*. (2010) who found SAT 1 and SAT 2 and Kalema-Zikusoka *et al*. (2005) who found SAT 3 in African buffaloes in QENP. Thus, there was indirect serological evidence that the FMDV circulating in buffaloes and cattle in this area are not the same and also absence of serotype C most likely indicating that it has been eradicated from Africa (Ayelet *et al.*, 2009; Roeder and Knowles, 2008). And it is not clear as to why the FMD virus may exist in cattle in QENP without clinical signs. The serological findings of this study still confirm that the serotypes responsible for most of the FMD outbreaks in cattle are mainly due to serotype O. However, antibodies were also detected against SAT 2 and SAT 3 in cattle.

Real-time PCR (rRT-PCR) confirmed presence of FMDV genome in OP fluids from 61.5% of buffaloes (72% of antibody positive buffaloes) and from 15% of the cattle (18% of the antibody-positive cattle). This confirmed serological findings in buffaloes presented in this work and previous work (Ayebazibwe *et al*., 2010). The number of positive cattle was higher than expected in an area without reported outbreaks for the last 5 years. This could be due to introduction of cattle from other areas depending on the age of the sampled cattle, be reminiscent carriers from the massive outbreak in 2006. A low level of subclinical circulation of FMDV cannot be excluded.

The high positive samples with FMDV in both cattle (3/20) and buffaloes (16/20) by real time PCR and the fact that few of these samples (buffaloes; 6/16 and cattle; 2/3) were able to have enough DNA materials to be detected with multi II PCR (+ or – diagnostic PCR) and the fact that one sequence from only buffalo samples was obtained is an indication that the real time reverse transcriptase PCR (rRT-PCR) is a more sensitive test. This was in agreement with previous studies by Shaw *et al*., 2004 and Feris *et al*., 2006 who found out that the rRT-PCR had superior sensitivity compared to other established methods used in diagnosis of FMD. It has also been published that rRT-PCR detects FMD virus in a sample with greater sensitivity than the conventional RT-PCR and other diagnostic procedures (Jeirani *et al.*, 2012).

To determine the FMDV serotypes present, all the samples that tested positive on Real-time PCR were further tested by multi-II PCR using two different primers (multi II SAT (F) and multi II F and single reverse primer multi II (R-1) (Balinda *et al*., 2010). The PCR products of approximately 100bp (DNA bands) with the multi II SAT (F) primer and the multi II R-1 primers observed in buffalo samples were suggestive of the FMDV SAT serotype cDNA. The absence of any PCR product with the multi II F and reverse primer multi II (R-1) indicated absence of FMDV cDNA for the other serotypes that is O, A, C and Asia 1 in buffaloes oro pharyngeal probang samples. This further confirmed the findings from previous work by Ayebazibwe *et al*. (2010) and Kalema-Zikusoka *et a*l. (2005) that FMDV SAT serotypes are circulating in African buffaloes in QENP.

Virus isolation was performed and a SAT 2 virus was recovered from one sample UGA/BUFF11/13, the VP1 region was amplified (648 bp) and sequenced. There was a low recovery of virus isolates 16% (1/6), this could have been due to long transportation time of samples in the field, the difficulties encountered to maintain the cold chain both during transportation and storage as it is well known that Kasese is a very hot area, the equator crosses QENP and it also lies in the rift valley with low humidity. These factors could have led to virus recovery. The results actually indicated the difficulty of isolating viruses from carrier animals since high CT values (meaning low quantities of DNA) are obtained. This makes it difficult to obtain good sequences from such samples. It could also be attributed to the short comings of the sampling technique and to the time sampling. Most times sampling was carried out when animals had already grazed thus animals would have a lot of ingesta in the pharynx, water and fluids these would affect the quality of probang sample.

The test sequence (UGA/BUFF11/13 Ruhweza *et al*., unpublished) was closely related to the previous SAT 2 isolate Buffalo 6 QE and Buffalo 10 QE (accession number: HM067705) (genebank accession number: HM067704) (Ayebazibwe *et al.,* 2010b) with nucleotide sequence similarity of 83% and 82% respectively, this indicated that they belong to the same topotype as suggested by previous scholars if the nucleotide difference is $\leq$ 20% (Balinda *et al.*, 2010; Knowles and Samuel, 2003; Samuel and Knowles, 2001b; Samuel and Knowles, 2001c).

The maximum likelihood method (Hasegawa *et al*., 1985) was used to determine the genetic relatedness between VP1 coding sequences. The phylogenetic tree indicated that the test sequence (UGA/BUFF 11/13) belonged to the East African buffalo lineage and was closely related to recently isolated SAT 2 buffalo virus from Kenya (KEN/ MMB14/ 12 Sabenzia *et al*., (unpublished) and previously isolated buffalo SAT 2 isolates from Uganda; Buffalo 6 QE (genebank accession number: HM067704) and Buffalo 10 QE (accession number: HM067705) (Figure: 6). This sequence was also distinct from the cattle SAT 2 isolates from Kenya and Uganda with an average sequence divergence of 37.3%. This implied that it was not related to the recently isolated SAT 2 virus (U/30/13 Namatovu *et al*., (unpublished) from Isingiro in Western Uganda. The 37.3% was greater than 5% indicating that test sequence and the recently outbreak isolated sequence (U/30/13 Namatovu *et al*., unpublished) from Isingiro from Uganda may not from the same epizootic. This could indicate that buffaloes may probably not be the source of the virus for the current SAT 2 out-breaks in Uganda. However if buffaloes harbor FMDV, the risk of transmission of FMDV to cattle still remains. It has been indicated that where FMD has been controlled in African buffaloes, it has also tendered to disappear in livestock as well (Thomson, 2002). However control of FMD in wildlife involves expensive measures like fencing off the park.

Evidence of prolonged FMDV SAT serotypes in buffaloes in QENP and absence of corresponding outbreaks in associated livestock indicates that perhaps control measures should independently target wildlife and livestock as it is un usual for infection to cross between livestock and wildlife species. In fact, FMDV serotype; O has never been isolated from wildlife in Uganda and neither SAT 3 has been isolated from cattle in Uganda.

The buffalo sample size was determined by Leslie and Kish formula and 358 samples were expected to be collected. However this was not possible as many there were many challenges encountered during the sampling exercise. These included; the sampling exercise was too expensive therefore only two cars were mostly used, making it hard to locate and also control the located herds not to run away before darting, most of the sampling was done during the hot season and this made it difficult to locate buffalo herd as most of them move to water points and shades that were hard to reach, sometimes located herds dispersed into inaccessible bushes and

thus making it hard to dart. These factors made it hard to dart buffaloes for sampling. However sampling was purposive and 36 buffaloes were darted and sampled. We tried as much as possible to sample from different herds and parts of the park.

CHAPTER SIX

6.0. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

- Non-structural protein ELISA and Real-Time PCR results indicated that buffaloes were more exposed than cattle. Serological results indicated; serotype; O in cattle and SAT 2 and SAT 3 in buffaloes. Serotype; SAT 1 could not be completely ruled out as indicated by the SPBE results.

- Although SAT 2 was obtained from buffaloes, previous isolates from cattle outbreaks in Uganda did not group closely with that of buffaloes in the present study.

- Much as the risk of transmission may be high at the Livestock Wildlife interface, it may not be true that they share the same viruses.

6.2. Recommendations

- I recommend that there is need to institute control measures in livestock and wildlife at the QENP interface.
- More surveillance for the different FMDV serotypes in cattle and buffaloes should be undertaken in QENP.
- The control of FMD should be laboratory based rather than on clinical symptoms.
- There is need to study epidemiological role of other wildlife species like the Uganda Kobs, impalas, in the spread and maintenance of FMDV in QENP.

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APPENDICES

Appendix I: Preparation of collecting medium for samples

Collecting medium for specimens of epithelium

Add 0.04 M phosphate buffer, 3.05 gm Na_2HPO_4.2 H_2O, 0.39 gm $KH_2PO_4$to 500 ml sterile distilled water, I ml I% phenol red, antibiotics . And adjust pH to 7.2-7.6 with HCI.

Probang and epithelium

To each 500 ml of 0.08 M or 0.04 M phosphate buffer add the following amounts of reconstituted antibiotics: Penicillin 2.5 ml (final concentration 1000 units/ml), Mycostatin 1.0 ml (final concentration 100 units/ml), and Neomycin 1.0 ml (final concentration l00 units/ml,) Polymyxin 0.5 ml (final concentration 50 units/ml).

Collecting medium for probang samples

Add 0.08 M phosphate buffer, 6.11 gm Na_2HPO_4.2H_2O, 0.78 gm KH_2PO_4 to 500 ml sterile distilled water, 1ml l% phenol red, antibiotics. And adjust pH to 7.2-7.4 with HCI.

Antibiotics reconstitution

Penicillin phial of 500,000 units add 2.5 ml sterile distilled water, Mycostatin phial of 500,000 units add 10 ml sterile distilled water, Neomycin phial of 500,000 units add 10 ml sterile distilled water, Polymyxin phial of 350,000 units add 7 ml sterile distilled water.

Appendix II: Preparation of laboratory reagents for testing of samples

ELISA buffer pH 7.2

0.5M NaCl	292.2g
14.5 mM Na_2HPO_4	25.844g
2.5 MmkH_2PO_4	3.4g
Tween 20	5.0ml
Distilled H_2O	10.0l

Sample dilution buffer

0.5M NaCl	29.22g
14.5 mM Na_2HPO_4	25.844g
2.5 MmkH_2PO_4	0.34g
Tween 20	0.50ml
NaN3	0.5g
Distilled H_2O	1.0l

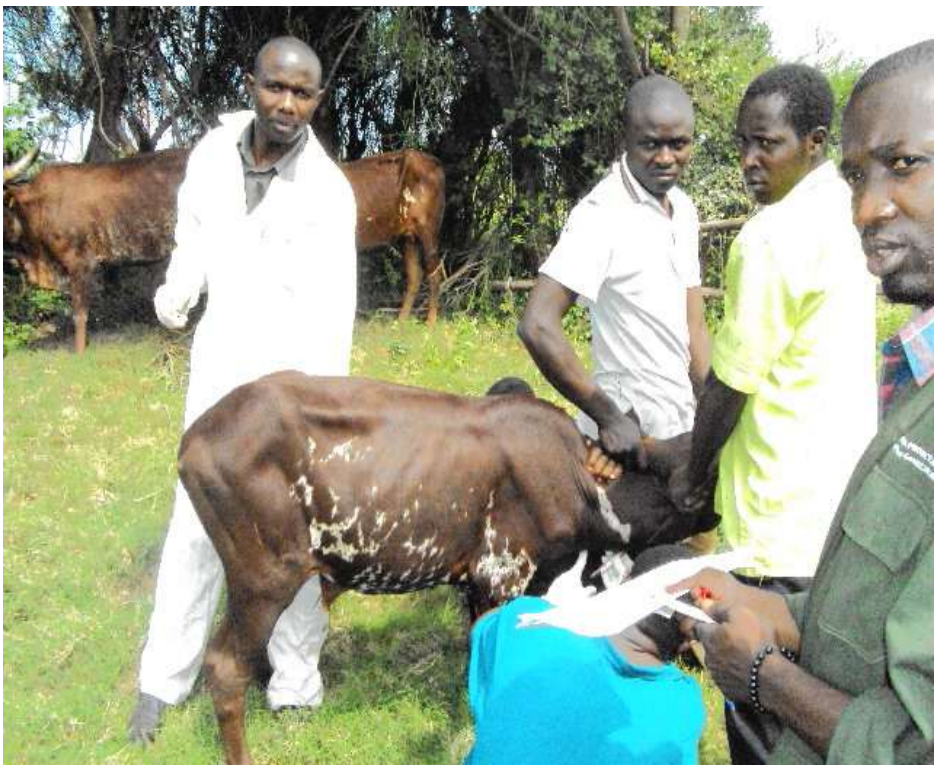
Add normal calf serum up to 10% before use

Buffer AVL containing Carrier RNA

Add 310ul of buffer AVE to tube containing 310ug of lyophilized carrier RNA and mix thoroughly. Then mix the solution with AVL buffer in the ratio of 1:1000. Store between 2-8^{o}C

Appendix III: Buffalo sampling in Katwe (QENP) (a), cattle sampling in Katwe-kabatooro (QENP) (b)

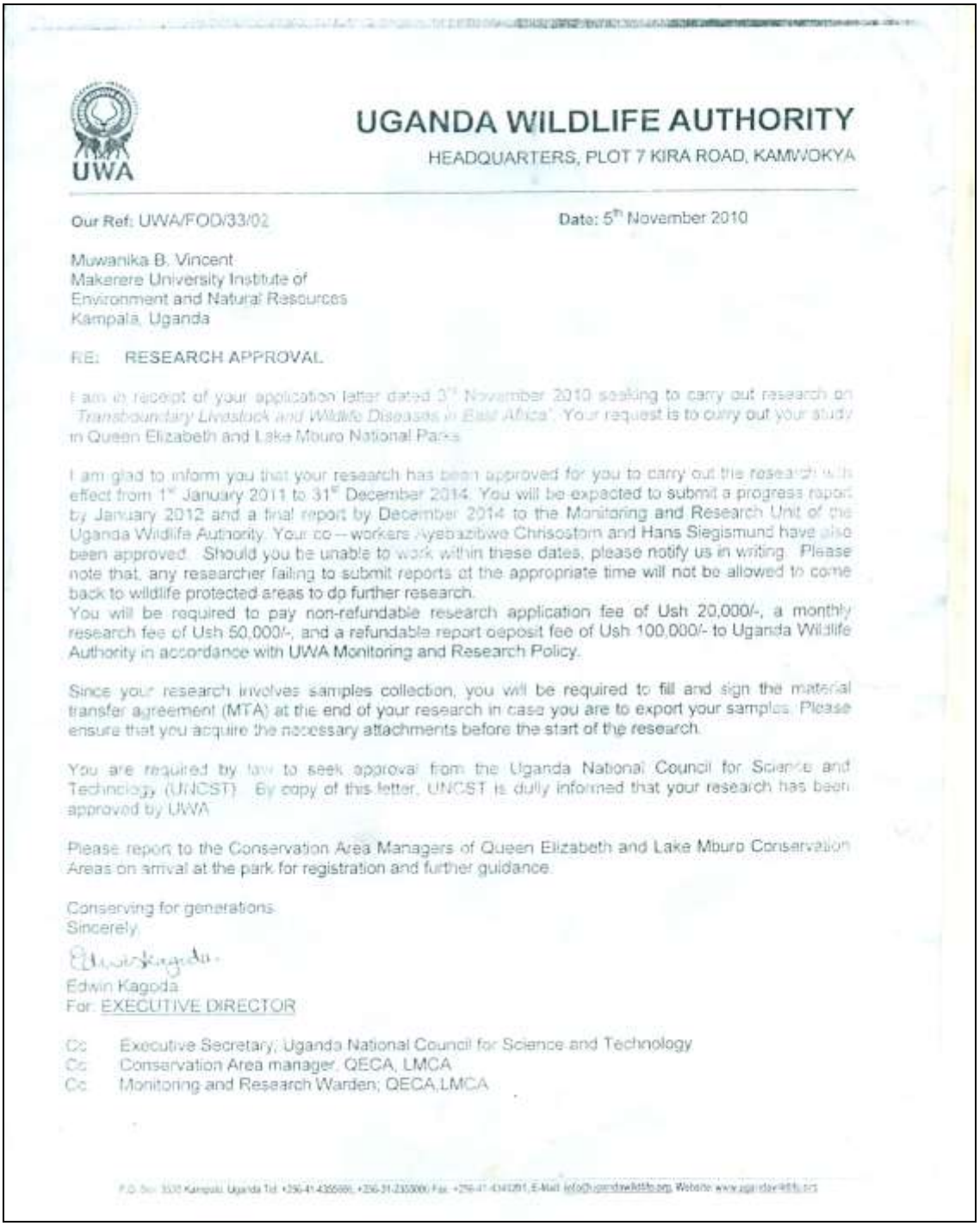
(a)



(b)

Appendix IV: Buffaloes and cattle grazing together in QENP in Katwe-kabatooro

Appendix V: The Park permit to collect samples from Queen Elizabeth National Park



UWA

UGANDA WILDLIFE AUTHORITY

HEADQUARTERS, PLOT 7 KIRA ROAD, KAMWOKYA

Our Ref: UWA/FOD/33/02 Date: 5th November 2010

Muwanika B. Vincent
Makerere University Institute of
Environment and Natural Resources
Kampala, Uganda

RE: RESEARCH APPROVAL

I am in receipt of your application letter dated 3rd November 2010 seeking to carry out research on *Transboundary Livestock and Wildlife Diseases in East Africa*'. Your request is to carry out your study in Queen Elizabeth and Lake Mburo National Parks.

I am glad to inform you that your research has been approved for you to carry out the research with effect from 1st January 2011 to 31st December 2014. You will be expected to submit a progress report by January 2012 and a final report by December 2014 to the Monitoring and Research Unit of the Uganda Wildlife Authority. Your co – workers Nyebazibwe Chrisostom and Hans Siegismund have also been approved. Should you be unable to work within these dates, please notify us in writing. Please note that, any researcher failing to submit reports at the appropriate time will not be allowed to come back to wildlife protected areas to do further research.
You will be required to pay non-refundable research application fee of Ush 20,000/-, a monthly research fee of Ush 50,000/-, and a refundable report deposit fee of Ush 100,000/- to Uganda Wildlife Authority in accordance with UWA Monitoring and Research Policy.

Since your research involves samples collection, you will be required to fill and sign the material transfer agreement (MTA) at the end of your research in case you are to export your samples. Please ensure that you acquire the necessary attachments before the start of the research.

You are required by law to seek approval from the Uganda National Council for Science and Technology (UNCST). By copy of this letter, UNCST is duly informed that your research has been approved by UWA.

Please report to the Conservation Area Managers of Queen Elizabeth and Lake Mburo Conservation Areas on arrival at the park for registration and further guidance.

Conserving for generations.
Sincerely,

Edwin Kagoda
For: EXECUTIVE DIRECTOR

Cc Executive Secretary, Uganda National Council for Science and Technology
Cc Conservation Area manager, QECA, LMCA
Cc Monitoring and Research Warden; QECA,LMCA

P.O. Box 3530 Kampala, Uganda Tel: +256-41-4355000, +256-31-260000 Fax: +256-41-4341291, E-Mail: info@uwa.or.ug, Website: www.ugandawildlife.org