INTER-EPIDEMIC MOLECULAR CHARACTERIZATION OF FOOT-AND-MOUTH DISEASE VIRUSES IN EASTERN AND NORTHERN UGANDA BETWEEN 2008 AND 2009

KASAMBULA LORDWIN (BBLT-MAK)

REG No: 2007/HD17/8971U

A THESIS SUBMITED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTERS OF SCIENCE DEGREE IN MOLECULAR BIOLOGY AND BIOTECHNOLOGY OF MAKERERE UNIVERSITY

FEBRUARY 2011

Declaration

I Kasambula Lordwin, hereby declare that this is a result of my own work and has never been submitted to this University or any other institution for any award. This has been submitted with the approval of my supervisors:

Name : KASAMBULA LORDWIN

Reg : 2007/HD17/8971U

Sign : ……..

Date : ……

Supervisors:

Dr. Charles Masembe (BVM, MSc. PhD)

Molecular Biology Laboratory,

Institute of Environment and Natural Resources

Makerere University.

Sign…….

Date ……

Prof Graham Belsham (PhD, DSc.)

Professor in Exotic Virus Diseases

Technical University of Denmark

National Veterinary Institute

Lindholm

4771 Kalvehave

Denmark

Sign.....

Date.....

DEDICATION

I dedicate this work to my dearest wife Joy Akiiki and beloved son Joel Kisembo Akiiki.

ACKNOWLEDGMENT

I would like to thank my supervisors Dr. Masembe Charles and Prof. Graham Belsham for accepting to supervise this work and for the valuable time they spent with me right from sample collection up to this report write up. I wish to thank my sponsors DANIDA, through the LIVESTOCK-WILDLIFE DISEASES IN EAST AFRICA PROJECT at Makerere University Institute of Environment and Natural Resources (MUIENR). Special thanks to the Director of MUIENR Prof. Frank Kansiime and the Project Manager Dr. Vincent Muwanika for timely release of funds needed for this project and for paying my tuition fees.

I would like to thank Dr. SN Balinda and Dr. AK Sangula for always giving me time to consult them. Thanks go to the entire staff of MUIENR for the encouragement they kept giving me during the study period. Thanks go to the Director of Joint Clinical Research Centre (JCRC) Prof. Peter Mugyenyi for permitting me to go for further studies. Special thanks go to the Head of Human Resources at JCRC Mr. Bitwire Herbert and all members of staff of JCRC for constant support and encouragement.

I thank my wife Ms Joy Akiiki and my son Joel Kisembo Akiiki for the care and support they gave me. My thanks also go to Prof. GW Lubega for the support he gave me through the course. I thank Ms Mary Frances Nakamya and Ms Nalugwa Allen for their guidance, support and encouragement.

I also thank Dr. Wilford Lordson Kirungi for the support he gave me. I can't forget to thank my fellow MMB students and fellow student Dr. S Lule who received the same fellowship. Lastly, I thank all the lecturers and staff of the Faculty of Veterinary Medicine.

List of abbreviations

cDNA	Copy Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
ELISA	Enzyme Linked Immunosorbent Assay
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
IRES	Internal ribosomal entry site
Kb	Kilo bases
MAAIF	Ministry of Agriculture animal industry and fisheries
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcriptase
SAT	South African Territories
SNAP	Simple and aqueous phases
SVD	swine vesicular disease
UTR	Untranslated Region

ABSTRACT

Nucleotide sequences of the VP1 coding region of foot-and-mouth disease viruses (FMDVs) obtained from FMD outbreaks in Eastern Uganda in April 2008 and in Northern Uganda in January 2009 were determined. Comparison of these VP1 nucleotide sequences with those of other FMDVs indicated the viruses from these outbreaks in the two regions belonged to FMDV serotype O. Bayesian Phylogenetic analyses of these sequences showed that the viruses from the two regions were closely related and belonged to topotype EA-2. Therefore the outbreaks in the two regions were due to the same serotype and strain indicating introduction of the virus from a single source. It is likely that the outbreaks in Northern Uganda were introduced from the Eastern region particularly across Lake Kyoga. This has significant implications on the effectiveness of FMD control measures in Uganda.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 Background

1.1.0 Foot-and-Mouth disease and its economic importance

Foot-and-mouth disease (FMD) is a highly infectious disease that affects domestic and wild cloven-hoofed animal species (Bastos *et al.*, 2003; Quian *et al.*, 2003). The disease has direct and indirect economic consequences resulting from constraints in international trade in animals and animal products originating from infected countries. It results in reduced productivity through reduced milk yield, loss of weight, abortions, delayed conception (James and Rushton, 2002). According to the 2007/2008 and 2008/2009 national budgets, the government of the Republic of Uganda spent over 24 billion and 17 billion Uganda shillings in these years (£7.5 million and £5.3 million respectively) to control FMD through short term control measures. These included ring vaccination, zoo sanitary measures, and quarantines to restrict the movement of livestock and livestock products to and from the affected areas as well as suspension of slaughtering of animals in abattoirs within the affected areas. The potential for high economic loss is exemplified by the devastating 2001 FMD epidemic in the United Kingdom that resulted in a total cost of over £5 billion (John, 2002). FMD is currently classified as an infectious disease (OIE, 2009).

1.1.1 Spread FMD

FMD is mainly spread through direct and indirect contact. Direct spread involves mechanical transfer of droplets from infected animals to susceptible ones. Indirect spread is through contaminated personnel from infected farms who may carry the virus to susceptible animals in another farm. Airborne transmission over long distances has been reported from pigs that exhale large quantities of the virus and can thus pass it to susceptible ruminants (Alexandersen *et al.*, 2002). This mode of FMD spread is only true for temperate region and is of no significance to the drier regions of East Africa (Alexandersen *et al.*, 2002).

1.1.2 The Virus

The etiological agent of FMD is a small, non-enveloped, positive-sense, single stranded RNA (8.4 kb in length) virus belonging to the genus *Aphthovirus* of the family *Picornaviridae* called foot-and-mouth disease virus (FMDV). There are seven immunologically distinct FMDV serotypes globally (Stanway *et al*., 2005). These serotypes are A, O, C, Asia1, SAT-1, SAT-2 and SAT-3. Apart from Asia1, the other six FMDV serotypes have each been reported on the African continent at some time (Kitching 1998; Knowles and Samuel, 2003).

The genome contains one open reading frame (ORF) encoding L^{pro} and capsid proteins: VP1, VP2, VP3 and VP4 (VP1-4) as well as nonstructural proteins: 2A, 3A, 3B, $3C^{pro}$ and $3D^{pol}$ (Anon, 2002). The genome is subject to a high rate of mutation because the FMDV RNA-dependent RNA polymerase lacks proof reading ability.

1.1.3 The situation of FMD in Uganda

In Uganda the first FMD outbreak was recorded in 1953 and since that time outbreaks have regularly occurred in cattle and other susceptible species. FMD is endemic in Uganda and according the Ministry of Agriculture Animal Industry and Fisheries (MAAIF), the number of FMD outbreaks has been on the increase over recent years. For example 1-15 FMD outbreaks per year were reported between 1996-1999 compared to 25-38 outbreaks per year between 2000 and 2007. Many recent studies have shown the predominance of FMDV serotype O and SAT-2 in this country (Balinda *et al*., 2009; Balinda *et al*., 2010; Ayebazibwe et *al*., 2010a; Mwiine *et al*., 2010). Other serotypes reported includeSAT-1 and SAT-3 (Vosloo *et al*., 2002; Kalema-Zikusooka *et al*., 2005 Balinda *et al*., 2009; Mwiine *et al*., 2010), serotype C that was last recorded in early 1971 Vosloo *et al*., 2002).

1.1.4

From April 2008 until near the end of that year, many outbreaks were reported in the Eastern region of the country particularly in Kamuli district. As a routine, quarantines were imposed coupled with ring vaccination using trivalent vaccine against serotypes O, SAT1 and SAT2 around the affected. Despite these interventions, FMD outbreaks were subsequently reported in over 10 districts in the Northern region of Uganda in January 2009. The major part of this region is recovering from the effects of the recent civil strife

of the Lord's resistance army war and many farmers in this region have embarked on restocking their livestock farms. This involves importing animals from other regions of the country, especially from cattle markets, including from the Eastern region that is separated from the Northern region by Lake Kyoga. The shores of this lake, on each side, have a number of landing sites and cattle markets where disease screening is almost nonexistent. Thus, these activities have the potential for spreading diseases, including FMD during outbreaks, in the nearby districts.

1.1.5 Molecular epidemiology of FMDV

The molecular epidemiology of FMDV has been extensively studied using the VP1 coding region of the virus genome (Knowles and Samuel, 2003). VP1, the most variable capsid protein includes a major immunogenic site of the virus has been used to genotype the seven serotypes of FMDV into geographically distinct groups called topotypes. Furthermore, comparison of VP1 coding sequences from isolates obtained during outbreaks provides evidence of relatedness between individual FMDV strains and hence the tracing of the spread and transmission of the virus from one region to another or across national borders (Samuel and Knowles, 2003).

In East Africa, the molecular epidemiology of FMD is not well understood (Vosloo, 2002). In Uganda, molecular characterization of FMDV is not routine. There is therefore limited data on the epidemiology of FMD yet FMD outbreaks are frequent and difficult to control. According the UBOS/MAAIF 2009, Uganda has a population of 11.4M cattle, 12.5M goats, 3.4M sheep, and over 3.2 M all of which are susceptible to FMD. Animals frequently cross Uganda's boarders to and from other neighboring countries. In addition, 4.6% of Uganda is occupied by National parks and there angulate occupants roam freely across national boarders and national parks thus. While these animals may be reservoirs of FMDV, there contribution to the introduction and maintenance of FMD is unknown (Ayebazibwe *et al*., 2010b)

1.1.6 Significance of the study

Due to the highly infectious nature of the virus and the accompanying economic constraints following an outbreak of the disease, surveillance and characterization of the virus is crucial. In this study, the VP1 coding region sequences were used to identify the

serotype and genetic relationship between the viruses responsible for the 2008/2009 Eastern and Northern Uganda FMD outbreaks with the aim of investigating the spread of the disease in Uganda. The results of this study have important implications for the currently used methods of FMD control in Uganda.

1.2 Problem statement

From 1996 to 2007, the number of FMD outbreaks has increased from 1-5 outbreaks to 25-38 outbreaks per year. From April up to the end of the year 2008 Eastern Uganda faced many FMD outbreaks. By January 2009 many outbreaks were reported spreading to more than 20 districts in Northern Uganda. Since molecular characterization of FMDV is not routine in Uganda, the serotype and the genetic relationships among the viruses responsible for the recent FMD outbreaks is not known. It is not known whether the outbreaks in the Eastern and Northern regions are due to a common source or they are new and independent introductions.

1.3 Aim and objectives

To characterize FMD viruses responsible for the outbreaks in Eastern and Northern Uganda in 2008 and 2009.

1.3.1 Specific objectives

i. To identify the FMDV serotypes involved in recent Eastern-Northern Uganda FMD outbreaks.

ii. To investigate the VP1 coding sequence relationship among the viruses responsible for the recent Eastern-Northern Uganda FMD outbreaks.

1.4 Justification

FMD is endemic in Uganda and billions of shillings are spent to control the spread of this disease through short term control measures but with limited success. Formulation of effective control strategies requires thorough understanding of the, molecular epidemiology of the disease and this can be done by nucleotide sequencing of the viruses. The aim of this study therefore was to identify the serotype and compare the VP1 coding

sequences of viruses collected from the 2008 and 2009 FMD outbreaks in eastern and northern Uganda respectively. The outcomes of this study have important implications on the effectiveness and enforcement of FMD control measures in Uganda.

CHAPTER TWO

Literature Review

2.1 Foot-and-mouth disease

Foot-and-mouth disease (FMD) is an acute systemic infection affecting cloven-hoofed animal species (Murphy *et al*., 1999; Bastos *et al*., 2003). FMD generally involves mortality below 5% but it is considered the most economically important disease of farm animals since it causes significant decreases in livestock productivity and trade in livestock products (Domingo *et al*., 1990; Domingo *et al*., 2002). The main route of infection of ruminants such as cattle is the inhalation of airborne virus, but infection via alimentary tract or skin lesions is also possible. Some of the clinical symptoms of FMD include fever, anorexia, weight loss, lameness, salivation and vesicular lesions (mouth and skin). An asymptomatic persistent infection can be established in ruminants for several years. Animals with this kind of infection are referred to as *carrier animals* and are important reservoirs of the causative virus. African buffalo (*Syncerus caffer*) are important carriers and are a possible source of FMD outbreaks by virus transmission to susceptible animals such as cattle (Woodsbury *et al*., 1995; Bruckner *et al*., 2002). Although FMD rarely causes death in adult animals, mortality rates are high in young animals (Doel, 1996). Recent outbreaks of the disease in a number of once FMD free countries particularly Taiwan (1997), United Kingdom (2001) have significantly increased public awareness of this highly infectious disease (Anon, 2002; Quian *et al*., 2003; Grubman and Baxt, 2004).

2.2 Economic consequences of FMD

FMD has very serious both direct and indirect economic effects including loss of productivity in terms of meat and milk, loss of weight, delayed conception (James and Rushton, 2002). Countries where FMD has occurred lose national trading status and markets for live animals and animal products hence losing a lot of revenue that would be generated from the livestock sector. The disease also interferes with agriculture and tourism. Additional costs include application of control measures such as quarantines, slaughter, compensation, vaccination as well as conducting scientific surveillance after an outbreak in order to prove that the disease and the virus have been eliminated (Prempeh

and Robert, 2001). The devastating economic implication of FMD are exemplified by the 2001 FMD outbreak in the United kingdom in which up to £5 billion was spent to compensate farmers and stamp out the disease (John, 2002).

2.3 Transmission of FMD

FMD is a highly contagious disease and can be transmitted in many ways leading to very rapid spread of the disease within farms and, to the surrounding farms, areas. The primary transmission modes are: direct contact between infected animals and susceptible animals, mechanical transmission by indirect means e.g. contaminated human clothing and animal feeds. Aerosol spread, including long distance wind-borne transmission under exceptional epidemiological and environmental conditions (Alexandersen *et al*., 2002).

Direct contact between infected and susceptible animals is the dominant mode of virus transmission and spread of the disease. Some ninety-five percent of outbreaks of FMD are the result of direct contact (Bannet and Cox, 1999). Infected animals release virus in exhaled air, all excretions and secretions, and from ruptured vesicles. As excretion may commence up to four days before the appearance of clinical signs, the movement of animals that are incubating the disease is of great epidemiological significance (Donaldson and Alexandersen, 2002). The disease may spread extremely rapidly in intensive farming areas because of high stocking density and the level of challenge both from infected animals and the environment. Conversely, disease spread in extensive grazing areas in hotter climates can be more insidious (Bannet and Cox, 1999). Congregation of animals, for example, at common grazing and watering points, at gatherings for vaccination, dipping, shearing, or through transhumance or nomadism, favour the spread of the disease to new herds and areas. The disease can also be disseminated very rapidly by movement of infected animals through livestock markets and shows (Donaldson and Alexandersen, 2002). The movement of infected sheep, that may show few, if any, clinical signs of the disease, can be pivotal in the spread of FMD. Such was the case in the epidemic in the United Kingdom in 2001. Most virus excretion by infected animals ceases some 4-6 days after the appearance of clinical signs. A

proportion of cattle, sheep, goats and possibly water buffaloes become chronic carriers (Donaldson and Alexandersen, 2002).

There is massive viral contamination of the environment around infected animals. This contamination may persist in the environment for a long time. Fomites are important in the spread of infection. Contaminated material may introduce virus into the skin or mucous membranes, e.g., via brushes and surgical instruments, or into food, e.g., via faeces, urine and contaminated fodder. Infected aerosols, e.g., slurry spray, may be produced from contaminated fomites. Those handling infected animals, such as farm workers, dealers and veterinarians, may carry the virus on their hands, underneath their fingernails, inside their nostrils and on their clothing and footwear. There are several known cases where veterinarians have carried infection from farm to farm. Personal disinfection is essential during an outbreak. The virus may also be carried mechanically on farm equipment and machinery, and on vehicles (particularly in the livestock compartment of trucks used for the transport of animals).

Infected aerosols are a significant mode of virus transmission. Aerosol transmission normally occurs when there is close contact between animals. When there is massive contamination of the environment, aerosols may also transmit infection to animals in neighboring paddocks or farms (Donaldson and Alexandersen, 2002). Whilst infected aerosols are most frequently generated by exhaled air, they can also result from splashing, exhaust from milk tankers or pressure hoses. Long-range windborne transmission of virus is an uncommon but important route of infection in certain environmental and epidemiological conditions. It is only likely to occur when pigs feature prominently in outbreaks because of the large quantities of virus that they excrete in their exhaled air (Gloster *et al*., 2007). However, this mode of FMD transmission is of significance in the temperate regions and of no significance in the dry regions such as East Africa.

2.4 FMD virus serotypes and their global distribution of FMD

The causative agent for FMD is the foot-and mouth disease virus (FMDV). FMDV is a prototype member of Aphthovirus genus of the family *Picornaviridae* (Samuel and

Knowles, 2001; Carrillo *et al.,* 2005). *Picornaviridae* are non-enveloped viruses with single-stranded RNA genome of positive polarity.

Serotypes A, C and O have a global distribution. They have been isolated in most parts around the world (Grubman and Baxt, 2004; Quian *et al.,* 2004). Outbreaks due Asia l have been restricted to Asia (Ayelet *et al*., 2009). FMD outbreaks due to SAT serotypes are known to occur in South African region spreading to the eastern and western regions of the continent (Bruckner *et al.,* 2002; Knowles and Samuel, 2003).

2.5 FMD virus serotypes in Uganda

FMD outbreaks occur annually in Uganda. Previous serological and molecular have shown FMD incursions due to serotype O, A, SAT-1 and SAT-2 in Uganda (Bastos *et al*., 2003, Kalema-Zikusooka *et al*., 2005; Ayebazibwe *et al*., 2010b; Mwiine *et al*., 2010a; Balinda *et al*., 2009;Balinda *et al*., 2010). Available data shows that the last serotype C was last diagnosed in Uganda in early 1971 (Vosloo *et al*., 2002). Previous studies have indicated that serotype SAT-3 has only been isolated from Ugandan African Buffaloes in 1970 and 1997 and has not been isolated in domesticated animals while only serological evidence of SAT-3 has been reported in cattle herds grazing in Queen Elizabeth National park (Hedger, 1972, Kalema-Zikusooka *et al*., 2005). However a more recent study has also shown serological evidence of SAT3 in cattle herds inside the Queen Elizabeth National Park (Mwine *et al.,* 2010). Recent studies recent studies show that FMDV serotype O and SAT-2 have been responsible for most of the FMD outbreaks in Uganda (Bastos *et al.,* 2003; Ayebazibwe *et al*., 2006; Balinda *et al*., 2009).

2.6 Foot-and-mouth disease virus genome

The virus was first recognized as a viral pathogen in 1898 (Loeffler and Frosch, 1898). The virus has single stranded positive sense RNA genome of about 8.4 kb depending on the serotype (Quian *et al*., 2003). It consists of a single open reading frame (ORF) flanked by highly structured 5' and 3' untranslated regions (5' and 3' UTRs), which are approximately 1300 and 100 nucleotides respectively (Knowles *et al*., 1998; Barco *et al*., 2000; Belsham and Martinez-Salas, 2004). The 5’ UTR consists of a 350-380 nucleotide short fragment (S), a 100-420 nucleotides poly C tract (90%) and approximately 700

nucleotides 5' terminus of the genomic long (L) fragment which contains three tandemly repeated pseudo knots, a stem loop *cis*-acting replication element (*cre*) and a type II Internal Ribosomal Entry Site (IRES). The FMDV 5' UTR plays an important role in cap independent translation initiation of viral polyprotein and viral genome replication (Martinez-Salas and Fenandez-Miragall 2004).

The 3' UTR is about 90 nucleotides long and is thought to contain *cis*-acting elements required for efficient genome replication (Carrillo *et al*., 2005).

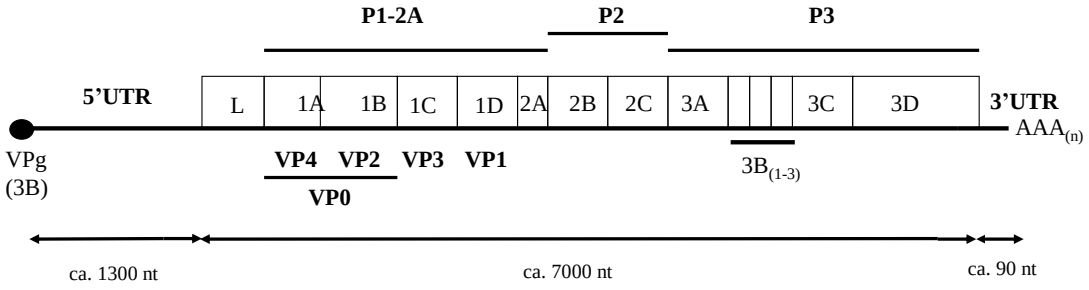

Figure 1. FMDV general Genome structure

Translation of the ORF results in an immature polyprotein which is initially cleaved by the P2A protease into P1, P2 and P3 peptides. Further cleavage results in VP1, VP2, VP3 and VP4. These four proteins form the capsid of the virus and are coded for by 1D, 1B, 1C, and 1A coding sequences respectively (Quian *et al*., 2003). The 1D is 627-639 nucleotides long and codes for VP1 which is a capsid protein containing 209-213 amino acid residue depending on the serotype. Comparison of 1D nucleotide or (VP1) protein sequences of isolates obtained from different regions provides evidence of the relatedness

of individual isolates of FMDV (Quian *et al*., 2003). The most important antigenic site of FMDV is in the VP1 (Quian *et al.,* 2003). It has multiple roles including receptor recognition, neutralization and antigenic diversity (Bastos *et al*., 2003). The VP1 coding sequence analysis can be used to determine the relatedness of FMDVs and thus evaluate the likelihood that a vaccine will provide protective immunity to the vaccinated animals since most FMDV vaccines are developed targeting the VP1 (Chenwen *et al*., 2007).

2.7 Molecular Epidemiology of FMDV in Uganda

The first FMD outbreak in Uganda was recorded in 1953 and since that time outbreaks have regularly occurred in cattle and other susceptible species. The disease has since that time frequently occurred in cattle and other susceptible animals. In Uganda, molecular characterization has not be routine and most of the outbreaks have not been recorded. Therefore, the molecular epidemiology of FMDV in Uganda has not been well documented. However, over the last 10 years molecular characterization has been carried on some of the frequent FMD outbreaks in Uganda. Reports indicate that over the recent years, FMD outbreaks due to serotypes O, SAT-1, SAT-2 and SAT-3 (Ayebazibwe, 2010; Mwiine et al., 2010; Balinda *et al*., 2009; Balinda *et al*., 2010), have been reported in Uganda. Other serotypes recorded in the past include serotype A and C (Vosloo *et al*., 2002). Serotype C was last reported in Uganda in 1970s, (Vosloo et al., 2002; Kalema-Zikusooka *et al*., 2005).

2.8 Control of foot-and-mouth disease in Uganda

The choice of control policy adopted by a given country depends on its FMD status and the risks of incursions of the disease (Ahl *et al*., 1991). Following an FMD outbreak Ugandan veterinary authorities enforce quarantines to restrict livestock and livestock product movement as the first control measure (Balinda *et al*., 2009). In addition the authorities impose a ban on slaughter in abattoirs for a period of six months. These measures are then followed by vaccination within and around the affected areas. The current FMD vaccine in use in Uganda is a trivalent vaccine that contains three serotypes including SAT-1 SAT-2 and O. However studies have recommended the inclusion of season and animal movement in the FMD control strategies (Ayebazibwe *et al*., 2010a).

2.9 Diagnosis of foot-and-mouth disease

Diagnosis of FMD is by Clinical signs, and in a laboratory by virus isolation, demonstration of the FMD viral antigens or nucleic acid in a sample tissue or fluid. Detection of virus specific antibodies can also be used. Additionally, antibodies to viral non structural protein can be used as indicators of infection irrespective of vaccination status (OIE, 2009).

2.9.1 Clinical diagnosis

Infection of susceptible animals with FMDV leads to the appearance of vesicles on the feet, in and lesion around the oral cavity and on the mammary glands (Barnett and Cox, 1999). Vesicles can also occur in other sites such as nostrils and pressure points on the limbs especially in pigs. The severity of clinical signs varies with the serotype and strains of the virus, the age, breed of the animals, the host species, and the degree of immunity (Barnett and Cox, 1999). Other signs include lameness, reduced milk production, salivation. These signs range from a mild infection to one that is severe and in some extreme cases death may occur. For example mortality from multifocal myocarditis is most commonly seen in young animals.

Hover, clinical signs alone are not sufficient since other vesicular diseases such as swine vesicular virus disease, blue tongue disease among others, may produce similar signs and a wrong diagnosis may be made. A wrong diagnosis will consequently lead to inappropriate FMD control measures and this leads to wastage of otherwise limited resources such as vaccines and equipment in resource constrained communities and countries. It can not even help in identifying the serotype and strain which are very crucial in vaccination program and this requires a laboratory based diagnosis.

2.9.2 Laboratory diagnosis

Several laboratory techniques for the detection and confirmation of FMD have been developed and are described in the OIE Manual of Diagnostic techniques (OIE, 2004).

2.9.2.1 Serological assays

Viral antigens can be detected using Enzyme Linked Immunosorbent Assay (ELISA). The demonstration of specific antibodies to structural proteins in nonvaccinated animals can be achieved by this technique (Crowther and Abu, 1979). Serological tests for the

detection of antibodies against FMD viruses irrespective of the vaccination status have been applied some studies (Berger *et al*., 1990). Although these tests were serotype specific, they were tideous to use for screening purposes especially in areas where FMD is endemic. Serological tests to detect FMDV non structural proteins (NSP) as well as FMDV structural protein of serotype O have been developed (Chenard *et al*., 2003; Sorensen *et al*., 2005). Like most other test serological test may not be of use in strain identification.

2.9.2.2 Virus isolation

FMDV infection can also be demonstrated by isolating the virus by cultures (OIE, 2004; OIE, 2009). The epithelium sample should be taken from the PBS/glycerol, blotted dry on absorbent paper to reduce the glycerol content, which is toxic for cell cultures, and weighed. A suspension should be prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium and antibiotics. Further medium should be added until a final volume of nine times that of the epithelial sample has been added, giving a 10% suspension. This is clarified on a bench centrifuge at 2000g for 10 minutes. Once clarified, such suspensions of field samples suspected to contain FMD virus are inoculated onto cell cultures or into unweaned mice. Sensitive cell culture systems include primary bovine (calf) thyroid cells and primary pig, calf or lamb kidney cells. Established cell lines, such as BHK-21 (baby hamster kidney) and IB-RS-2 cells, may also be used but are generally less sensitive than primary cells for detecting low amounts of infectivity (Clarke and Spier, 1980). The sensitivity of any cells used should be tested with standard preparations of FMD virus. The use of IB-RS-2 cells aids the differentiation of swine vesicular disease (SVD) from FMD (as SVD virus will only grow in this cell type) and is often essential for the isolation of porcinophilic strains, such as O Cathay. The cell cultures should be examined for cytopathic effect (CPE) for 48 hours. If no CPE is detected, the cells should be frozen and thawed, used to inoculate fresh cultures and examined for CPEs for another 48 hours. Unweaned mice are an alternative to cell cultures and should be 2–7 days old and of selected inbred strains. Some field viruses may require several passages before they become adapted to mice (Skinner, 1960). In the case of OP fluids, pre-treatment with an equal volume of chloro-

fluoro- carbons may improve the rate of virus detection by releasing virus from immune complexes. This may not be of use in identifying the serotypes involved.

2.9.2.3 Nucleic acid detection

The presence of viral genomic material can be detected using RT-PCR assays. RT-PCR can be used to amplify genome fragments of FMD virus in diagnostic materials including epithelium, milk, serum and probang samples (Amarel *et al*., 1993). RT combined with real-time PCR has sensitivity comparable to that of virus isolation and automated procedures enhance sample throughput (Reid *et al*., 2001; Reid *et al*., 2003). Specific primers have been designed to distinguish between each of the seven serotypes. *In situ* hybridization techniques have been developed for investigating the presence of FMD virus RNA in tissue samples. These techniques are only in use in specialized laboratories, although simplified systems for potential field-use are under development (Callahan *et al*., 2002). Nucleic acid tests can identify the serotype and strains involved in FMD outbreaks providing a tool for tracing the spread of the virus, more detailed characterization and vaccine selection.

2.9.3 Sample collection for laboratory diagnosis of FMD

In animals with a history of vesicular disease, vesicular fluid, the epithelial tissue, milk, blood and probang samples are sufficient to establish a diagnosis to confirm the observed clinical signs. Body organs such as heart or other organs of fatal cases are also useful for virus isolation (House and House, 1989). For laboratory diagnosis, the tissue of choice is the epithelium. Ideally at least 1g of epithelial tissue should be collected from un-raptured or recently ruptured vesicles. Epithelium should be collected and placed in transport medium composed of equal amounts of glycerol and 0.04M phosphate buffer pH (7.2-7.6) with some antibiotics. Samples should be kept refrigerated on ice until received by the laboratory.

2.9.4 Laboratory Diagnosis of FMD in Uganda

Rapid identification of the causative agents is a key element in any control strategy (Anon, 2002). For the case of FMD, a number of detection methods have been developed and published. These among others include viral nucleic acid detection, detection of antigens and the host immune response or antibody detection (Anon, 2002). Each of these

methods has its own weaknesses depending on the purpose intended for its use. In Uganda today, the presence of FMDV in samples is detected by two methods. These include Enzyme Linked Immunosorbent Assay (ELISA) and nucleic acid detection methods such as RT PCR, VP1 amplification and sequencing. ELISA is the commonest method being used at MAAIF and at the Makerere University Institute of Environment and Natural Resources (MUIENR) Molecular Biology Laboratory. In addition to ELISA, PCR-based methods are used at MUIENR and plans are underway to apply the same techniques at MAAIF.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study sites

Between April 2008 and January 2009, probang and swab samples were collected from cattle with clinical signs of FMD. These samples were collected from Kamuli district (Eastern Uganda) in April 2008 and from Northern Uganda districts of Amolatar, Apac, Dokolo, Lira, Gulu, and Pader (Figure 2) in January 2009. Probang and swab samples were respectively collected and stored in phosphate buffered saline (PBS) and RNAlater (Ambion USA). The samples were then placed on ice until they were transported to the laboratory. The samples were then stored at $^{-}80^{o}$C until RNA extraction was performed.

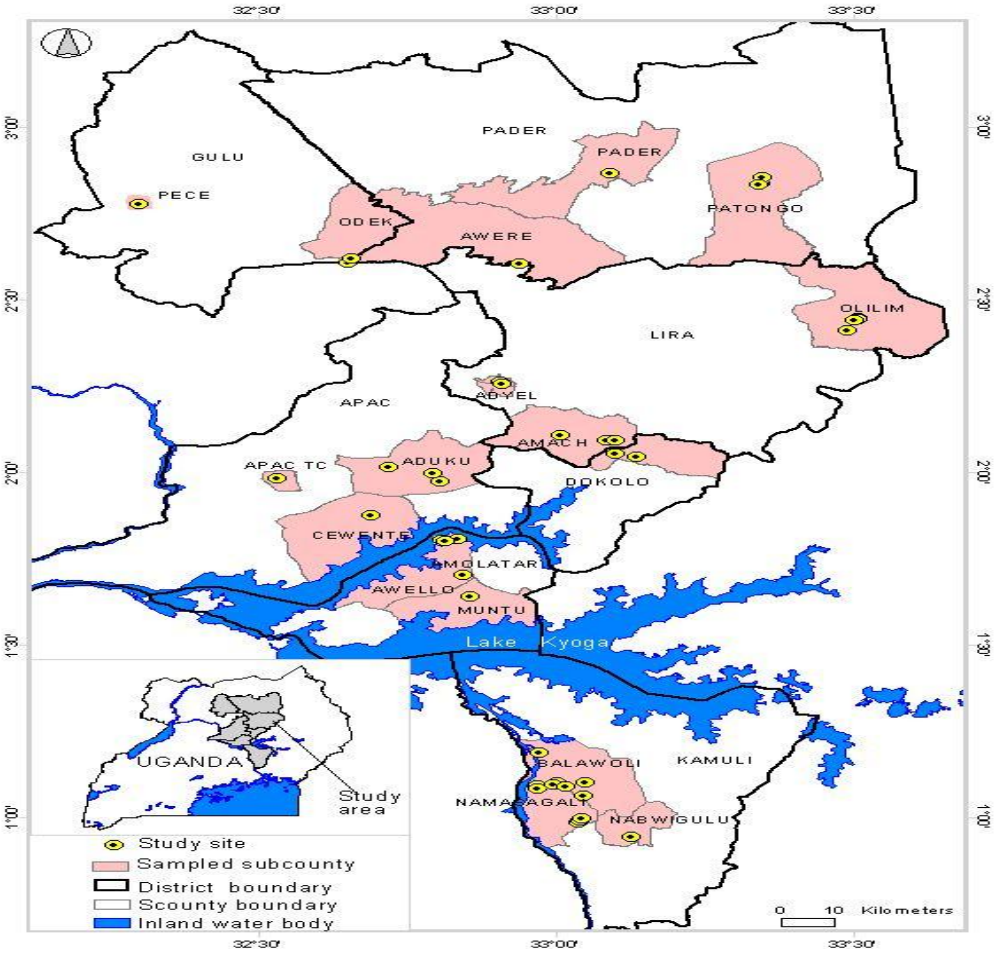

Figure 2. Map showing areas where samples were collected in various districts of Uganda. Inset is a map of the republic of Uganda

3.2 RNA extraction and RT-PCR

Total RNA was extracted from probang and swab sample supernatants using the QIAmp RNA extraction kit (Qiagen Inc) following the mini spin protocol according to the manufacturer's instructions. The extracted RNA was reverse transcribed using the Ready-To-Go You-Prime First-Strand Beads cDNA synthesis kit (Amersham Biosciences) according to the manufacturer's instructions using random hexamers. To confirm the presence of FMDV cDNA, a standard diagnostic PCR (+ or - PCR) targeting the cDNA corresponding to the 5' untranslated region of the FMDV RNA was carried using two forward primers: Multi-II (F) and Multi-II SAT F and one reverse primer Multi-II(R-1) (Reid *et al*., 2002).

Multi-II (F): 5'CAC(T/C)T(T/C)AAG(G/A)TGACA(T/C)TG(G/A)TACTGGTAC-3' and Multi-II SAT F: 5-CAC(T/C)T(T/C)AAG(G/A)TACACTCTG(G/A)GACTGGTAC-3'and one reverse primer Multi-II (R-1): 5'-CAGAT(C/T)CC(G/A)AGTGT/AC(I)TGTT-3' (Balinda *et al*.,2009). This PCR was carried out using Multi-II PCR assay using 2X TaqMan Universal PCR Master Mix (PE Biosystems). The reaction mixture composed of 10pmol/ µL of one of the forward primers and reverse primers 2X TaqMan Universal Master Mix, RNase free water and 7 µL of cDNA in a 25 µL reaction volume. The reactions were carried out in a TECHNE TC-412 PCR machine (Techne Cambridge UK), under the following conditions: 50°C for 2 minutes for UNG digestion, 95°C for 10 min for TaqGold activation, 95°C for 15 sec for denaturation, 60°C for 60 sec. These three steps were repeated for 40 cycles and a subsequent hold temperature of 5°C. The production of a 96-100bp product was determined by 2% agarose gel electrophoresis after ethidium bromide staining under UV light using a ΦX174 maker (Amersham Biosciences, UK).

To confirm the serotype, all samples that had the expected product size were subjected to an additional PCR. The amplification of cDNA corresponding to the 1D coding region was carried out using TaqMan Gold RT-PCR kit (Applied Biosystems).

For FMDV SAT-1 and SAT-2, two SAT specific primers were used. These were:

SAT-1D209F: (5`-CCACATACTACTTTTGTGACCTGG-3') and FMD-2B208R (5`-ACAGCGGCCATGCACGACAG-3') (Balinda *et al*., 2009) were used as forward and

reverse primers respectively to generate PCR products of 730bp and 715bp for serotypes SAT-1 and SAT-2 respectively. For serotype O, two primer sets were used in two separate PCRs to generate overlapping fragments (termed as fragment five and fragment six) which when combined include the entire ID coding region (Figure 3). For Fragment five, forward primer 8A-PN84 (5`-TACTACCCAGTACAGCG-3`) and reverse primer 8-A-PN-85 (5`-GGAGCACCCGAAGCTGCA -3`) were used. For fragment six, the forward primer 8A-PN98 (5`-GCATCCACTTACTACTTTGC-3`) and the reverse primer 8A-PN64 (5-'GGAGATCTGGAGTCCAACC-3') (Balinda *et al*., 2009) were used to amplify the 1D region that codes for VP1 (Figure 3). The reaction mixture composed of the following: 1.5mM $MgCl_2$, 200µM dNTPs, and 1 X PCR buffer, forward and reverse primers at a concentration of 0.2 pmol/µL, 1.2U Taq Gold DNA polymerase in a 50 µL reaction volume.

For FMDV SAT1 and SAT2, the amplification reaction was done under following conditions: 95°C for 5 minutes for TaqGold activation, 94°C for 15 seconds, 60°C for 1 minute, 72°C for 2 minutes for strand extension. The three steps were repeated for 40 cycles followed by a final extension temperature of 72°C for 10 minutes and a subsequent hold temperature of 5°C using Ependorf Mastercycler Gradient thermocycler.

For FMDV serotype O, the amplification of fragment five was done under the following conditions: 95°C for 5 minutes for TaqGold activation, 94°C for 15 seconds for denaturation, 55°C for 1 minute for primer annealing, 72°C for 2minutes for strand extension and these three steps were repeated for 40 cycles followed by a final extension temperature of 72°C for 10 minutes and a subsequent hold temperature of 5°C using Eppendorfs Mastercycler Gradient thermocycler. Fragment six was amplified under the following conditions: 95°C for 5 minutes for TaqGold polymerase activation, 94°C for 15 seconds, 55°C for 1 minute, 72°C for 2 minutes, for 40 cycles and a final extension temperature of 72°C for 10 minutes and a subsequent hold temperature of 5°C using Eppendorfs Mastercycler Gradient thermocycler (Hamburg). The PCR products were confirmed by 2% agarose gel electrophoresis after ethidium bromide staining and viewing under UV light alongside a ΦX174-RF DNA maker (Amersham Biosciences, UK).

3.3 Purification of PCR products and sequencing

Excess oligonucleotide dNTPs and primers were removed using the QIAquick purification kit (Qiagen, Germany). The purified PCR fragments were sent to Macrogen (Seoul, South Korea) where they were directly sequenced on both strands using the same primers used in the PCRs under Big Dye terminator conditions using an automated DNA sequencer to obtain the complete VP1 sequences.

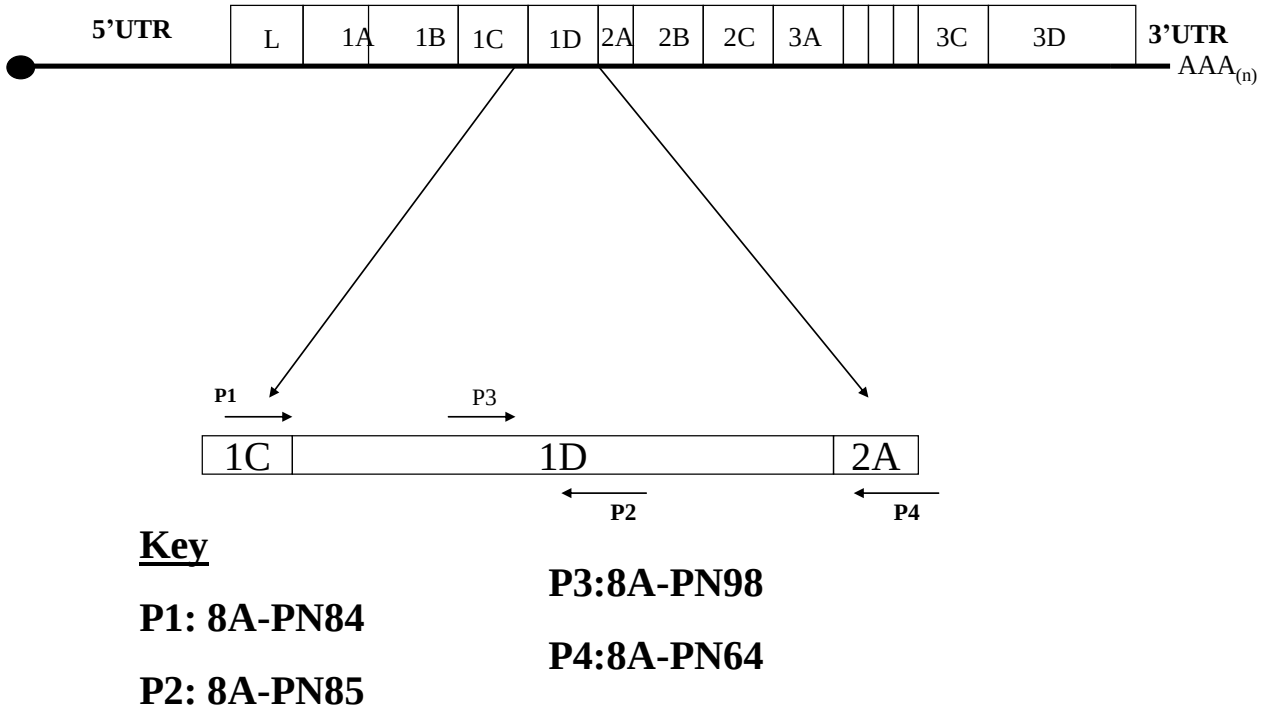

Figure 3. Primer positioning during the 1D amplification process

3.4 Data analysis

3.4.1 Sequence editing and assembling

The chromatograms of both forward and reverse strands were visually analyzed using SEQUENCHER 4.8 computer software (Gene Code Corporation, USA) and assembled into contigs resulting in overlaps. The consensus nucleotide sequences were exported to BioEdit (Hall 1999) computer program and manually aligned using the same program. Serotype reference sequences were obtained from the NCBI Genebank (www.ncbi.nlm.nih.gov). Multiple sequence alignments were made using ClustalW (Thompson *et al*., 1994) incorporated in Mega 4.0 (Tamura *et al*., 2007) computer software and the sequences trimmed to a usable 639 nucleotides covering almost the full VP1 of serotype O.

3.4.2 Identification the serotype using the generated sequences

Sequence comparisons were made using BLAST sequence search that is also available from NCBI website (www.ncbi.nlm.nih.gov) using default search parameters.

3.4.3 Phylogenetic analysis

To assess the evolutionary relationships between the FMDV sequences, phylogenetic trees were constructed. The selection of molecular evolutionary models was made using the hierarchical likelihood ratio test that is implemented in the computer program Modeltest ver. 3.7 (Posada, 2008), and Bayesian inference was performed using the Bayesian methods available in the computer program MrBayes ver 3.11 (Ronquist and Huelsenbeck, 2003) assuming a General Time reversible model of nucleotide substitution with invariable sites

To further confirm the BLAST search results, a phylogenetic tree based on complete VP1 sequences of representative serotypes A, C, O, SAT-1 and SAT-2 obtained from the Genbank was constructed. In addition, a phylogenetic tree consisting of representatives of eight topotypes of FMDV type O was constructed.

CHAPTER FOUR

RESULTS

4.1 Confirmation of FMD and sample distribution

Diagnostic PCR assays amplified cDNA fragments corresponding to part of the FMDV 5' untranslated region confirmed the presence of FMDV as being responsible for the observed disease. A total of 59 (Table 1), samples were collected and tested for the presence of FMDV RNA. Twenty three (23) samples representing 40% of the total samples were positive for FMD. Twelve (12) sample were collected from Apac and 8 (67%) were positive for FMD. Sixteen (16) samples were collected from Amolatar and 3 (18%) were positive for FMD. Three (3) samples were collected from Dokolo and 1 (33%) was positive for FMD. Two samples were collected form Gulu and all of them were negative for FMD. From Lira 14 samples were collected and 7 (50%) were positive. Eight samples from Kamuli were studied and 2 (25%) were positive for FMD. From Pader, 4 samples were collected and 2 (50%) of them were positive for FMD.

Table1. The distribution of samples and their diagnostic PCR test status

District	Sample ID	+/- Test for SAT	+/-Test for type O
Apac	APC1	-	-
	APC2	-	-
	APC3	-	+
	APC4	-	+
	APC5	-	+
	APC6	-	+
	APC7	-	-
	APC9	-	+
	APC10	-	+
	APC11	-	+
	APC12	-	+
	APC13	-	-
Amolatar	AMO1	-	-
	AMO2	-	-
	AMO3	-	+
	AMO4	-	-
	AMO5	-	-
	AMO6	-	+
	AMO7	-	-
	AMO8	-	-
	AMO9	-	-
	AMO10	-	-
	AMO11	-	-
	AMO13	-	-
	AMO14	-	-
	AMO15	-	-
	AMO16	-	-
	AMO19	-	+
Dokolo	DOK1	-	+
	DOK2	-	-
	DOK3	-	-
Gulu	GUL1	-	-
	GUL2	-	-
Lira	K1	-	+
	K2	-	+
	K3	-	+
	K4	-	-
	K5	-	-
	K10	-	-
	K13	-	+
	K14	-	-
	K15	-	-
	K19	-	+
	ABE1	-	-
	ABE2	-	-
	ABE3	-	+
	ABE4	-	-
KAMULI	KMC1	-	-
	KMC3	-	+
	KMC4	-	+
	KMC6	-	-
	KMC15	-	-
	KMC22	-	-
	KMC27	-	+
	KMC28	-	-
Pader	PAD1	-	-
	PAD3	-	-
	PAD4	-	+
	PAD9	-	+

4.2 The VP1 coding region Sequence characteristics

Pair-wise comparison between the VP1 coding region of all the samples showed that the sequences shared greater than 99% nucleotide sequence similarity. Some sequences only differed by simply nucleotide substitutions (Figure 4), and thus there were no amino acid differences among the samples were observed. Moreover, some of the samples from Kamuli and northern Uganda shared 100% VP1 nucleotide sequence similarity and in such samples a representative of each haplotype was selected for further analysis. Therefore, although these samples were collected from different districts and different outbreaks as well as different regions (Eastern and Northern region), they were genetically similar.

Figure 4 VP1 coding region sequence comparison between sequences analyzed in this study in this study. The only variable sites indicating synonymous nucleotide substitutions are shown in green and blue.

4.3 Identification of the serotype

Nucleotide sequence comparisons conducted using BLAST searches indicated that all the sequences obtained from the 2008/2009 FMD outbreaks in Eastern and Northern Uganda had the greatest sequence similarity to FMDV isolates of serotype O FMDV isolates. A comparison of the test sample nucleotide sequences with other selected FMDV sequences is shown in Table 2. The Ugandan serotype O sequences from the Genbank had the highest nucleotide sequence similarity ranging from 92-94% to the test sample sequences. The Genebank sequences from other countries had lower sequence similarity.

Table2. Description of FMD virus sequences used for comparison in this study.

Accession No.	Virus name	Serotype	Topotype	Year	Country	% nucleotide Identity
AJ296327	O/UGA/5/96	O	EA	1996	UGANDA	84
DQ165077	**O/UGA/3/02**	**O**	**EA-2**	**2002**	**UGANDA**	**94**
AY349953	O/UGA/3/03	O	EA	2003	UGANDA	83
AY349954	O/UGA/4/03	O	EA	2003	UGANDA	83
AY344623	O/UGA/1/79	O	EA	1979	UGANDA	84
AY344620	**O/UGA/11/00**	**O**	**EA-2**	**2000**	**UGANDA**	**94**
AY344630	O/UGA/9/74	O	EA	1947	UGANDA	85
AY344618	O/UGA/29/96	O	EA	1996	UGANDA	85
AJ303511	O/KEN/83/79	O	EA-1	1979	KENYA	82
DQ165072	0/K77/78	O	EA1		KENYA	84
DQ165073	O/KEN/5/2002	O	EA-2	2002	KENYA	90
AJ303514	O/KEN/2/95	O	EA	1995	KENYA	85
AJ296320	O/TAN/7/98	O	EA	1998	TANZANIA	89
DQ165075	O/SUD/2/86	O	EA	1986	SUDAN	85
DQ165076	O/SUD/1/99	O	EA-3	1999	SUDAN	85
AY283383	O/ETH/3/2004	O	EA-3	2004	ETHIOPIA	84
FJ798139	O/ETH/1/2007	O	EA-3	2007	ETHIOPIA	84
FJ798139	O/ETH/27/2007	O	EA-3	2007	ETHIOPIA	80
AY283370	O/ETH/1/79	O	EA-3	1979	ETHIOPIA	84
EU553840	O/EGY/3/93	O	ME-SA	1993	EGYPT	84
DQ165074	O/MAL/1/98	O	EA-2	1998	MALAWI	89
AJ303485	O/CIV/8/99	O	WA	1999	COTE DE VOIR	82
DQ165071	O/GNA/4/94	O	WA	1994	GHANA	80
AJ294906	O/CAM/11/94	O	SEA	1994	CAMEROON	83
AJ318829	O/CAM/4/2000	O	SEA	2000	CAMEROON	83
AJ294911	O/HKN/21/71	O	CATHY	1971	HONGKONG	82
AJ294919	O/HKN/6/83	O	CATHY	1983	HONGKONG	80
AJ294986	O/HKN/19/73	O	CATHY	1973	HONGKONG	81
AJ294916	O/HKN/33/71	O	CATYY	1971	HONGKONG	81
AJ194919	O/HKN/6/83	O	CATHY	1973	HONGKONG	80
AJ303501	O2/BRESCIA/ITL/47	O	EURO-SA	1947	ITALY	79
EU583837	O/BUL/1/91	O	EURO-SA	1991	BULGARIA	84
EU553843	O/BURDOF/FRG/87	O	EURO-SA	1987	GERMANY	84
AJ004645	O/VENEZUELA/51	O	EURO-SA	1951	VENEZUELA	84
AJ318832	O/CHA/4/99	O	ME-SA	1999	CHAINA	84
AJ303509	O/JAV/5/72	O	ISA-2	1972	INDONESIA	82
AJ303500	O/ISA/1/62	O	ISA-1	1962	INDONESIA	83
AJ318841	O/IRQ/26/2000	O	ME-SA	2000	IRAQ	84
AJ318824	O/BAR/1/99	O	MESA	1999	BAHRAIN	83
AF308157	O/YUNLIN/97	O	ME-SA	1997	TAIWAN	83
AJ318825	O/BAR/8/98	O	ME-SA	1998	BAHRAIN	83
DQ164862	O/BAR/6/99	O	ME-SA	1999	BAHRAIN	85
DQ165037	O/BHU/2/2002	O	ME-SA	2000	BHULTAN	85
AY442010	SAT1/UGA/3/74	SAT1	VI	1974	UGANDA	59
AY442011	SAT1/UGA/7/99	SAT1	VI	1999	UGANDA	60
AY442008	SAT1/TAN/2/77	SAT1	I(NWZ)	1977	TANZANIA	59
AY441999	SAT1/SUD/9/74	SAT1	VI	1974	SUDAN	59
FJ498154	SAT1/ETH/3/2007	SAT1	IX	2007	ETHIOPIA	61
AF367099	SAT2/MAL/3/75	SAT2	VI	1975	MALAWI	56
AY343938	SAT2/ETH/1/90	SAT2	VI	1990	ETHIOPIA	56
AY343945	SAT2/KEN/2/87	SAT2	IV	1987	KENYA	58
AY343970	SAT2/TAN/1/75	SAT2	IV	1975	TANZANIA	59
AY254444	A/CAR/5/2000	A	AFRICA	2000	CEN. AFR. REP	68
FJ208757	A/EGY/1/2006	A	AFRICA	2006	EGYPT	68
FJ798150	A/ETH/4/2007	A	AFRICA	2007	ETHIPIA	68
EF208769	A/IRN/11/2005	A	AFRICA	2005	IRAN	64
EF208773	A/KEN/29/2009	A	AFRICA	2009	KENYA	68
FJ798152	C/ETH/6/2005	C	AFRICA	2005	ETHIOPIA	67
FJ798153	C/ETH/7/2005	C	AFRICA	2005	ETHIOPIA	67
EU553871	C/MOR/8/83	C	AFRICA	1983	MOROCCO	67

4.4 The serotype

The complete VP1 coding sequences of FMDV serotypes A, C, O, SAT1, SAT2 and the study sample sequences were used to construct a sequence similarity tree (Fig.5). This phylogenetic analysis shows that the 2008/2009 outbreak strains had the greatest sequence similarity to other FMDV serotype O viruses and formed a clade with them.

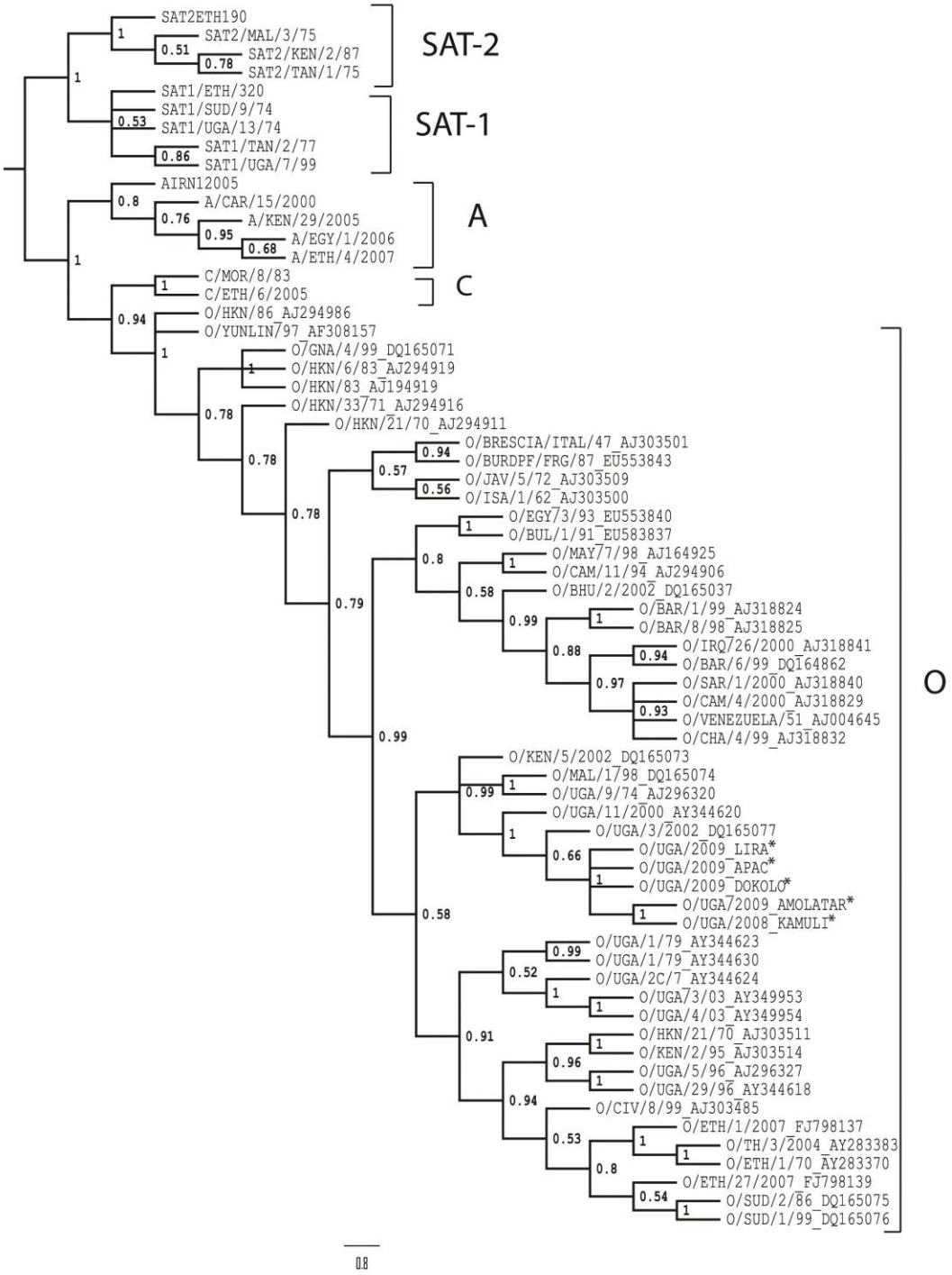

Figure 5. Phylogenetic tree constructed by Bayesian analysis of the VP1 coding region of FMDV type O, A, C, SAT-1 and SAT-2. Samples isolated during this study appear arsterics (*)

4.5 The topotype

Representative complete VP1 coding sequences of eight FMDV serotype O East African topotypes and the study sample sequences were phylogentically compared (Figure 6). The 2008/2009 outbreak strains had the greatest sequence similarity to isolates from Kenya (DQ165073), Malawi (DQ165074) and Uganda (DQ165077, AY344620 and AJ296320) and formed a sub-clade with them. Viruses in this sub-clade belong to EA-2 topotype. The outbreak strains formed a cluster with isolates from Uganda (DQ165077 and AY344620). Therefore the study sequences belong to EA-2 topotype

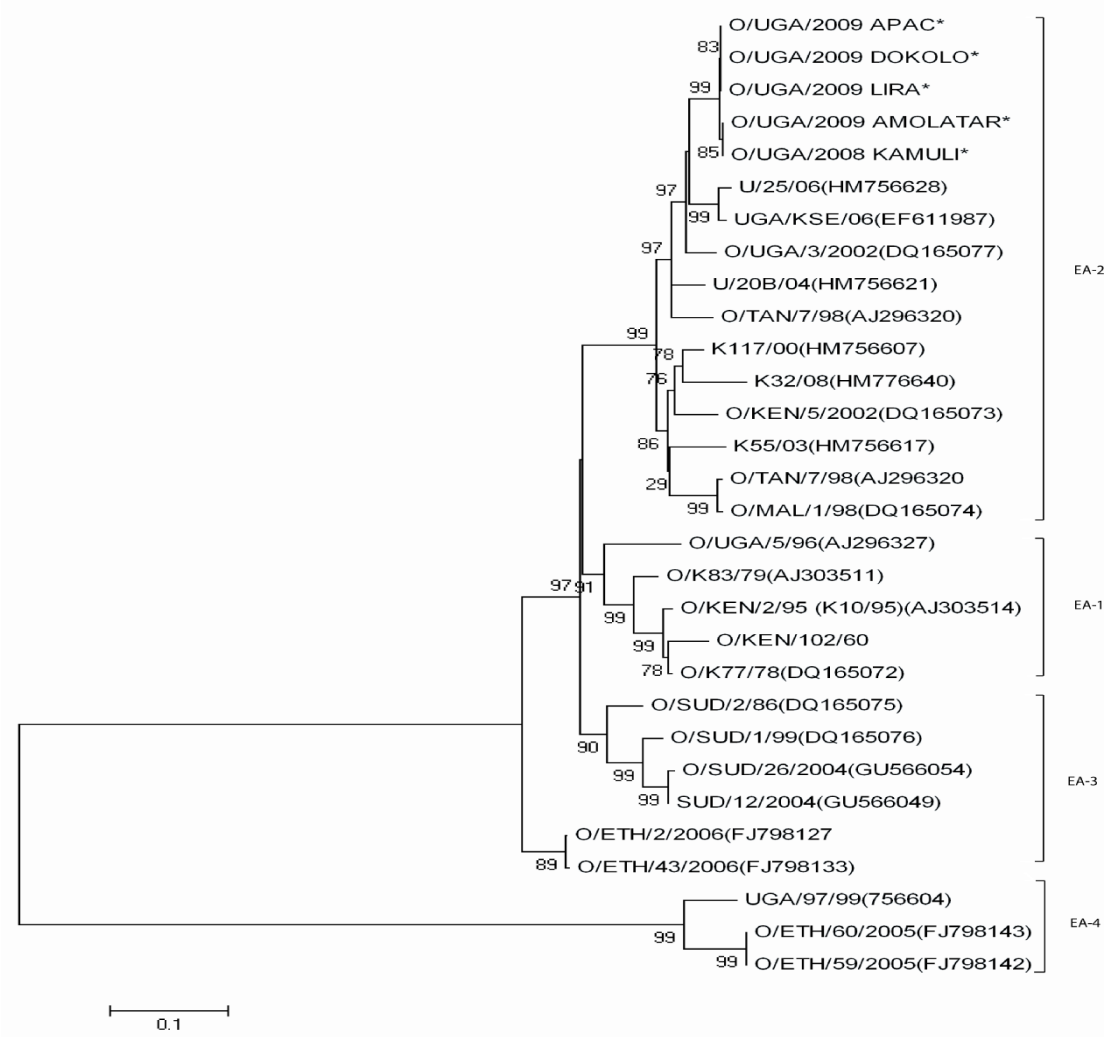

Figure 6. Neighbour joining phylogenetic analysis of VP1 of FMDV serotype O East African (EA) topotypes. This study sequences appear with arsterices. O/K77/78_DQ165072 is the current vaccine strain in use in Uganda.

CHAPTER FIVE

DISCUSSION

This was an inter-epidemic analysis of the VP1 coding region sequences in Uganda of viruses that caused FMD outbreaks in Eastern and Northern Uganda in 2008 and 2009 respectively. These sequences were obtained directly from RNA extracted from clinical specimens and this avoided the potential effects of passaging through cell cultures when some parts of the FMDV genome can change due to adaptation to cell culture (Cottam *et al*., 2006). Therefore the sequences analyzed should be a true reflection of the outbreak virus sequences.

Pair-wise comparison between VP1 coding sequences of viruses obtained from these outbreaks showed that all the sequences analyzed shared greater than 99.2% nucleotide sequence identity. No transversions in the VP1 coding region were observed and some sequences shared 100% nucleotide sequence identity. In addition there were no amino acid differences in the predicted VP1 protein across all the sequences from these outbreaks. Therefore, all the samples, although collected from different districts and to a great extent from different regions, they were very closely related.

Primers designed to amplify SAT-1 and SAT-2 strains failed to yield any products and only serotype O specific primers amplified the complete VP1 sequences. This finding therefore indicated that the SAT serotypes were not involved in these outbreaks. In line with this, nucleotide sequence comparisons conducted using BLAST searches indicated that all the sequences obtained from the 2008/2009 Ugandan FMD outbreaks had the greatest sequence similarity to FMDV isolates of serotype O. The sequence relatedness between the 2008/2009 Ugandan sequences and the serotype O genbank sequences ranged from 79% to 94% (Table 2). This confirmed that FMDV serotype O was responsible for the outbreaks in eastern and northern Uganda. This is consistent with previous studies which have indicated that serotype O is highly prevalent in Uganda (Ayebazibwe *et al*., 2001b; Balinda *et al*., 2010; Mwiine *et al*., 2010). Complete VP1 coding sequences including representatives of serotypes A, C, SAT1, SAT2 and O, were subjected phylogenetic analysis. From phylogenetic analysis all the 2008/2009 sequences grouped with typical FMDV serotype O strains and formed a clade with them. This

further confirmed that serotype O was solely responsible for FMD outbreaks in Uganda between April 2008 and January 2009.

The molecular epidemiology of serotype O has been well studied and on the basis of VP1 sequence data collected from around the world, 11 topotypes have been identified worldwide (Knowles and Samuel 2003). In East Africa, four topotypes including EA-1, EA-2, EA-3 and EA-4 have been reported (Ayelet *et al*., 2009). EA-1 and EA-2 have been reported in Kenya and Uganda with the last 10 year but EA-2 strains have been the most predominant. The only EA-4 incursion into Uganda occurred in 1999 (Ayelet *et al*., 2009). EA-3 has not yet been reported in Uganda but only occurred in Kenya in 1980s but have bee frequently observed in Ethiopia.

To investigate the topotype involved in the 2008/2009 Ugandan FMD outbreaks, phylogenetic analysis indicated that the sequences from these outbreaks formed clade with isolates from Kenya (DQ165073), Malawi (DQ165074) and Uganda (DQ165077, AY344620 and AJ296320). The viruses in this sub-clade belong to the EA-2 topotype (Ayelet *et al*., 2009). This finding further supports the most recent study that has indicated that EA-2 as responsible for most of the type O outbreaks in Uganda (Balinda *et al*., 2010).

FMD outbreaks occurred in Eastern Uganda earlier in 2008 and they were followed by outbreaks in Northern Uganda in January 2009. The sequence identity between the 2008 Eastern Uganda outbreak isolates and the 2009 Northern Uganda outbreak was over 99.2%. A high nucleotide sequence identity between viruses from the two outbreaks indicates that the two outbreaks were due to the same strain or outbreaks are from a common source (Knowles and Samuel, 2003). Overall, all the 24 VP1 coding sequences from the 2008 and 2009 FMD outbreak viruses were almost identical with just a few synonymous nucleotide within the VP1 coding sequences. Some of the 2008 and 2009 outbreak virus sequences shared 100% identity.

This high level of VP1 coding sequence identity between the viruses obtained from these outbreaks across the two regions is a clear indication that the outbreaks in the Northern region originated from the Eastern region most likely by the movement of live animals across. This means that control measures put in place following FMD outbreaks in

Kamuli district were not adequate so infected animals crossed from the east to the north and caused an outbreak in this region. More recent studies in Uganda have identified risk factors encouraging the spread of FMD for one area to another including season and movement of infected animals from one region to another (Ayebazibwe 2010a). At the time this study was carried out, the northern region was experiencing a dry season and animals were on constant move in search for water and pusture. Such movement predisposes animals to infectious diseases such as FMD and encourages its spread.

FMD is not zoonotic and has low mortality rates. For this reason, many people especially in Uganda perceive FMD to be only a trade sensitive disease. International conventions require that all countries participating in international trade of animal and animal products abide by the rules that ensure sanitary guarantees for this trade. This requirement however is in conflict with the socio-economic practices of farmers who do not directly participate in international trade but rear animals such as cattle for a variety of other values other than trade. Some of these include ceremonial uses such as dowry and ploughing plus the production manure, meat and milk. Since farmers have different perceptions regarding the value of their animals, eradication of FMD, in Uganda becomes very difficult. Application of more efficient control measures such as stamping out are not carried out. Therefore there are two methods of FMD control in Uganda. These include vaccination and quarantines.

The capacity to investigate the transmission pathways using genetic data is of great value and helps to avoid the difficulties and uncertainties in identifying them using other methods. In this study VP1 coding region sequences were used to investigate the genetic relationship between viruses that caused outbreaks in Eastern and Northern Uganda. The findings from this study show that the viruses were genetically identical in this region. VP1 region is 639 nucleotides long and makes less than 10% of the FMDV genome. Therefore VP1 sequences alone may not contain sufficient genetic information to enable the reconstruction of transmission pathways at the highest resolution. High power resolution can be achieved through complete genome analysis to resolve transmission histories with the benefit of getting improved precision for epidemiological investigations especially for rapidly mutating viruses such as FMDV (Sibnarayan, 2007).

CHAMPTER SIX

CONCLUSIONS RECOMMENDATIONS

6.1 Conclusions

There were multiple FMD outbreaks in Eastern and northern Uganda. These outbreaks were caused by FMDV serotype O that belongs to EA-2 topotype. It is likely that the control measures put in place following an outbreak in Kamuli district in 2008 were not adequate so the disease spread from the East to the North through live animal movement.

6.2 Recommendations

It is probable to suggest that EA-1 vaccine strain that is currently being used to control FMD in Uganda does not offer complete protection against the EA-2 strains that are causing the current FMD outbreaks in this country. This has serious implication for FMD control through vaccination. It is therefore necessary to obtain definitive evidence for this possible incomplete protection that may include neutralization test.

The association between the continuous usage of the EA-1 vaccine strain and the increasing number of FMD outbreaks in Uganda poses a challenge to MAAIF on whether to continue using this expensive vaccine with limited success, or consider preparation of a vaccine stock derived from EA-2 that have increasingly caused FMD outbreaks in Uganda over the recent years.

Molecular characterization of the FMDV should be carried out following each FMD outbreak so that the vaccine strain and field strains can be assessed and efforts to match the field strains and the vaccines should be attempted.

Vaccination should be carried out at regular intervals using vaccine prepared from circulating strains.

Uganda should consider setting up an FMDV vaccine testing/matching laboratory that should routinely evaluate the vaccines and field or circulating strains.

Where an outbreak has occurred, strict Quarantines should be enforced to avoid the spread of the disease to new FMD free areas.

References

Ahl R, Haas B, Lorenz RJ, Wittman G (1991) Alternative potency test of FMD vaccines and results of comparative antibody assays in different cell systems and ELISA, European Commission for the Control of Foot-and-mouth disease, Session of the Research Group of the Standing Technical Committee, Lindholm, Denmark pp 51.

Alexandersen S, Brotherhood I, Donaldson AI, (2002) Natural aerosol transmission of Foot-and-mouth disease virus to pigs: Minimal infection dose for strain O1 Lausanne. *Journal of Epidemiology and Infection*, **128**: 301-312.

Amarel-Doel CMF, Owen N., Ferris NP, Kitching R, Doel TR (1993) Detection of foot-and-mouth disease viral sequences in clinical specimens and ethyleneimine-inactivated preparations by the polymerase chain reaction. *Vaccine*, **11**: 415–421.

Anon (2002) Infectious diseases in livestock. A policy document 15/20. The royal Society, London. *Lippincott, Williams and Wilkins Philadelphia*, 685-715.

Ayebazibwe C, Kirsten T, Mwine FN, Muwanika VB, Ademun Okurut AR, Siegismund HR, Alexandersen S (2010a) Patterns, risk factors and characteristics of reported and perceived foot-and-mouth disease (FMD) in Uganda. *Tropical Animal Health*, **42**:1547-1559.

Ayebazibwe C, Mwine FN, Kirsten T, Balinda SN, Muwanika VB, Ademun Okurut AR, Belsham BJ, Norma P, Siegismund HR, Alexandersen S (2010b) The role of the African buffalo (*Syncerus caffer*) in the maintenance of foot-and-mouth disease in Uganda, Veterinary Research, **6**: 54.

Ayelet G, Mana M, Gelaye E, Berhe GE, Tesfaye R, Mesfin S, Nigel PF, Jamma W, Hutching GH, Knowles NJ (2009) Genetic characterization of foot-and-mouth disease viruses. Ethiopia 1981-2007. *Journal of Emerging Infectious diseases*, **15:** (9), 1409- 1417.

Balinda SN, Belsham GJ, Masembe C, Sangula AK, Siegismund HR, Muwanika VB (2009) Molecular characterization of SAT 2 foot-and-mouth diseases virus from post-outbreak slaughtered animals: implications for disease control in Uganda. *Journal of Epidemiology and Infections*, **138**: 1201-1210.

Balinda SN, Sangula AK, Rasmus H, Muwanika VB, Belsham GJ, Masembe C, Siegismund HR (2010) Diversity and transboundary mobility of serotype O foot-and-mouth disease virus in East Africa: Implication for vaccination policies. *Infection, Genetics and Evolution*, doi:1016/j.meegid.2010.06.017

Barco A, Feduchi E, Carrasco L (2000) Poliovirus Protease 3C pro kills cells by apoptosis. *Journal of Virology*, **266**: 352-360**.**

Bastos ADA, Anderson EC, Bengis RG, Keet DF, Winterbach HK, Thomson GR (2003) Molecular epidemiology of SAT-3 type foot-and-mouth diseases. *Journal of Virus Genes*, **27:** (3), 283-290.

Barnett PV and Cox SJ (1999) The role of small ruminants in the epidemiology of foot-and-mouth disease. *Veterinary Journal*, **158:** 6-13.

Belsham GJ and Martinez-Salas E (2004) Genome organization translation and replication of Foot-and-mouth disease virus RNA. Current perspective. *Horizon Biosciences Norfolk, UK* 19-52.

Berger HG, Straub OC, Ahl R, Tesar M, Marquardt, (1990) Identification of foot-and-mouth disease virus replication in vaccinated cattle by antibodies to non-structural virus proteins. *Vaccine*, **8**:213-216.

Bruckner GK, Vosloo W, Du Plessis BJA, Kloeck PEG, Cannoway L, Ekron MD, Weaves DB, Dickson CJ Schrauder FJ, Marais T. Mogajane ME (2002) Foot-and-mouth Disease: The experience of South Africa. *Scientific and Technical Reviews of office International des Epizooties*, **21:** (3), 751-754.

Callahan JD, Brown F, Csorio FA, Sur JH, Kramer E, Long GW, Lubroth J, Ellis SJ, Shoulars KS, Gaffney KL, Rock DL, Nelson WM (2002) Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *Journal of American Veterinary Medical Association*, **220:** (11), 1636–1642.

Carrillo C, Tulma ER, Delhon G, Lu Carreno A, Vagnozzi A, Kutish GF, Rock DL(2005) Comparative genomics of foot-and-mouth disease. *Journal of Virology*, **79:** (4), 6487-6504.

Chenard GK, Miedema K, Moone P, Schrijver RS, Deker A (2003) A solid-phase Blocking ELISA for the detection of type O foot-and-mouth disease virus antibodies suitable for mass serology. *Journal of virology Methods*, 107:89-98.

Chenwen X, Zahid R, Diwen L, Songhua H (2007) Enhancement of Serological Immune Responses to Foot-and-Mouth Disease Vaccine by a Supplement Made of Extract of Cochinchina Momordica Seeds. *Clinical and Vaccine Immunology,* 1634-1639.

Clarke JB and Spier RE (1980) Variation in the susceptibility of BHK populations and cloned cell lines to three strains of foot-and-mouth disease virus. *Archives of . Virology,* **63:** 1–9.

Cottam EM, Haydon DT, Panton JP, Gloster J Wilesmith JW, Nigel RP, Hutchings GH, Donald PK (2006) Molecular Epidemiology of the Foot-and-mouth disease Virus out break in the United Kingdom in 2001. *Journal of Virology,* **80**:11274-1128.

Crowther ER and Abu EEME (1979) Detection and quantification of foot-and-mouth disease virus by enzyme labeled Immunosorbent assay techniques. *Journal of General virology*, 42:597-602.

Doel TR (1996) Natural and vaccine induced immunity to foot-and-mouth disease. *Scientific and technical Review Office International des Epizotes,* **15**: 883-911.

Domingo E, Mateu MG, Martinez MA, Dopazo J, Moya A, Sobrino F(1990) Genetic variability and antigenic diversity of foot-and-mouth disease virus. *Journal* of *Applied Virus Research,* **2**: 233-266.

Domingo E, Baranowski E, Escarmis S (2002) Foot-and-mouth disease. *Journal of Comparativ*e *Immunology, Microbiology and Infectious Disease,* **25:** (5), 297-308.

Donaldson AI, Alexandersen S (2002) Predicting the spread of foot-and-mouth disease Virus by airborne virus. *Review and Scientific Technical, Office International des Epizotes,* **21**: (3), 569-575.

Gloster J, Doel C, Gubbins S, Paton DJ (2007) Foot-and-mouth disease: Measurements of aerosol emission from pigs as a function of virus strain and initial dose. *The Veterinary Journal,* **177**: (3), 374-380.

Grubman MJ, Baxt B (2004) Foot-and-mouth disease. *Clinical Microbiology Review,* **17:** (2), 465-493.

Hall TA (1999) BioEdit. A user friendly biological sequence alignment editor and analysis program for windows 98/98 NT. *Nucleic Acids Symposium Series,* **41**: 95-98.

Hedger S (1972) Foot-and-mouth disease in the African buffalo (*Syncerus caffer*). *Journal of Comparative Pathology*, **82**:19-28.

House C and House JA (1989) Evaluation of techniques to demonstrate FMDV in bovine tongue epithelium: comparison of the sensitivity of cattle, mice, primary cell cultures, cryo-preserved cell cultures and established cell lines. *Journal of Veterinary Microbiology*, **20**: 99-109.

James AD, Rushton J (2002) The economic s of foot-and-mouth disease. Review Scientific Technical Office International des Epizotes **21**: 637-644

John B (2002) The 2001 outbreak of foot-and-mouth disease. Report by the Comptroller and Auditor General; National Audit office, HC 939 Session 2001-2001: 21 June

Kalema-Zikusooka G, Begins RG, Michel AL., Woodford MH (2005) A preliminary investigation of tuberculosis and other diseases in African buffalo (*Syncerus caffer*) I Queen Elizabeth National Park, Uganda. Onderstepoort Journal of Veterinary Research, **72**: 145-151.

Kitching RP (1998) A recent history of Foot-and-mouth disease. *Journal of comparative pathology*, **118**: 89-108.

Knowles NJ, Ansell DM, Samuel AR (1998) Molecular comparison of recent foot-and-mouth disease type A virus form West Africa with historical and reference virus strains. Report of the session of the Research Group OS Standing Technical Committee of the European Commission of Foot-and-Mouth Disease, Aldershot, United Kingdom. Rome: FAO, *Appendix* **4:** 41-48.

Knowles NJ and Samuel AR (2003) Molecular epidemiology of Foot-and-mouth disease virus. *Journal of Virus Research,* **91:** (1), 65-80.

Loeffler F, Frosch P (1898) Report of the Commission for Research on Foot-and-mouth Disease. *Journal of Parasitology,* **23**: 371-397.

Martinez-Salas E, Fernadez–Miragall O (2004) Picornavirus IRES: Structure function relationship. *Current Pharmacology Disease* **10**: 3757-3767.

Mwiine FN, Ayebazibwe C, Olaho-Mukani W, Alexandersen S, Balinda SN, Masembe C, Ademun Okurut AR, Christensen LS, Sorensen KJ, Tjornehoj K, (2010) Serotype specificity of antibodies against foot-and-mouth disease virus in cattle in selected districts in Uganda. *Transboundary and Emerging Diseases,* 57: 365-374.

Murphy FA, Gibbs EP, Horzineck MC (1999) Picornaviridae. *International Veterinary Virology,* **417**-518.

OIE (2004) Foot-and-mouth disease. In: Mannual of standards for Diagnostic Tests and Vaccines for Terrestrial Animals 5^{TH} Edn. Paris, OIE.

OIE (2009) Foot-and-mouth disease. In: Mannual of standards for Diagnostic Tests and Vaccines for Terrestrial Animals 6^{T} Edn. Paris, OIE, http/www.oie.int/eng/norms/mannual-2008-pdf/2.01.01 FMD.pdf

Posada D (2008) Model test: Phylogenetic Model Averaging. *Journal of Molecular Biology and Evolution,* **25**:1253-1256.

Prempeh H, Robert M (2001) Foot-and-mouth disease. The human consequences. The health consequences are slight, the economic ones are huge. *British Medical Journal,* 3: (22), 566-572.

Quian F, Chen X, Ma O, Liu Y, Ding M, Collins AR, Lung-Sang K, Xing J, Lok-Ting L, Cheung-Hoi, AY, Chen J (2003) Serotype and VP1 Sequence of Foot-and-mouth disease virus from Hong Kong. *Biochemical and Biophysical Research communications,* **30:** (2003), 715-721.

Reid S, Ferris NP, Hutchings GH., Zhang Z, Belsham GJ, Alexandersen S (2001). Diagnosis of foot-and-mouth disease by real-time fluorogenic PCR assay. *Veterinary Records,* **149**:621–623.

Reid SM, Grierson SS, Ferris NP, Hutchings G H, Alexandersen S (2003) Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *Journal of Virology Methods,* **107:** (2), 129–139.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian Phylogenetic inference under mixed models. *Journal of Bioinformatics*. **19**:1572-1574.

Samuel AR, Knowles NJ (2001) Foot-and-mouth disease virus type O exhibit genetically and geographically distinct evolutionary lineages (topotypes). *Journal of virology*, **82**: 609-621.

Sibnarayan D (2007) Selecting a genetic region for molecular analysis of Hepatitis B Virus transmission. *Journal of Clinical Microbiology*, **45**: 687-689.

Skinner HH (1960) Some techniques for producing and studying attenuated strains of the virus of foot and mouth disease. *OIE*, **53**: 634–650.

Sorensen KJ, de Stricker K, Dyrting KC, Grazioli S, Haas B (2005) Differentiation of virus infected animals form vaccinated animals using blocking ELISA based on Baculovirus expressed FMDV 3ABC antigen and 3ABC antibody. *Archives of Virology*, **150**:805-814.

Stanway G, Brown F, Christian P, Hovi T, Hypia T (2005) Family *Picornaviridae*. Eighth Report of the International committee on Taxonomy of viruses. London: Elsevier/Academic Press 2005. Pp 757-778.

Tamura K, Dudley J, Nei M, Kumar S. MEGA4 (2007): Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**:1596–9.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTALW: Improving sensitivity of progressive multiple sequence alignment through sequence weighing .position specific gap penalties and weight matrix choice. *Journal of Nucleic acid Research* 22:4673-4680.

UBOS/MAAIF (2009) Uganda Bureau of Statistics/ Ministry of Agriculture Animal Industry and Fisheries –Summary report of the National Livestock Census-2008, Natinal Livestock Census, Uganda.

Woodsbury RL (1995) A review of the possible mechanisms for the persistence of foot-and-mouth diseases virus. *Journal of Epidemilogy and infections*, **114**: 1-13.