RISK FACTORS AND MOLECULAR EPIDEMIOLOGY OF FOOT AND MOUTH DISEASE VIRUS AND OTHER ANIMAL PATHOGENS INFECTING AFRICAN BUFFALOES, CATTLE AND GOATS IN EASTERN RWANDA.

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A thesis submitted in fulfilment for the Degree of Doctor of Philosophy in Biotechnology in the Department of Biochemistry, University of Nairobi

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DECLARATION

I, Jean Claude UDAHEMUKA, declare that this thesis is my original work and has not been presented for a degree or any other award in any university. To the best of my knowledge and brief, this thesis contains no material previously published or written by another person except where due reference is made.

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DEDICATION

In loving memory of my parents.

To my wife and children for their love and support.

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LIST OF ABBREVIATIONS AND ACRONYMS:

Abbreviation **Term**

- AI: Artificial insemination.
- ANP: Akagera National Park.
- AU-IBAR: African Union Interafrican Bureau for Animal Resources.
- BEAST: Bayesian Evolutionary Analysis Sampling Trees.
- BLAST: Basic Local Alignment Search Tool.
- BoLA: Bovine Leukocyte Antigen.
- CI: Confidence interval.
- CTL: Cytotoxic T lymphocytes.
- DDBJ: DNA Data Bank of Japan.
- DNA: Deoxyribonucleic Acid.
- DRC: Democratic Republic of Congo.
- DTU: Technical University of Denmark.
- EBI: European Bioinformatics Institute.
- ELISA: Enzyme-Linked Immunosorbent Assay.
- EMBOSS: European Molecular Biology Open Software Suite.
- EuFMD: European commission for the control of Foot-and-Mouth Disease.
- FAO: Food and Agriculture Organization.
- FMD: Foot-and-Mouth Disease.
- FMDV: Foot-and-Mouth Disease Virus.
- FOTIVAX^{TM}: Kenya Foot-and-Mouth Disease Vaccine.
- FRET: Forster (or fluorescence) Resonance Energy Transfer.
- HLA: Human Leukocyte Antigen.

- HNB: Hydroxylnaphtol blue.
- IEDB-AR: Immune epitope database analysis resource.
- IRES: Internal Ribosome Entry Site.
- KEVEVAPI: Kenya Veterinary Vaccines Production Institute.
- KNP: Kruger National Park.
- LFD: Lateral Flow Device.
- MEGA: Molecular Evolutionary Genetics Analysis.
- MHC: Major Histocompatibility Complex.
- MINAGRI: Ministry of Agriculture.
- mPCR: multiplex polymerase chain reaction.
- NGS: Next-Generation Sequencing.
- NSP: Non-Structural Proteins.
- OIE: Organisation Internationale des Épizooties.
- ONT: Oxford Nanopore Technologies.
- OPF: Oropharyngeal fluid.
- OR: Odds Ratio.
- PacBio SMRT: Pacific Biosciences Single-Molecule Real-Time sequencing.
- PCP-FMD: Progressive Control Pathway for the control of Foot-and-Mouth Disease.
- PDB: Protein Data Bank.
- PhD: Doctor of Philosophy.
- PVS: Protein Variability Server.
- QGIS: Quantum Geographic Information System.
- qPCR: quantitative polymerase chain reaction.
- RBSP: Risk-Based Strategic Plan.
- RFU: Relative fluorescence units.

- RGD: Arginine-Glycine-Aspartic acid.
- RNA: Ribonucleic Acid.
- RT-qPCR: Reverse Transcription quantitative Polymerase Chain Reaction.
- RT-LAMP: Reverse Transcription Loop-mediated isothermal AMPlification.
- RT-PCR: Reverse Transcription Polymerase Chain Reaction.
- SAT: Southern African Territories.
- SOLiD ligation: Sequencing by Oligonucleotide Ligation and Detection.
- SPSS: Statistical Product and Service Solutions.
- SRA: Sequence Read Archive.
- SYBR green: asymmetrical cyanine dye used as a nucleic acid stain in molecular biology.
- UK: United Kingdom.
- USDA: United States Department of Agriculture.
- UTR: Untranslated region.
- VP1: Viral Protein 1.
- WOAH: World Organization for Animal Health
- WRLFMD: World reference laboratory for Foot-and-Mouth Disease.

ABSTRACT

Foot-and-Mouth Disease Virus (FMDV) is a positive-sense RNA virus of the family of the picornaviridæ causing the Foot-and-Mouth Disease (FMD). Although FMD is endemic in Rwanda, there are information gaps with regards to risk factors, seroprevalence and molecular epidemiology of FMDV strains circulating in Eastern Rwanda. The objectives of this study were to identify FMD risk factors, seroprevalence and establish molecular profiles of FMDV in cattle, goats and African buffaloes in Eastern Rwanda.

The study was performed in two main parts, first the development of questionnaires to establish risk factors for FMD outbreaks. Descriptive statistics were determined and odds ratios were calculated to determine the effects of risk factors on the occurrence of FMD. Quantum Geographic Information System (QGIS) was used to produce thematic maps on the proportion of putative risk factors for FMD per village. Secondly, surveillance of FMD-susceptible African buffaloes, was also carried out in Akagera National Park. A competitive ELISA kit manufactured by innovative diagnostics (ID.Vet, Grabels, France) was used to detect antibodies against the non-structural protein 3ABC of FMD in sera of animals from 3 districts of the Eastern province in Rwanda. Field and vaccine strains of FMDV were detected using RT-PCR and RT-LAMP assays and thereafter sequenced using the Ion Torrent Next-Generation Sequencing platform (Thermo Fisher Scientific). Epitopes-mapping was done *in silico* using the VP1-3 proteins of FMDV isolated in East Africa based on a 3D model (PDB ID:5aca) using the PyMol software (Schrödinger, 2021).

Questionnaire data revealed that 85.31% ($p < 0.01$) of the farms experienced more outbreaks during the dry season than the wet season. Univariate analysis revealed that mixed farming (OR = 1.501, $p = 0.163$), and natural breeding method (OR = 1.626; $p = 0.21$) were associated with the occurrence of FMD indicating that the two risk factors could be responsible for FMD outbreaks in the farms. The occurrence of FMD in the farms was found to be significantly

associated with a lack of vaccination of calves younger than 12 months in herds (OR = 0.707; $p = 0.046$).

The overall seroprevalence of FMD in the study area was 9.36% for cattle and 2.65% for goats. The phylogenetic analysis revealed the introduction of SAT2, lineage II traditionally known to be in Southern Africa (Zimbabwe). On sequence analysis, FMDV was not detected in buffalo samples. Nevertheless, other pathogens such as orf virus and bacteria were detected suggesting that cattle in the study area may be infected with other pathogens apart from FMDV due to interactions between wildlife and domestic ruminants. *In silico* analysis revealed that VP1, VP2 and VP3 surface proteins of the East African - FMDV isolates could induce humeral and cell-mediated immunity if administered in cattle. The Wu-Kabat variability index identified variable regions, including the known GH loop, and conserved regions in the VP1, VP2 and VP3 peptides. Epitope mapping revealed 3D-structures having various epitopes located on VP1, VP2 and VP3 model. In conclusion, it appears that some animals were exposed to FMDV with some animals being infected with the disease. Furthermore, the vaccination of all age group would improve the control of FMD. Finally, there appears to be an introduction of SAT 2 strain from Southern Africa into the East African region. Further studies are recommended on molecular epidemiology of FMD virus on buffaloes and livestock in Eastern Rwanda. Although, the generated models appeared to be antigenic, more detailed studies are needed to confirm these predictions. The *in silico* analysis proposes epitopes and *in vitro* analysis of vaccines showed the presence of SAT2 serotype in the used vaccine. It appears that African buffaloes in Akagera National Park did not have FMDV. Nevertheless, further studies are required to validate these findings.

Chapter 1 : INTRODUCTION

1.1. Background of the study

The majority of the population of Rwanda is involved in agriculture, therefore increasing the export of animal products is very important(Central Intelligence Agency, 2019). However, due to the endemicity of animal infectious diseases such as Foot-and-Mouth Disease (FMD), there is a barrier to the trade of meat and other animal products. Part of the strategy outputs is to improve the meat and milk industry to meet international standards and reach the level of freedom from FMD in the Progressive Control Pathway for Foot-and-Mouth Disease (PCP-FMD) to export to international, more lucrative, markets.

There exist seven serotypes of FMDV namely O, A, C, Asia-1 including SAT 1, 2 and 3. All the isolates identified worldwide so far have been reported to belong to one of the seven serotypes (Jamal & Belsham, 2013). Though the isolates may belong to the same serotypes, some circulating FMDV have been shown to be of different genotypes. Due to the high variability in VP1 sequences, the protein has been used to classify isolates of the same serotype into topotypes. For serotypes other than SATs, if the difference in VP1 sequences doesn't exceed 15%, then they are considered to belong to the same topotype (Jamal & Belsham, 2013); however, for SATs, this threshold has been kept at 20% (Ayelet et al., 2009).

The SAT 2 is composed by fourteen topotypes; and widely distributed across Sub Saharan Africa from Gambia to Ethiopia and from Sudan to South Africa. These fourteen topotypes can be divided into three geographic regions including Southern African region (Bastos et al., 2003a), West African region (V and VI)(Sangare, 2002) and the Eastern-Central African countries (VII-XIV) (Sahle, 2004; Sangare, 2002). Topotype IV is found both in Southern Africa (Malawi) and East African countries such as Burundi, Kenya, Tanzania and Ethiopia. For SAT 3, four (I, II, III and IV) out of six topotypes have been isolated in Zimbabwe,

Botswana, Zambia and South Africa with the topotypes V and VI having been isolated in Uganda (Bastos et al., 2003a; Reid et al., 2001). However, some of the topotypes including VI, VII and VIII for SAT 1; VI, XI, XII, and XIV for SAT 2; III and V for SAT 3 may be extinct because they have not been isolated since 1994 (Di Nardo et al., 2011; Knowles et al., 2010), SAT 3 is mainly restricted to buffaloes and is not considered as a real threat for a cross-border spread (Hammond et al., 2012).

FMD is caused by a virus of the family of the Picornaviridae of the Aphtovirus genus. FMDV is an icosahedral single-stranded RNA virus of 25-30 nm diameter (Acharya et al., 1989; USDA, 2020) and is inactivated by temperature above 50ºC, pH <6.0 or >9.0, humidity, other climatic factors and common disinfectants such as sodium carbonate (4%), sodium hydroxide (2%) and citric acid (0.2%) (Krug et al., 2012) and the solar UV (254nm) light (El din Mahdy et al., 2015). After death of infected animals, FMDV is inactivated in the muscles by acidification due to the *rigor mortis* state but survives in lymph nodes and bone marrow because of poor acidification in those tissues (WOAH, 2013).

FMD is mainly transmitted by direct contact with an infected animal grazing on contaminated pasture especially near communal watering point. The disease can also be transmitted to a susceptible animal through air. During the 1980s, a case was identified where an infected animal from a farm in France transmitted FMD to another farm in the UK with FMDV to have probably by crossed the English Channel in the air (Meyer & Knudsen, 2001).

FMD is considered to be a livestock disease that has the highest impact on economy, it does also affect transboundary trade and modern farming worldwide (Depa et al., 2012). This is because the disease is infectious and can persist in farms for a long time thereby impacting livestock production negatively (Knowles & Samuel, 2003). All the 7 serotypes have been reported geographically in Africa, Asia, Europe with serotypes C, A and O reported in these regions (Rweyemamu et al., 2008). The SAT serotypes are only found in Sub-Saharan Africa,

with some sporadic outbreaks in Middle East and Greece for SAT 1 (Knowles & Samuel, 2003) and SAT 2 has been reported in Yemen, Libya and Egypt (Valdazo-González et al., 2012). In 1984 and 2000, Greece is the only non-Asian country reported to have experienced outbreaks of Asia type 1-FMD (Ansell et al., 1994; Jamal & Belsham, 2013). Based on the present serotypes in different countries, seven pools have been identified for FMD worldwide, based on the serological and molecular investigations and these pools are geographically grouped (Sumption et al., 2012).

Recently, strains traditionally known to be in one part of the world have been identified in other parts of the world. The presence of East African topotypes of serotype O in other parts of the world other than East Africa is a reflection of the dynamics of animal movements among other risk factors. Therefore, there is a dire need of constant evaluation of the circulating virus to assure the effectiveness and matching of the circulating genetically evolving virus.

FMD testing is done at both field and laboratory levels. Some tests have been developed to screen and confirm an FMDV infection in the field(Bearinger et al., 2011; Olasagasti & de Gordoa, 2012). However, these field tests alongside with clinical observations still need to be confirmed in the laboratory with other tests. Also, when choosing a test to perform different characteristics (sensitivity and specificity) have to be taken into consideration such as their performance (limit of detection, use of different sample types), the time it takes to give results, its scalability, the cost, the simplicity and the results interpretation, and the biosafety concerns (Anil et al., 2021; Ochwo et al., 2023).

The lateral flow device (LFD) is a simple and quick device that can detect FMD within less than 10 minutes and can be inactivated by soaking it in 0.2% citric acid for 15 minutes for a safer transport from the field to the laboratory (Ferris et al., 2008). They have an advantage of confirming FMD on the pen-side and after a simple inactivation of the virus, a deep immersion in the citric acid can inactivate the virus, it can be transported to the laboratory for molecular

testing without the laborious and costly triple packaging. If enough genetic material is collected on this strip, it can be used for molecular testing (WOAH, 2022b).

In the case of FMD diagnosis timing is crucial, for instance if one needs a live virus that can even be cultivated later, samples have to be taken within eight days after infection. Different techniques have been developed according to how samples are intended to be exploited. Molecular detection can either use the Loop Mediated Isothermal amplification (LAMP) or the polymerase chain reaction (PCR). The LAMP is a simple and rapid way of amplifying nucleic material at a constant temperature and doesn't require a gel imaging step. LAMP provides results faster and with fewer requirements. For many techniques used to detect FMDV, RT-qPCR presents advantages of being time saving, reliable and sensitive. In 1991, the development of primers has helped in detecting nucleotides of several serotypes of FMDV at an early stage of an outbreak (Meyer et al., 1991).

Later with the introduction of automated PCR methods, the time of detection has been reduced and the accuracy to detect RNA has been increased. In this regard, automated PCR methods have been recommended as the gold standard test for molecular diagnostic of FMD and many other pathogens(Gu et al., 2023). For SAT serotypes, targeting 3D region is more sensitive that targeting the 5' UTR (King et al., 2006).

In 2016, Bachanek-Bankowska and her colleagues (Bachanek-Bankowska et al., 2016) developed an RT-qPCR for some East African countries (Kenya, Uganda, and Tanzania), without strains from Rwanda, this leads to a need of such work for the strains circulating in Rwanda as well to fill in the gap.

The control of FMDV differs from country to country and depending on the epidemiology status. Countries can choose a method according to its policies. For countries that are FMD freedom they can afford to slaughter all animals that infected and those suspected (Depa et al., 2012), another method to manage an outbreak used by other countries would be continuous

vaccination (Domingo et al., 2002). In Sub-Saharan Africa, many countries prefer the vaccination method because the compensation to farmers would cost a huge amount of resources. This is because countries in Sub-Saharan Africa are resource poor and therefore, cannot advocate for culling. There is also the issue of the reservoir in wild animals, which means culling by itself would not work as the new and susceptible herd would simply get infected by the first herd of buffalo or wildebeest that they encounter.

1.2. Problem statement

Foot and Mouth Disease (FMD) causes major economic losses to livestock production industry with losses estimated to range between $6.5 and $21 billion globally (Knight-Jones & Rushton, 2013). Foot and Mouth Disease (FMD) poses a significant threat to the livestock industry in Rwanda, a country heavily reliant on agriculture and livestock as a source of income and nutrition. Despite the presence of various control measures, the disease continues to persist and spread in Rwanda, impacting the livelihoods of millions of farmers. The problem arises from several factors, including the gaps on risk factors, the seroprevalence, the role played by different species (cattle, small ruminants, pigs and wild animals), the role played by transboundary animal movements, the circulating strains and the effectiveness of the used vaccines.

Bachanek-Bankowska *et al.* (2016) developed RT-qPCR that could detect FMDV strains circulating in East African countries such as Kenya, Uganda and Tanzania. However, this molecular detection method has not been used for the detection of the Rwandan strains of FMDV. Therefore, there is a need to apply the same method in order to establish whether the test can also be used to detect FMDV strains in Rwanda (Bachanek-Bankowska et al., 2016).

This thesis aims to address the critical gaps in understanding and implementing FMD control strategies in Rwanda. It has investigated the risk factors, the seroprevalence, the molecular epidemiology of FMD and the vaccinology part. The ultimate goal is to provide evidence-based

recommendations to enhance FMD control efforts, protect livestock-dependent livelihoods, and contribute to the overall development of Rwanda's livestock sector.

This study investigated the risk factors for the incursion, spread and persistence of FMD. the seroprevalence of FMD was also determined using the Enzyme-Linked Immunosorbent Assay (ELISA). Finally molecular techniques such as the polymerase chain reaction (PCR), the Loop-mediated isothermal amplification (LAMP), the Next-Generation Sequencing (NGS) and molecular modelling were also used to study the molecular epidemiology of FMD.

1.3. Study justification

For Rwanda to progress to stage 3 of Progressive Control Pathway (PCP-FMD) with a goal of achieving the stage of "FMD free without vaccination" (stage 5) and boost the meat import/export industry, proper research projects and regional coordinated efforts are needed. The findings from this study will help all stakeholders to effectively choose the right vaccines for the animals and reach a point of controlling and further control of FMD. It has been shown that African buffaloes (*Syncerus caffer),* which are carriers of FMDV (reservoirs), play a big role in transmitting the virus to cattle and vice-versa, hence this area surrounding Akagera National Park (ANP) was chosen. By aligning my results with the data of previous outbreaks, conclusions on how genotypes have changed over time will guide the choice of vaccines to be adopted, and develop policies that are fit for FMD control in Rwanda.

Understanding risk factors assists policymakers in strategically establishing policies that have optimal impacts with fewer resources. Likewise, molecular diagnostics assist in understanding several aspects of the dynamics of the disease such as animal movements, the role played by the wildlife, the mutations of the virus and the vaccines to be used.

This research will offer valuable insights into how Rwanda can better combat Foot and Mouth Disease, ultimately improving the resilience of its livestock industry and ensuring food security for its growing population.

1.4. Objectives

1.4.1. General study objective

To establish risk factors and genetic diversity of FMDV isolated from African buffaloes and cattle in Eastern Rwanda.

1.4.2. Specific objectives

i. To determine risk factors and the seroprevalence of FMD outbreaks in Eastern Rwanda.

ii. To identify FMDV strains and other animal pathogens isolated in African buffaloes in Eastern Rwanda.

iii. To determine the genetic diversities of FMDV infecting cattle in Eastern Rwanda.

iv. To identify epitopic peptides from capsid proteins of FMDV SAT 2 to improve FMDV vaccines and analyze the vaccine strains.

1.5. Null hypotheses

- Not including all age groups in vaccination increases the risk of cattle to get infected with FMDV.
- There is no diversity of the strains causing FMDV outbreaks in Eastern Rwanda.

Chapter 2 : LITERATURE REVIEW

2.1 Spatial and temporal risk factors for foot and mouth disease

Spatial risk identification is of paramount importance in any disease control, the key example being the 1854 London Cholera outbreak (Brody et al., 2000). FMD is a highly transmissible animal viral disease. The rate at which FMD is transmitted across a certain territory depends on different factors such as the landscape heterogeneity of the area among many others (Keeling et al., 2001). Depending on the amount of the virus released in the air, aerosols, clouds, the direction and speed of the wind, and the temperature and humidity rate of the atmosphere, the virus can travel many kilometres (Simmer & Volz, 2007). A striking example is the FMD transmission across the English Channel from France to the UK in 1981, this was described that pigs released enough virus in the air that they reached the British coast still having an infective dose of the virus though by the time they were analysed the dose had reduced to non-infective concentrations. Together with the transmission from the former German Democratic Republic to Denmark in 1982 and with a combination of meteorological data and other outbreaks, a computer model to describe the airborne spread of the FMDV was able to be developed (J. Sørensen et al., 2000).

Large farms are considered to be more susceptible to the disease (Keeling et al., 2001) although there is a lack of enough work determining the full economic cost of FMD in endemic countries, it is obvious that the bigger the farm, the bigger the loss to the farmer (Knight-Jones & Rushton, 2013). However, James *et al.* indicated that FMD would have a higher impact in dairy and pig farms compared to sheep farms in the UK (James & Rushton, 2002). There is a need to describe if this would be the case in endemic countries like Rwanda.

The study of landscape heterogeneity has also to take in consideration the different domestic species present in the area. For instance, cattle transmit the virus slightly more than sheep and

this difference may be due to biological parameters but mostly to the contact rate between human/vehicle and species (Keeling et al., 2001).

2.2. Foot and mouth disease virus and the role of wild animals

Domestic and wild animals, are susceptible to the FMDV (Belsham et al., 2022). Susceptible wild animals include among others; African buffaloes (Syncerus caffer), Wild Boars (Sus scrofa), and Impala (Aepyceros melampus) but the highest and most consistent antibody titres have been recorded in African buffaloes (Hedger, 1976).

In Southern Africa it was established that African buffaloes are carriers of FMD virus. For instance, in the Kruger National Park almost all buffaloes aged 2 years and above are infected with FMDV and the infection can last for a lifetime period (Vosloo et al., 2002a).

The physical separation using electrical fences has been successful in controlling livestock-wildlife contacts. It has been observed that the gap from the damage caused by floods to the electrical was responsible for a SAT 1 outbreak (Brückner et al., 2002). Studies have demonstrated a relationship between the virus circulating in livestock and wild animals with a special accent on buffaloes (Thomson, 1995; Vosloo et al., 2001).

In East Africa, there is still a gap in identifying the role played by African buffaloes in FMD epidemiology, but based on the buffalo population density, it can be assumed that the role played by buffaloes for FMD epidemiology in East Africa would be similar to the Southern African pattern (Tekleghiorghis et al., 2014). In Uganda (Queen Elizabeth National Park), serotype SAT 3 has been identified in buffaloes in calves grazing around the park (Bastos et al., 2003b). In Kenya, SAT 1 and SAT 2 viruses were successfully isolated from A. buffaloes. An earlier study had found no difference of strains of isolates from buffaloes and cattle (Anderson et al., 1979) and a more recent report found that the strains circulating in livestock were genetically different from those isolated in buffaloes (Wekesa et al., 2015). Likewise, Casey-Bryars in her thesis did not find a consistent proof of risk factors for the contact with

wildlife (buffaloes) and cattle in Tanzania (Casey-Bryars, 2016). Later on in 2020, Omondi et al. reported opposing facts suggesting that strains in cattle and buffalo in Kenya were related (Omondi et al., 2020). There is no known study reporting on FMDV isolates from buffaloes in Rwanda or Burundi.

Wild pigs such as boars are seen as reservoirs of many pathogens that can infect domestic animals, humans and other wildlife animals (Meng et al., 2009; Perrat et al., 2022). Animals that can be FMD virus positive after 28 days post infection are considered as carriers (Bronsvoort et al., 2016). The general perception is that pigs are not FMD carriers but some studies have shown an exception of identifying FMD viral RNA in blood samples at 28 days post infection (Mezencio et al., 1999). The available data show that wild boars (*Sus scrofa*) are susceptible to FMDV but are not carriers (Breithaupt et al., 2012). Furthermore, the 2011 FMD outbreak in Bulgaria demonstrated how wild boars constitute a major threat for FMD in livestock (Alexandrov et al., 2013). There is limited knowledge on the role played by wild pigs in FMD dynamics, this may be due to the difficult of sampling in wild animals. But innovative sampling methods such as the use of pathogen sampling wild animals with baits (pSWAB) can help in tackling this challenge as the cost-effective and most practical method (Ballesteros et al., 2011; Dietze et al., 2016; Mouchantat et al., 2014). In East Africa and Rwanda, there is the Giant Forest Hogs (*Hylochoerus meinertzhageni*) subspecies *H. m. meinertzhageni* in the national parks. The distribution is illustrated in figure 2-1. Hogs are at risk of getting infected with FMD due to swill feeding (Jamal & Belsham, 2013).

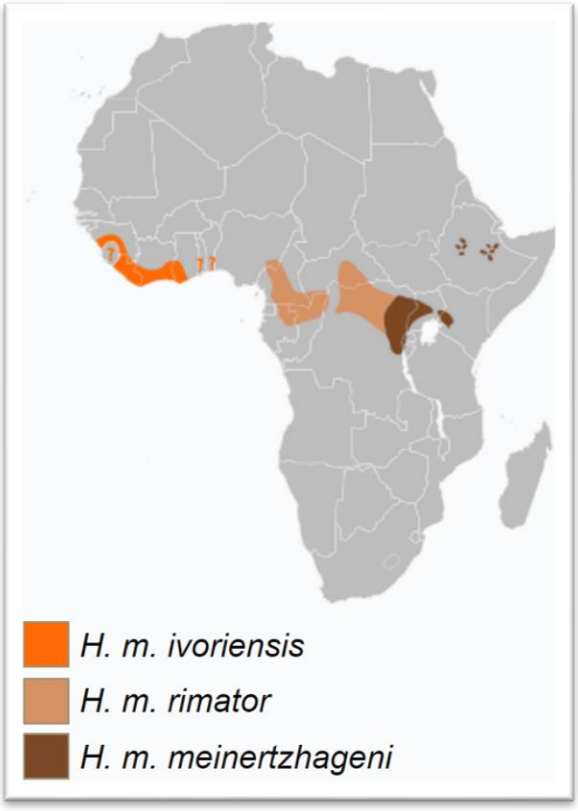

Figure 2-1: Hylochoerus meinertzhageni subspecies distribution in Africa (Wilson & Mittermeier, 2011)

Studies conducted in the Kruger National Park (KNP), South Africa, assessed the possibility of FMD natural transmission via Impala (*Aepyceros melampus*) and African buffaloes (*Syncerus caffer*) using VP1 sequences (coding for the major antigenic sites) isolated from Impala and Buffaloes they demonstrated inter- and intraspecies transmission of the circulating strains in those wild species (Bastos et al., 2000; Vosloo et al., 2009). Furthermore, it was shown that strains responsible for FMD outbreaks in livestock were related to the strains identified in buffaloes (Vosloo et al., 2002b).

2.3. Detection and molecular characterization of foot and mouth disease virus

The molecular characterization FMDV is very important since it allows to distinguish FMD with other vesicular diseases that share similar clinical symptoms on one hand, and it allows serotyping and phenotyping on the other hand. The VP1 sequence analysis has been used to determine serotypes and topotypes but also to understand the evolutionary story of circulating strains in a certain area.

2.3.1. Polymerase chain reaction

FMD diagnostics is very important for the control and eradication campaigns especially for countries where FMD is endemic (Rémond et al., 2002). In 1992, Rodriguez and his team realized the first typing of FMDV by RT-PCR (Rodriguez et al., 1992). For the reverse transcription qPCR, there are two methods developed, one targeting the 3D RNA part (Callahan et al., 2002) and another the IRES of the 5’ untranslated region (Reid et al., 2002). Detection by TaqMan has been preferred (Reid et al., 2003). Serotype-specific assays to characterize all the seven serotypes have been developed targeting VP1 (Knowles et al., 2017). Globally isolates collected from both livestock and wild animals have been characterized using both classic and real-time PCR (Giridharan et al., 2005; Lloyd-Jones et al., 2017; Paixão et al., 2008; Reid et al., 2009).

To improve the assays' capacity, multiplex Polymerase Chain Reaction (mPCR) assays using multiple primer sets have been developed to detect more than one pathogen (Das et al., 2022; Vangrysperre & De Clercq, 1996), or several serotypes (Giridharan et al., 2005; Sareyyüpoğlu & Burgu, 2017). Real-time quantitative PCR (qPCR) has been combined with fluorescent-emitting compounds (such as SYBR green) to measure the number of amplicons during an amplification process in real time (Smith & Osborn, 2009). FMDV detection using RT-qPCR have used several compounds, TaqMan (Callahan et al., 2002; Reid et al., 2002), FRET (Moonen et al., 2004), and SYBR green (Rios et al., 2018). Although SYBR green is economical, signals generated by FRET and TaqMan from unspecific amplification are negligible (Wong et al., 2020).

2.3.2. Loop-Mediated Isothermal Amplification (LAMP)

The Loop-Mediated Isothermal Amplification technique was designed as an alternative to PCR assays where the targeted nucleic acid is amplified using four primers without changing the temperature (Notomi, 2000) or sometimes a set of six primers (Shao et al., 2010) as described in figure 2-2.

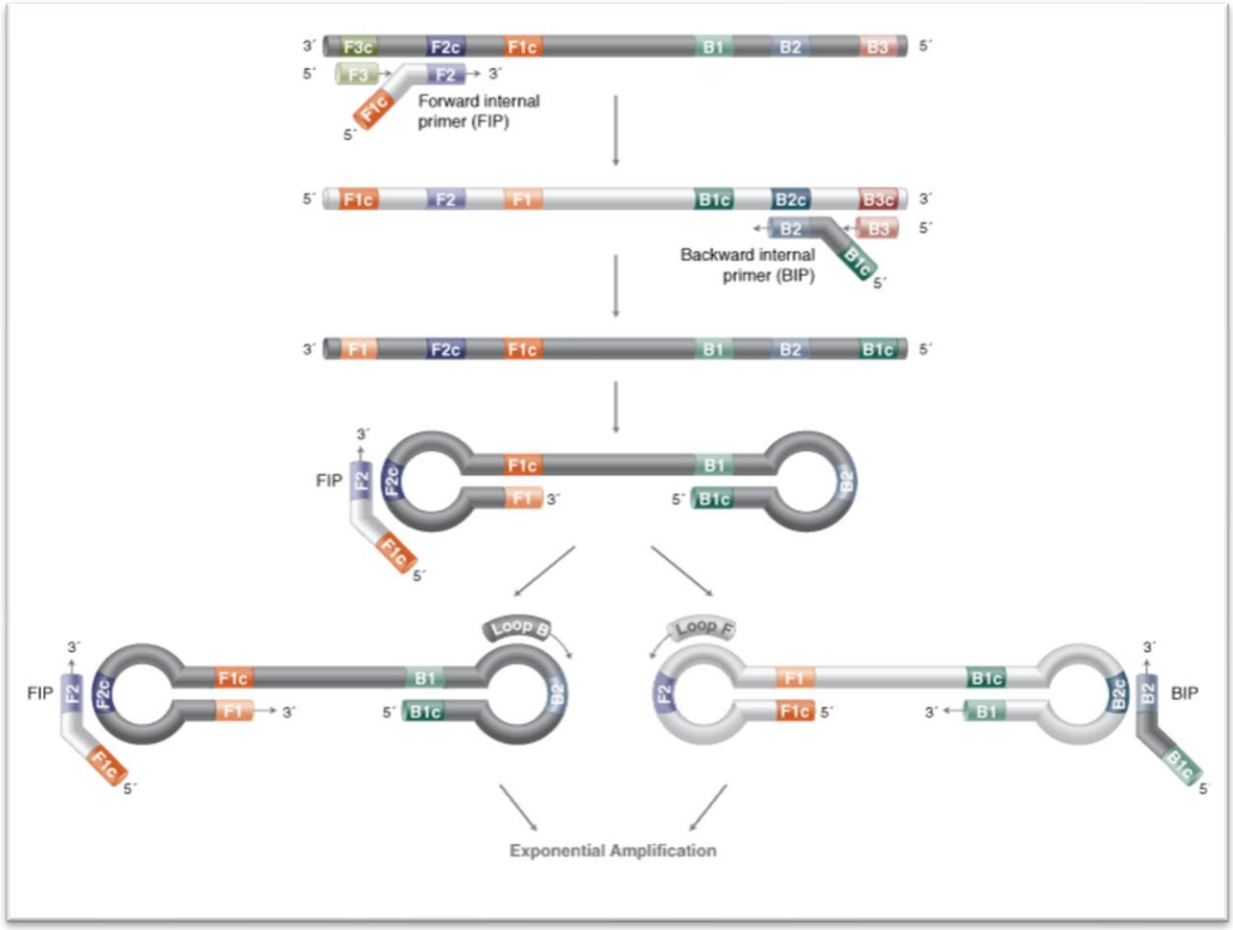

Figure 2-2: Between 4 and 6 sets of primers are used for LAMP to recognize 6-8 regions of target DNA (New England Biolabs, 2022).

A reverse-transcription-based assay (RT-PCR) was validated and could detect the FMD virus in less than an hour where the positive results could be seen by the naked eye if a dye like the hydroxyl naphthol blue (HNB) is added (Dukes et al., 2006; Li et al., 2009; Ranjan et al., 2014). Yamazaki et al., later on, developed and evaluated a multiplex LAMP assay for the molecular detection of FMD viruses (Yamazaki et al., 2013).

As a rapid, portable and hence field-deployable, low-cost, and simple to run, LAMP is a good alternative, especially in areas with fewer resources as a point-of-care detection assay (Parida et al., 2005; Sen & Ashbolt, 2011). Although LAMP is a such superb technique, it has a limitation in its sensitivity to cross-contamination by detecting non-specific amplification (Soroka et al., 2021). However, a study has reported the effect of Carboxamide and N-alkyl carboxamide additives in reducing non-specific amplification for FMD LAMP-based detection (Ghaith & Ghazaleh, 2021). The improvement of the assay to detect FMD has been reported in other parts like a study that investigated the detection of FMDV serotypes O and A strains of pool 1 region and RT-LAMP (Lim et al., 2018). Therefore, there is a need for developing and improving such techniques in FMD pool 4 region and other endemic areas especially for SAT serotypes.

2.3.3. Sequencing and Phylogenetic Analysis of Foot and Mouth Disease

In the early 1990s, it was possible to obtain Sanger sequencing results for FMD within a period of 3 days (Marquardt & Adam, 1990). Field sequencing and characterization of the VP1 protein played a pivotal role in developing strategic control measures by understanding how the disease strains circulated between geographic regions (Klein, 2009; Knowles & Samuel, 2003). Phylogenetic analyses revealed how strains of Asia 1, O and A serotypes circulated in different countries or regions of Asia by studying how nucleotide sequences were related (Muthuchelvan et al., 2001; Sanyal et al., 2004; Valarcher et al., 2009). The VP1 sequencing revealed the role played by animal movements in the epidemiology of FMDV between North Africa and Middle

East countries (ElMoety et al., 2013; Knowles et al., 2016; Samuel et al., 1999) or the proximity of wild animals and livestock in Southern Africa (Vosloo et al., 2001).

Several sequencing technologies and associated bioinformatics have been used to sequence the whole or partial genome of FMDV. In the 1970s, a first-generation sequencing technology based on the selective incorporation of chain-terminating dideoxynucleotides was developed (Sanger et al., 1977). Later on second-generation technologies emerged based on different detection methods including the 454 pyrosequencing (Roche), sequencing by synthesis (Illumina) (Ramirez-Carvajal et al., 2018), sequencing based on SOLiD ligation (Life Technologies) and semi-conductor sequencing (Ion Torrent) (Forth et al., 2020; Moon et al., 2019) generating greater throughput and eliminating capillary electrophoresis (Cottam et al., 2009; Singanallur et al., 2019; VanBorm et al., 2015).

The third-generation sequencing technologies brought in simplicity, scalability, low cost, long reads and no need for prior DNA amplification, hence removing potential bias related to amplification (VanBorm et al., 2015). The PacBio RS (Pacific Biosciences) was the first platform to be used to sequence single DNA molecules in real time without necessity of prior amplification (Belák et al., 2013) and PacBio SMRT has been used to study the polyclonal antibody responses to FMDV in African buffaloes and cattle (Philp, 2017). The Oxford Nanopore Technology (ONT) Sequencing platform is also a third-generation sequencing technology that enables real-time nucleic material (DNA or RNA) analysis by capturing changes to an electrical current of nucleic acids passing through pores (Deamer et al., 2016). A method for fast and reliable FMD diagnostics by ONT was developed that can be used both in laboratory and the field (Brown et al., 2021; Hansen et al., 2019) and ONT has been used on another picornavirus, the Senecavirus A (Tan et al., 2020). Other technologies have been used on FMD virus such as Single cell sequencing (10X) could provide more insights in the virus persistent infection (Yuan et al., 2022). The FMD sequencing results have been

phylogenetically analyzed using different techniques such as the Neighbour-Joining method (Saitou & Nei, 1987) to create phylogenetic trees and classify isolates (Knowles et al., 2017; Samuel & Knowles, 2001) and/or the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (Abeyratne et al., 2018; Knowles & Samuel, 2015) using tools such as Molecular Evolutionary Genetics Analysis (MEGA) (Tamura et al., 2021). A Bayesian phylogenetic inference is computationally more efficient and incorporates a model of evolution by considering prior information about a population and can use a tool like BEAST: Bayesian Evolutionary Analysis by Sampling Trees (Drummond & Rambaut, 2007). This analysis has been useful in studying the evolutionary dynamics of FMDV in different areas (Abeyratne et al., 2018; Bae et al., 2021; Brito et al., 2018).

2.4. Retrospective genetic analysis of foot and mouth disease virus circulating in East Africa (pool 4) for 10 years

The world reference laboratory for the foot-and-mouth disease (WRLFMD) has been at the frontline of characterizing isolated strains in different parts of the world. Based on VP1 sequencing, FMD endemic countries have been classified into pools and the East African countries are considered as being part of Pool 4 (Rweyemamu et al., 2008). The East Africa (Pool 4) comprises of the following countries: Burundi, Comoros, DRC, Djibouti, Eritrea, Ethiopia, Kenya, Rwanda, Seychelles, Somalia, South Sudan, Tanzania and Uganda as illustrated in figure 2-3. The appendix 1 describes different strains that have been isolated in Pool 4 for a period of 10 years as reported in different WRLFMD reports (WRLFMD, 2022). Furthermore, a study identified several serotypes, serotype A (topotype Africa lineage G-I), SAT 2 (topotype IV) as being responsible for FMD outbreaks in 2016 in Burundi (Garcia et al., 2022). In Sudan, serotypes O, A and SAT 2 were identified (Raouf et al., 2022).

Figure 2-3:Map of countries in the FMD Pool 4 (WRLFMD, 2022)

2.5. FMDV vaccines

2.5.1. Epitope prediction and modelling of capsid proteins and a multi-epitope vaccine design

Seven distinct serotypes have been identified and geographically localized with seven pools (Brito et al., 2017) and SAT 2 has been responsible for several outbreaks in Eastern Rwanda (Udahemuka et al., 2020). Vaccination against one serotype does not confer immunity against other serotypes (Rowlands et al., 1983). Therefore, high potency multivalent vaccines have been developed (Waters et al., 2018) and a continuous study of molecular epidemiology at the regional and national level is of paramount importance to have regional-tailored vaccines. Poor understanding of circulating strains leads to limited vaccine matching and vaccine failure (FAO/EuFMD, 2020). The peptide-based vaccines paradigm has been proposed as an alternative to the currently used inactivated vaccines (Defaus et al., 2020; Forner et al., 2021; C. Wang et al., 2002). Using the Bovine Leukocyte Antigen (BoLA) system, restricted Cytotoxic T lymphocytes (CTL) epitopes were predicted using the NetMHCpan 2.8 tool and the Immune Epitope Data Base Analysis Resource (IEDB-AR) and therefore producing sequences fit for potent vaccine design for both serotype A and Asia 1 (Mohan et al., 2022).

Vaccines and vaccination have proved to be efficient to control animal infectious diseases including FMD in South America (Naranjo & Cosivi, 2013; Saraiva & Darsie, 2004) and vaccines are considered as the main tool to control FMD in Sub-Saharan Africa (Hunter, 1998). East Africa, has the most complicated situation of FMD and efforts have been put together to produce region-tailored vaccines based on field-circulating strains (Hammond et al., 2021; Udahemuka et al., 2020). Using a large database of Major Histocompatibility Complex (MHC) systems (i.e. HLA for humans or BoLA for cattle), researchers have managed to develop computational tools able to predict the binding of peptides to MHC molecules such as the NetMHCPan (Nielsen et al., 2007) and improve it to be run on an online server (Nielsen &

Andreatta, 2016; Reynisson et al., 2020). FMD VP1 is an important immunogenic protein and has been considered to be included in the epitope prediction (Liu et al., 2011; Momtaz et al., 2014). Other structural proteins have been considered as well for *in silico* B and T cell epitope prediction (Bari et al., 2015; Raza et al., 2019). After identifying residues that would constitute good epitope candidates, it is very important to know if in the 3 directional confirmation they would still be accessible to antigens (Lo et al., 2021; Shih et al., 2022; Uttamrao et al., 2021). For the 3D visualization of proteins, there are several bioinformatic tools such as PyMol (Schrödinger, 2021), or RasMol (Bernstein, 2009).

2.5.2. Vaccine analysis

Thanks to the crystallographic studies, the FMDV capsid structure is known (Lea et al., 1994). Five FMDV neutralizing antigenic sites are known through monoclonal antibody (mAb) (Aktas & Samuel, 2000; Crowther et al., 1993; Grazioli et al., 2013; Kitson et al., 1990; Mahapatra et al., 2011) and those sites are illustrated in figure 2-4.

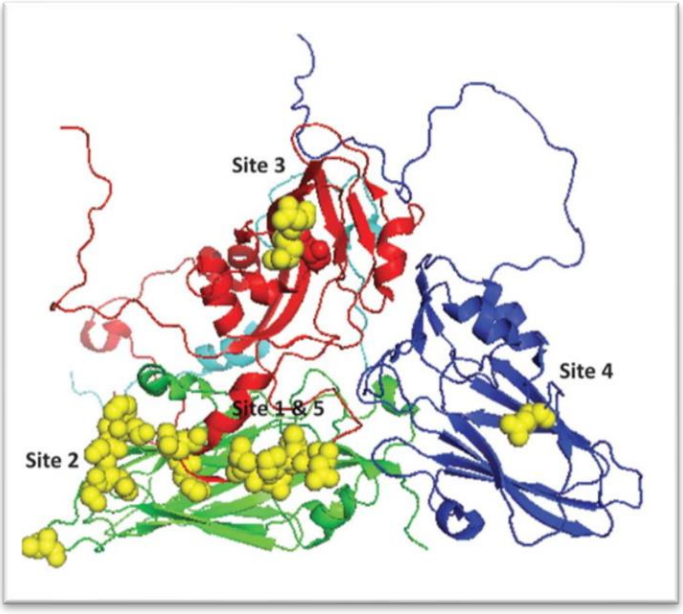

Figure 2-4: O1 BFS coordinates 3D structure (1FOD, reduced. The 5 antigenic residues are highlighted in yellow. Reproduced from (Mahapatra & Parida, 2018).

Due to the high variability in FMDV, there is no universal vaccine. In many areas more than one serotype are expected to cause outbreaks and sometimes one outbreak may be due to two or more serotypes (Garcia et al., 2022). This emphasizes the need for developing and using multivalent vaccines. Moreover, within the same serotype there may be no cross protection (Mahapatra et al., 2008; Mattion et al., 2004). There is always a need to study how the field strains match the vaccine strains. This can be done by calculating the antigenic relationship values (r1-values) (Kitching et al., 1988) where the 0.3 value is considered as the threshold above which there a close relationship between vaccine strains and field strains (Cao et al., 2021).

$$\text{r1-value}=\frac{\text{Antibody titre of vaccinal serum against field isolate (heterologous)}}{\text{Antibody titre of vaccinal serum against vaccine strain (homologous)}}$$

Other methods include extracting the RNA from the inactivated purified vaccine and phylogenetically compare it with the field strains (Jain et al., 2021; Je et al., 2018; Paraguison et al., 2010) or more innovatively use the artificial intelligence by using *in silico analysis* and trials using peptides towards BoLA class II (Borley et al., 2013; Glass, 2004; Liao et al., 2013; Raza et al., 2019; Udahemuka et al., 2022). The prediction of epitopes has been evaluated for strains circulating in different place to design peptide vaccines (Musa et al., 2017; Raza et al., 2019).

Chapter 3 : SPATIAL AND TEMPORAL RISK FACTORS FOR FOOT AND MOUTH DISEASE IN EASTERN RWANDA

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Risk factors for the incursion, spread and persistence of the foot and mouth disease virus in Eastern Rwanda

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Abstract

Background: Identification of risk factors is crucial in Foot-and-mouth disease (FMD) control especially in endemic countries. In Rwanda, almost all outbreaks of Foot-and-Mouth Disease Virus (FMDV) have started in Eastern Rwanda. Identifying the risk factors in this area will support government control efforts. This study was carried out to identify and map different risk factors for the incursion, spread and persistence of FMDV in Eastern Rwanda. Questionnaires were administered during farm visits to establish risk factors for FMD outbreaks. Descriptive statistical measures were determined and odds ratios were calculated to determine the effects of risk factors on the occurrence of FMD. Quantum Geographic Information System (QGIS) was used to produce thematic maps on the proportion of putative risk factors for FMD per village.

Results: Based on farmers' perceptions, 85.31% (with $p < 0.01$) experienced more outbreaks during the major dry season, a finding consistent with other reports in other parts of the world. Univariate analysis revealed that mixed farming (OR = 1.501, $p = 0.163$, CI = 95%), and natural breeding method (OR = 1.626; $p = 0.21$, CI = 95%) were associated with the occurrence of FMD indicating that the two risk factors could be responsible for FMD outbreaks in the farms. The occurrence of FMD in the farms was found to be significantly associated with lack of vaccination of calves younger than 12 months in herds (OR = 0.707; $p = 0.046$, CI = 95%).

Conclusions: This is the first study to describe risk factors for persistence of FMDV in livestock systems in Rwanda. However, further studies are required to understand the role of transboundary animal movements and genotypic profiles of circulating FMDV in farming systems in Rwanda.

Keywords: Foot-and-mouth disease, Risk factors, Maps, Geospatial, QGIS, Eastern Rwanda, Foot-and-mouth disease spread

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3.1. Introduction

FMD is a highly contagious viral disease caused by a picornavirus known as FMDV (Grubman & Baxt, 2004). FMD affects cloven-hoofed animals including domestic and wild animals (G. V Weaver et al., 2013). Cattle, sheep, goats and pigs are the most important domestic animals affected by the disease. In wildlife, at least 70 species of wild and captive animals including African buffaloes (*Syncerus caffer*) are affected (G. V Weaver et al., 2013). The East African region is considered to have the most complicated situation with regard to the control of FMD. This is due to interactions between domestic and wild animals susceptible to FMD, uncontrolled transboundary animal movements and high genetic diversity of FMDV in the region (Rweyemamu et al., 2008). The Akagera National Park is home to many FMD susceptible wild animals, which are at risk of interacting with livestock in farms adjacent to ANP. Rwanda has experienced many outbreaks of FMD with serotypes O reported in 1960, 1998, 2004 2008, 2009 and 2010. On the other hand, the first outbreak of SAT 2 was reported in 1992 with the subsequent outbreaks being reported in 1996–1997, 2000–2001, 2004, 2005, 2006, 2008, 2009, 2010, 2013, 2015 and 2017 (AU-IBAR, 2013; Humanitarian, 2001; Mbaraga, 2017; Ntirenganya, 2017; Rwembeho, 2015). The other outbreaks of FMD involving serotypes A and SAT 1 occurred in 2008, 2009, 2010 (AU-IBAR, 2013) and again serotype SAT 1 in 2012–2013 (FAO/EuFMD, 2017).

Despite the reports of the previous outbreaks of the disease, little is known about risk factors responsible for these outbreaks. Understanding these risk factors is needed for the development of a Risk Assessment Plan necessary for the advancement in the PCP-FMD stages (Sumption et al., 2012).

The Eastern Province of Rwanda is the largest province having 9813 km^2 with a predominantly sedentary farming system. The livestock population in Eastern Province is composed of approximately 500,000 cattle, 500,000 goats, 13,000 sheep and 130,000 pigs (MINAGRI,

2015). This province neighbours Uganda and Tanzania and uncontrolled transboundary animal movements are likely to occur here. Moreover, the province receives low rainfall and is characterized by the absence of water bodies such as rivers. Consequently, many farmers in this province tend to use communal watering points thereby encouraging the congregation of animals and eventually contributing to the spread of FMD outbreaks (Elnekave et al., 2015). In this regard, this area has been known to be a hotspot for most of the FMD outbreaks in Rwanda for the last two decades (MINAGRI, 2015). The dry season seems to be the period during which FMD outbreaks are more likely to occur in Eastern Rwanda and surrounding areas in Uganda and Tanzania (FAO/EuFMD, 2018; Kerfua et al., 2018; WOAH, 2022a). During the dry season, there is a shortage of pasture and water; thereby forcing most livestock farmers to move their animals in search of pasture and water. This encourages contacts between infected and non-infected animals during an outbreak of FMD. Spatial distribution of FMD risk factors is not known in Rwanda at this stage. Therefore, this study established spatial and temporal distribution of FMD as well as risk factors for the disease outbreak.

3.2. Materials and methods

3.2.1. Study area

Nyagatare and Gatsibo districts experience low rainfall amounts with fewer rivers and are home to ANP, which has a considerable number of domestic and wildlife animals including African buffaloes (Syncerus caffer). The study area falls in the triangle neighbouring three countries namely, Tanzania, Rwanda and Uganda indicating risks for uncontrolled transboundary movements of animals between these countries. Since 1994, almost all of the reported FMD outbreaks (1996–1997, 2000–2001, 2004, 2005, 2006, 2013, 2015 and 2017) in the country have happened in Eastern Rwanda.

3.2.2. Study design

A questionnaire was designed [see Additional file 1] based on previously published papers on risk factors. Also, the Food and Agriculture Organization's European Commission for the Control of FMD online document on FMD Investigation was used and is available at https://eufmdlearning.works/ (FAO/EuFMD, 2018). The questionnaire covered several risk factors including farms not vaccinating calves less than 12 months of age, mixed farms keeping small and large ruminants (Megersa et al., 2009), breeding systems, seasonality of FMD outbreaks and farming systems (Bronsvoort et al., 2004a; Dukpa et al., 2011), farm adjacent to each other (Boender et al., 2007) as well as the wildlife-livestock interface (Bronsvoort et al., 2004a). In this study, zero-grazing stands for the system where livestock are reared inside the farm and water is available within the farm boundaries. Semi-zero grazing is for farmers who feed their animals within their farms' boundaries but move their animals to communal watering points. Lastly, free-ranging is for farmers who graze and water their animals outside their farms. Most farmers in the area cultivate fodder mainly Napier grass (93.2%) (Mazimpaka et al., 2017).

3.2.3. Target population and questionnaire administration

This study applied a cross-sectional study design and data were collected from Nyagatare and Gatsibo villages based on their proximity to ANP. A questionnaire (Appendix 2) was administered to all cattle farmers within the selected area covering a 20 km distance from the electric fence of the ANP. This area covered the wildlife livestock interface where domestic and wildlife animals, especially African buffaloes, are likely to interact. Interviews were carried out between May 2018 and August 2018. The information collected was documental on a paper-based questionnaire translated in Kinyarwanda and later entered in a spreadsheet of the Statistical Product and Service Solutions (SPSS Inc., IL, USA) version 16.0. The questionnaire had both open-ended and closed questions and was pre-tested on a smaller

number of respondents to check for the clarity of the questions. Before each interview, respondents gave their verbal consent to proceed with the interview, after being briefed on the objectives and the expected outcomes of the study. The geographical coordinates of the farms were recorded using a Smartphone Application (Global Positioning System Coordinates Finder® by EzgApps). Using the geographical coordinates, maps were created in QGIS v. 2.18.22 (Las Palmas, USA).

3.2.4. Data analysis

Data were coded in SPSS (SPSS Inc., IL, USA) for analysis, the codes were extensively revised to make sure that they are relevant based on the responses given by the farmers during the questionnaire administration. The descriptive statistics including proportion, means and categories of risk factors were generated. Data were also summarized using graphs. Inferential statistics such as the chi-square ($\chi 2$) test were used to analyse the variables using the SPSS software (SPSS Inc., IL, USA). Where applicable, univariable and multivariable analyses were performed to estimate odds ratios (OR), using a criterion of whether a farm had experienced an FMD outbreak in the last 5 years with a confidence interval (CI) of 95% because there was no serological data.

3.2.5. Availability of data and materials

The dataset backing the findings is available on:

https://figshare.com/articles/dataset/FMD_Risk_Factor_consolidated_data/12756134.

3.3. Results

One hundred and eighty-four farmers were interviewed in 19 villages of Nyagatare and Gatsibo districts. However, considering that, some farmers did not answer all the required questions and hence unsuitable for analysis, only 143 responses were used in this study. Among the 143 respondents, 36 (25.17%) of them reported having had at least one FMD outbreaks within the

last 5 years in their farms. There was no active outbreak of FMD in the visited farms during the interviewing period, the last outbreaks had occurred between May 2017 and February 2018 and were caused by serotypes SAT 2 (Ntirenganya, 2017; Udahemuka et al., 2022).

3.3.1. Vaccinating calves younger than 12 months

By the time of this field visit, Rwanda has been vaccinating against FMD using FOTIVAX™ from Kenya (KEVEVAPI) once a year. It is a tetravalent vaccine containing serotypes A, O, SAT 1 and SAT2 with a recommendation of vaccinating twice or thrice a year for better protection (Grubman & Baxt, 2004). This study found that 57/142 (40.15%) of farmers did not vaccinate calves which are younger than 12 months of age. The findings also revealed an association between farmers reporting not to vaccinate calves under 12 months and the occurrence of FMD outbreaks in their herds (OR = 0.707) (Table 3-1).

Table 3-1: Multivariable model for risk factors for the occurrence of FMD outbreaks in herds raised in Eastern Rwanda

Parameter description	Estimate	Standard error	Significance	Odds ratio
Intercept	1.964	0.1336	0	7.13
Vaccinate calves under 12 months	− 0.347	0.1738	0.046	0.707
Not vaccinating calves under 12 months	0			

3.3.2. Presence of small ruminants

In this study, small ruminants such as goats and sheep being kept together with cattle were reported in all villages as shown in the map below as illustrated in figure 3-1. Among the 143 respondents, only 13/143 (9.09%) kept cattle only while 129/143 (90.21%) were mixed farms and one respondent did not respond to this question. Analyses showed that there are more chances (OR = 1.50) for FMD outbreaks to occur when there are sheep and goats on the same farm as indicated in table 3-2.

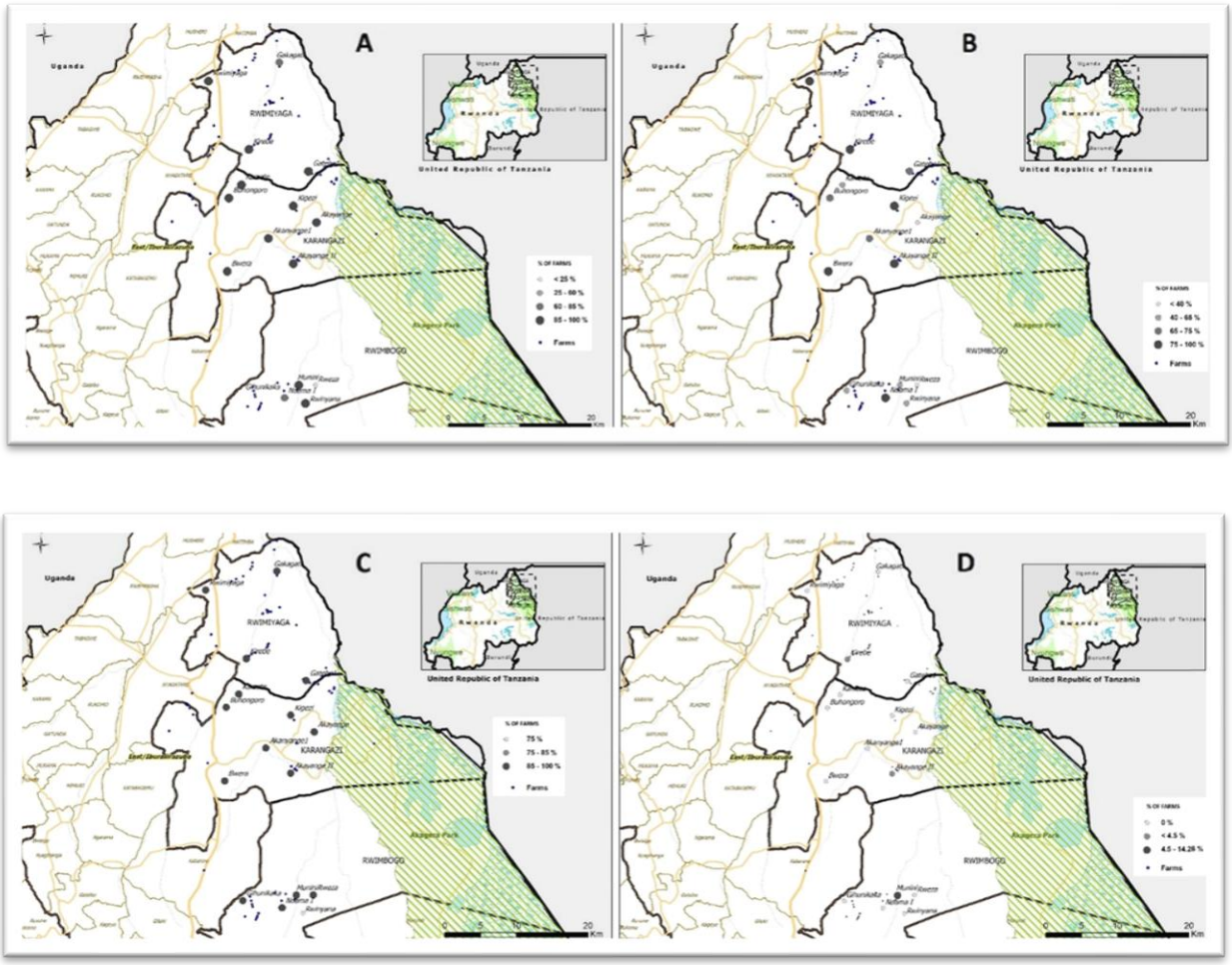

Figure 3-1: a. Map showing the proportion of mixed farms, farming system, closeness of farms and the non-fenced farms in each village.

Table 3-2: Univariable model for risk factors for the occurrence of FMD outbreaks in herds raised in Eastern Rwanda.

Parameter description	Estimate	Standard error	Significance	Odds ratio
Natural breeding	0.486	0.3876	0.21	1.626
Artificial insemination	0			
Vaccinate calves under 12 months	− 0.347	0.1738	0.046	0.707
Not vaccinating calves under 12 months	0			
Presence of small ruminants	0.406	0.291	0.163	1.501
Absence of small ruminants	0			
Farm adjacent to another	−0.257	0.583	0.659	0.773
Farm isolated	0			
Not zero-grazing	−0.204	0.186	0.274	0.816
Zero-grazing	0			
Farm fenced	−0.255	0.712	0.720	0.775
Farm not fenced	0			
Farm adjacent to the park	−1.255	0.571	0.028	0.285
Farm not adjacent to the park	0			

3.3.3. Breeding methods

The impact of the breeding systems on the spread of the disease was evaluated. It was found that only 7 farmers out of 143 do use Artificial Insemination (AI) as a breeding method. Furthermore, the use of natural breeding methods increased the odds of FMD outbreak (OR = 1.626,) as compared to farms which were using artificial insemination (AI) as a breeding method ($p = 0.21$, $n = 143$) (Table 3.2). Due to the low number of farmers using AI, these results might be inconclusive and further analyses are necessary.

3.3.4. Seasonality of FMD outbreaks and farming system

In this study, the influence of seasonality on outbreaks of FMD was also determined. A majority (85.31%) of the farmers interviewed reported that FMD is more likely to occur during the long dry seasons (the major one from June to September and a less severe one from December to February) than in the wet season. It was found that the majority (71.33%) ($p < 0.01$) of the farmers practising semi-zero grazing system and fewer (27.97%) practising strict zero grazing. All the farmers did not practice free-range farming system. One respondent did not provide answers to the interview question on farming system.. Strict zero-grazing was practised in three villages only. In other villages, the majority of cattle move daily in search of water and animals from different farms are more likely to congregate at watering points. Rwimiyaga, Kirebe, Kigezi, Bwera and Akanyange II villages located in Nyagatare district and Ndama village located in Gatsibo district had more than 75% of the farmers practicing semi-zero grazing (Fig. 3-1b). Not practicing zero grazing system does not significantly increase the outbreak occurrence, and cannot be considered as a risk factor (Table 3.2). In addition, farmers practicing zero grazing reported higher incidence of outbreaks than those practicing semi-zero grazing system as indicated in table 3-3.

Table 3-3: The proportion of farms for each farming system practised in Nyagatare and Gatsibo districts of Eastern Rwanda and the impact on FMD outbreaks.

	Farming system	
	Zero-grazing	Semi zero-grazing
	Number of farms (%)	
At least one outbreak in the last 5 years		
Yes	16/40 (40)	20/102 (19.6)
No	24/40 (60)	82/102 (80.4)
Total	40 (27.97)	102 (71.33)

3.3.5. Proximity of farms to each other

It was found that majority of the farms (140/143 [97.9%]) were adjacent to each other (Fig. 3-1c), a situation that can lead to increased transmission of FMD in case there is an outbreak in one of the farms. Some (2.1%) of the visited farms were not fenced. None of the three farms that were not adjacent to another farm reported having had an FMD outbreak in the previous 5 years. When tested against the criterion of either having had at least one FMD outbreak in the farm the results were not statistically significant (OR = 0.773, $p = 0.659$).

3.3.6. Wildlife-livestock interface as a risk factor

Of the 27 farms located in Akanyange II village of Nyagatare district, 88.9% of them were adjacent to the ANP, indicating that domestic animals from this village had the highest chances of interacting with wildlife (Fig. 3-2). Overall, 36/143 farms were adjacent to ANP. Among these, 11.1% (4/36) reported having experienced FMD outbreaks in the previous 5 years against the remaining 88.9% (32/36) who reported not to have had FMD in their farms in that period. For the other group of farms, 30.5% reported having had FMD outbreaks while 69.5% have not had FMD outbreaks in their farms in that period (OR = 0.285, p = 0.028,).

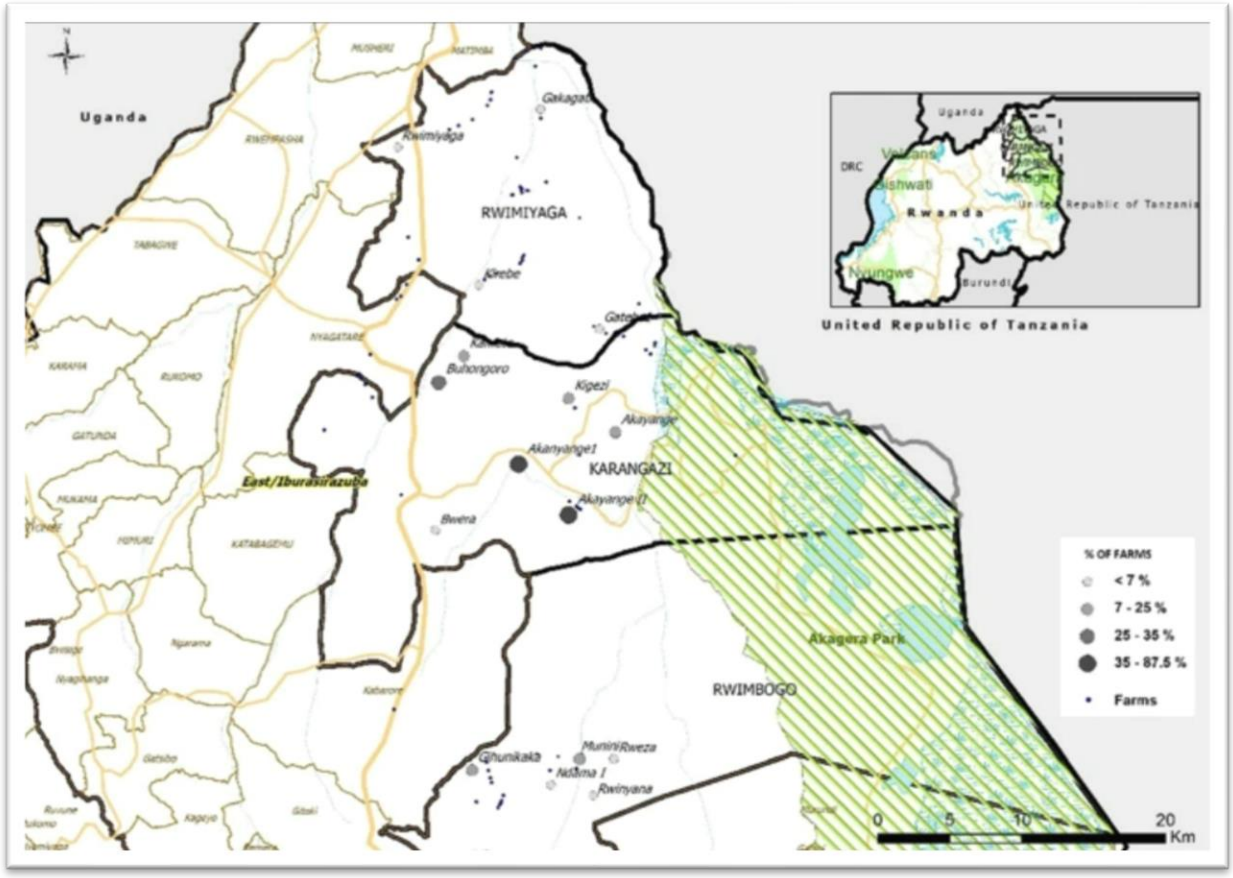

Figure 3-2: Map showing the proportion of farms at the interface with ANP per village in Eastern Rwanda (map created using QGIS v. 2.18.22 (https://qgis.org/downloads/)).

3.3.7. Maps of selected risk factors

The risk factors such as farms that kept cattle, sheep and goats together (mixed farms), farming systems, non-fenced farms, closeness of various farms to each other and farms adjacent to the ANP were mapped. As shown in Fig. 3-1a, most of the farms in the villages had a mixed-farming system (raising cattle, sheep and goats together) with 129 out of 143 (90.21%) mixed farms. Mixed farms are evenly distributed throughout the Nyagatare and Gatsibo districts except for two villages in Nyagatare district, which had less than 85% of mixed farms. The figure 3-2 shows the proportion of farms practising semi zero-grazing and free-ranging per village. Observation shows that villages near rivers would have more farms not practising strict zero-grazing (> 75%). This is the case for Rwimiyaga, Kirebe, Kigezi, Akayange II and Bwera villages of Nyagatare district and Ndama I village in Gatsibo district.

As the Fig. 3-1d demonstrates, the fencing of the farms is well practised with an exception of Munini (> 4.5%) village of Gatsibo district having a higher percentage of unfenced farms. Rwinyana village of Gatsibo district is the only one among all the villages with fewer farms (< 75%) that are not close to each other (Fig. 3-1c). Akayange I and Akayange II villages in Nyagatare district are the ones with a higher proportion of farms at the interface with ANP (Fig. 3-2). These two villages are also near a major road network linking ANP and other parts of the country. An outbreak in these two villages would easily spread to other parts due to its central position in Eastern Rwanda and the nearby road network.

3.4. Discussion

3.4.1. Vaccinating calves younger than 12 months

The tetravalent (A, SAT 1, SAT 2 and O) Kenyan vaccine has been used to vaccinate cattle in Eastern Rwanda once a year. In the case of an FMD outbreak, a ring vaccination targeting cattle in the area has been practised. FMD vaccines are provided to farmers and subsidised by

the government and this mitigates the risk of leaving behind some farms unvaccinated. Approximately 40% of the respondents were found not to vaccinate calves younger than 12 months during the vaccination programs. There may be a perception that calves are less impacted by FMD hence leading farmers to be reluctant in including them into vaccination programs (Nyaguthii et al., 2019). A further study to understand what would have made a farmer not to include calves into vaccination programs is needed. This study revealed that failure to vaccinate calves younger than 12 months significantly increased the risks of FMD occurrence in the farms. This finding is consistent with previous studies in which vaccination of calves below 12 months have been found to enhance the protection of herds against FMD outbreaks (Dekker et al., 2014; Elnekave et al., 2015). It is therefore recommended to conduct more vaccinations a year as per the manufacturer's instructions and regular vaccine matching studies. Furthermore, it is recommended to conduct a sensitization to farmers to include animals of all ages in vaccination and at least two vaccination campaigns a year with booster doses.

3.4.2. Mixed farms

Mixed farms (cattle with small ruminants) as a risk factor can be a result of the fact that small ruminants show less severe clinical signs of FMD and are not usually included in vaccination programs (Barnett & Cox, 1999; Kitching & Hughes, 2002). Balinda and colleagues (2009) reported a high prevalence of FMDV in small ruminants in Uganda and this has been linked to FMD outbreaks in cattle (Balinda et al., 2009). The findings are in agreement as the above findings in other places. Furthermore, in the study area, it was observed that small ruminants are reared separately from cattle, they tend to go more often and further outside the farm for grazing and watering. However, the results above lack a statistical significance to assert the above facts in this case. The low FMD rate may be because the non-mixed farms were small-

scale dairy farms (Nyaguthii et al., 2019). Therefore, considering the vaccination regime and the high number of small ruminants in Eastern Province, a deeper investigation on the role played by small ruminants could provide a basis for better control of FMD. From this, policy-makers would decide on whether to conduct more regular vaccination of only cattle with high-quality vaccines or if it is necessary to also include small ruminants in vaccination. Due to a cultural taboo for traditional cattle pastoralists to keep pigs, farmers in area reported not to keep pigs. Further studies should focus on the role played by pigs in other regions of Rwanda.

3.4.3. Breeding methods

Testing and monitoring of bulls to provide pathogen-free semen could reduce the risk (Guérin & Pozzi, 2005). Indeed, it was observed that the AI centres used by the farmers usually screen the bulls for a range of animal infectious diseases including FMD; thereby reducing the risk of the disease transmission. However, further studies on the role of AI on the FMD transmission risk in Eastern Rwanda is still needed to confirm my results.

3.4.4. Seasonality of FMD outbreaks and farming system

Previous studies have documented that common watering point can provide a means for trans-farm transmission and spread of FMD (Bronsvoort, et al., 2004b; Waret-Szkuta et al., 2011) and this is usually observed during prolonged droughts as reported in one study in Tanzania (Kivaria, 2003). According to this study, the trend in the area was to have a common valley dam where animals from different farms go to drink. The daily gathering of animals from different farms, as it was observed during this study, was an important trend that can be responsible for disease spread. Other previous studies have also reported that herd contacts at watering points can be a risk for the introduction, spread and persistence of FMD (Abbas et al., 2014; Bayissa et al., 2011; Bronsvoort et al., 2004b; Wungak et al., 2016). A previous study has established that uncontrolled cattle movements in East Africa are one of the risk factors responsible for the transmission and spread of FMD in the region (Baluka et al., 2013). This

study reports that during the the dry season the risks of FMD are high, These period coincides to what was reported by Kerfua et al. (Kerfua et al., 2018) along the Uganda-Tanzania border and the OIE records (J. Weaver et al., 2019; WOAH, 2022a). To mitigate this problem, digging water dams or well within the farms seems to be an appropriate solution. Access to water on the farm is not always easy and affordable, especially to small-scale farmers. In India and Ethiopia, a subsidised system of solar-powered pumps has been adopted to provide water to the farms (Bird et al., 2016; Giordano et al., 2019).

3.4.5. Proximity of farms to each other

The transmission and spread of FMD during outbreaks tend to be faster in farms located close to each other (Lyytikäinen et al., 2011, 2015; Rossi et al., 2017; Y. Wang et al., 2013). Though many farms are adjacent to another farm, these results were not found to be conclusive. Hence, further investigations should look into this aspect. This may be because airborne transmission is much less when compared to direct contact transmission seen in communal watering points. Previous studies have suggested that low humidity and high temperature could be responsible for reduced transmission of FMDV (J. Sørensen et al., 2000; Sutmoller et al., 2003).

3.4.6. Wildlife-livestock interface

Previous studies in Southern African countries have reported that wildlife, especially African buffaloes, are carriers of FMD virus indicating that the animals could be a source for the transmission of the virus (Condy et al., 1985; Paton et al., 2018; Thomson et al., 1992, 2013; Thomson & Vosloo, 2004; Vosloo et al., 1996). This often happens when cattle graze near the parks in the Southern African countries, especially during the dry season. In Southern African countries, the problem of wildlife-livestock interaction has been solved by fencing the national parks found in these countries to minimize contacts between wildlife and livestock (Jori & Etter, 2016). Therefore, in order to reduce the human-wildlife-livestock interactions in

Rwanda, the fencing approach has also been adopted particularly in Eastern Rwanda (The New Times, 2013). In this case, the Rwanda Development Board used an electric fence to separate the ANP from the livestock farmers. However, as observed, some buffaloes were left outside the fenced park during the fencing programme. Nevertheless, there was not an immediate effect observed for farms adjacent to the ANP when compared to other farms in the study, probably park fencing mitigated this risk. Up to date, no laboratory-based results is pointing to the role played by buffaloes in Rwanda is available. A Pirbright research group suggested in 2018 that the role of African buffaloes in the transmission of FMD in East African might be different from what has been reported in Southern African countries. The latter study proposed a different control measure involving vaccination of cattle before an outbreak, as "a region-tailored" solution (Casey-bryars et al., 2018). This proposal is supported by the findings, in which it was surprisingly found that more farms have had FMD than the ones adjacent to the ANP fence. This can be linked to several factors such as that farms adjacent to the park are far from borders with less effect of transboundary animal movements. Moreover, ANP has been fenced since 2013 reducing the wildlife-livestock contact.

3.4.7. Maps of selected risk factors

The mapping of risk factors is an important tool for understanding the epidemiology of FMD. The mapping has been used for generating a spatio-temporal distribution of the risk factors as reported in different parts of the world (AU-IBAR, 2014; Carvalho et al., 2015; Cottam et al., 2008; Wungak et al., 2016). Generating thematic maps of risk factors has also been reported to be of paramount importance in modelling and zoning of the disease in some countries (AU-IBAR, 2014; Picado et al., 2011). There is no previous study done to map the risk factors in Eastern Rwanda.

Rwinyana village of Gatsibo district is the only one among all the villages with fewer farms (< 75%) that are not close to each other (Fig. 3-1c). Akayange I and Akayange II villages in Nyagatare district are the ones with a higher proportion of farms at the interface with ANP (Fig. 3-2). These two villages are also near a major road network linking ANP and other parts of the country. An outbreak in these two villages would easily spread to other parts due to its central position in Eastern Rwanda and the nearby road network. For instance, infected cattle crossing roads to the watering points would leave infectious material behind that can attach to the tire surfaces of passing by vehicles. There is a need to confirm if farmers living near major roads move their animals more often than the farmers living far from major roads. In Nyagatare district, only 6% of the farmers have watering-points on their farms (Mazimpaka, 2017). It seems that the use of on-farm watering-points would reduce the dependence on communal water points and hence reduce the odds of FMD transmission between different herds as reported previously (Megersa et al., 2009; Miller et al., 2018).

3.5. Conclusion

It is concluded that vaccinating calves under 12 months would protect the herds from the incursion of FMD. Therefore, vaccination programs that target both older and younger cattle, particularly following manufacturers' instructions are recommendable. Besides, farmers are aware that dry seasons are riskier than rainy seasons as long as FMD outbreaks are concerned. The proximity of farms to ANP or other farms including mixed farms that rear both cattle and small ruminants appear not to be a risk factor.

Chapter 4 : DETECTION AND GENETIC CHARACTERIZATION OF FOOT AND MOUTH DISEASE VIRUS AND OTHER PATHOGENS INFECTING CATTLE, BUFFALOES AND GOATS AT THE INTERFACE WITH AKAGERA NATIONAL PARK BETWEEN 2017 AND 2020

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Investigation of foot and mouth disease virus and other animal pathogens in cattle, buffaloes and goats at the interface with Akagera National Park 2017 – 2020

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Abstract

Background: Foot-and-Mouth Disease Virus (FMDV) is a positive-sense RNA virus of the family of the picornaviridae that is responsible for one of the livestock diseases with the highest economic impact, the Foot-and-Mouth Disease (FMD). FMD is endemic in Rwanda but there are gaps in knowing its seroprevalence and molecular epidemiology. This study reports the FMD seroprevalence and molecular characterization of FMDV in Eastern Rwanda.

Results: The overall seroprevalence of FMD in the study area is at 9.36% in cattle and 2.65% in goats. We detected FMDV using molecular diagnostic tools such as RT-PCR and RT-LAMP and the phylogenetic analysis of the obtained sequences revealed the presence of FMDV serotype SAT 2, lineage II. Sequencing of the oropharyngeal fluid samples collected from African buffaloes revealed the presence of *Prevotela ruminicola, Spathidium amphoriforme, Moraxella bovoculi Onchocerca flexuosa, Eudiplodinium moggii, Metadinium medium* and *Verrucomicrobia bacterium* among other pathogens but no FMDV was detected in African buffaloes.

Conclusions: We recommend further studies to focus on sampling more African buffaloes since the number sampled was statistically insignificant to conclusively exclude the presence or absence of FMDV in Eastern Rwanda buffaloes. The use of RT-PCR alongside RT-LAMP demonstrates that the latter can be adopted in endemic areas such as Rwanda to fill in the gaps in terms of molecular diagnostics. The identification of lineage II of SAT 2 in Rwanda for the first time shows that the categorised FMDV pools as previously established are not static over time.

Keywords: FMD, RT-LAMP, RT-PCR, Buffaloes, FMDV, SAT 2, Seroprevalence, Rwanda

Background

Foot-and-Mouth Disease Virus (FMDV) is a positive-sense RNA virus of the family of the picornaviridae [1]. Based on the most variable part of the capsid, the VP1, FMDV is classified into seven serotypes (SAT1, SAT2, SAT3, O, A, C and Asia1) which are also subdivided into topotypes [2, 3]. Vaccination against one serotype does not confer protection against a different serotype and multivalent vaccines are often used [4–6]. Due to the constant change of this virus, a consistent molecular analysis is of paramount importance to improve the vaccines. Understanding the serological and molecular epidemiology of FMDV in Rwanda is very important because East Africa is considered to have the most

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4.1. Introduction

FMDV is a positive sense RNA virus of the family of the picornaviridæ (Domingo et al., 2002). Based on the most variable part of the capsid, the VP1, FMDV is classified into seven serotypes (SAT 1, SAT 2, SAT 3, O, A, C and Asia1) which are also subdivided into topotypes (Ayelet et al., 2009; Jamal & Belsham, 2013). Vaccination against one serotype does not confer protection against a different serotype and multivalent vaccines are often used (Brooksby, 1982; Cartwright et al., 1982; Mattion et al., 2004). Due to the constant change of this virus, a consistent molecular analysis is of paramount importance to improve the vaccines. Understanding the serological and molecular epidemiology of FMDV in Rwanda is very important because East Africa is considered to have the most divergent FMD situation in the world (Rweyemamu et al., 2008). In this study, the FMDV strains responsible for FMD outbreaks in Rwanda in 2017 and FMD seroprevalence in 2020 were investigated. Molecular diagnostics were performed using Reverse Transcription Polymerase Chain Reaction (RT-PCR) and the pen-side Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) chargeable and portable machine, the Axxin T8 isothermal instrument. This was complemented by sequencing the strains responsible for the 2017 FMD outbreaks in Eastern Rwanda. Moreover, the serological situation of FMDV in large and small ruminants from the Eastern Province of Rwanda is hereby presented. These data will be crucial in policy guidance but also in studying risk factors again later. African buffaloes (*Syncerus caffer*) known to be natural wildlife reservoirs of FMDV were sampled (Hedger, 1972; Vosloo et al., 2002a). Though FMDV was not isolated in African buffaloes, other pathogens and commensals were identified. There was no published research on FMD seroprevalence in Rwanda and the latest available FMD molecular characterization results were from the 2001 FMD outbreak (National Center for Biotechnology Information, 2005a, 2005b). This study aims to identify genetic

diversities of FMDV strains and other animal pathogens isolated in African buffaloes and cattle in Eastern Rwanda.

4.2. Materials and methods

Seven (n=7) healthy-looking mature African buffaloes were randomly immobilized inside the ANP in the Nyamirama area. They were darted with a tranquiliser from a 4x4 vehicle using a JM Special dart gun with a 13 mm barrel loaded with a 2 mL Pneu-Dart and then injected with a 3.8 cm barbed needle using a spring-loaded pole syringe (Dan-Inject). The animals were darted in the hindquarters with 8 mg etorphine and 48 mg azaperone (Hoffman et al., 2018), which brought them down in sternal recumbence. After sedation, their faces were covered up to the ears with a cloth tissue, to avoid early wake-up. A dose of 150 mg of Ketamine was administered to increase muscle relaxation and jaw-bone movements. During the sampling, they received an additional 200 mg ketamine IV and 2 mg etorphine combined with 40 mg azaperone IM to delay their waking up. After sample collection, these buffaloes were roused with up to 20 mg diprenorphine and 100 mg naltrexone given intravenously (Clarke et al., 2018; Hoffman et al., 2018). Throughout the sample collection, the buffaloes' breathing was monitored and no animal needed a respiratory stimulant or partial antagonism. Oropharyngeal fluids (OPF) and scraps were collected using a probang cup and transferred to sterile tubes containing an equal amount of transport media. A probang cup was inserted in the OP tract and vigorously passed it with back-and-forth movements at least 5-10 times between the anterior portion of the oesophagus and the back of the pharynx. Tubes had equal amounts of glycerol and 0.04 M phosphate buffer (pH 7.2–7.6) containing a 1× antibiotic–antifungal mixture (Thermo-Fisher Scientific, Johannesburg, South Africa) (Paton & King, 2014; WOAH, 2022a). Between OPF collection from one animal to the next, the probang cup was cleaned and sanitized using the WOAH three-bucket washing system (Paton & King, 2014; WOAH, 2022a). The probang cup was washed in a bucket containing 0.3% citric acid, rinsed it in

another bucket with water and lastly disinfected the cup in Phosphate-buffered Saline as illustrated in figure 4-1. The sample tubes were topped up to contain an equivalent volume of transport medium to that of the sample.

Oropharyngeal samples were also collected from crossbred Ankole cattle using the probang cup as described for the buffalo samples above. This sampling was done in Gatsibo and Nyagatare districts during the 2017 FMD outbreak. In addition, 16 blood samples were collected from Ankole crossbred cattle from Nyagatare during the 2022 FMD outbreak.

For both outbreaks (2017 and 2022), samples were collected from animals presenting FMD clinical signs such as blisters on the mouth or the foot. Samples were collected in accordance with guidelines provided in the USDA's Foreign Animal Disease Investigation Manual (USDA, 2017) and approved by the Rwanda Agriculture and Animal Resources Development Board. Samples were transported in cool boxes on ice from the field to the Virology Laboratory of the Rwanda Agriculture and Animal Resources Development Board located in Kigali, Rwanda. Samples were stored at -20°C until further processing or -80°C for a longer storage period. The animals used in this study were African buffaloes (*Syncerus caffer*) from the ANP, healthy-looking Cattle and Goats (for serology) from individual farms in Eastern Rwanda and symptomatic Cattle from infected farms in Eastern Rwanda.

Figure 4-1: The three-bucket system to change a probang cup from one animal to another.

4.2.1. Serological analyses

A total of 1011 sera samples comprising of 823 from cattle and 188 from goats were analyzed using the competitive ELISA kit (ID Screen®). The competitive ELISA was used to detect specific antibodies to 3ABC-NSP of FMDV circulating in blood. The detailed protocol outlined in the manufacturer's manual was followed (Mesfine et al., 2019).

4.2.2. RNA extraction and cDNA synthesis

In 2017, RNA was manually extracted using the PureLink Viral RNA/DNA Kit according to the manufacturer's instructions. 200 µL of OPF sample were added to the Kit's Master Mix (proteinase K, lysis buffer, carrier RNA, and 100% ethanol). A two-step wash using a wash buffer solution was performed, and eluted in 60 µL nuclease-free water was collected and transferred to 1.5 ml tubes. The eluate was treated with DNase using the Turbo DNA-free Kit and kit manual, to remove host genomic DNA. Thereafter, 50 µL of DNA-free eluate were collected and transferred to 1.5 mL tubes for downstream analyses. The nucleic material in the collected solutions was quantified using the Quantus™ Fluorometer. The SuperScript VILO cDNA Synthesis Kit and kit manual were used for the conversion of RNA extracted manually, and 20 µL from that extract using the KingFisher Duo machine, to cDNA. 10 µL of RNA eluate was added to a master mix containing 5X VILO reaction mix, 10X SuperScript™ enzyme mix and nuclease-free water. Complementary DNA synthesis was achieved at 42°C for 60 minutes. Thereafter, the cDNA was cleaned up using the Macherey-Nagel™ Nucleospin Gel and PCR Clean-up Kit and kit manual. Finally, 50 µL cDNA were added to Buffer NE, Buffer NT1, and Buffer NT3 following the methodology detailed in the kit manual. The total RNA was extracted using the MagMAX™ Viral/Pathogen Binding Solution following the manufacturer's instructions on the Thermo Fisher Scientific KingFisher™ Flex Purification System, with 96 Deep-well Head (Thermo Scientific SKU 5400630). The complementary DNA (cDNA) was

synthesized using the LunaScript® RT SuperMix Kit (New England Biolabs, USA) following the kit's manual.

4.2.3. Amplification of synthesized cDNAs by qRT-PCR

The cDNAs synthesized were amplified using qRT-PCR using primers and probes targeting VP1 gene of the FMDV. These primers are designed to detect all the seven serotypes of FMDV and do not amplify other viruses including viruses similar to FMDV responsible for vesicular diseases (Knowles et al., 2017). The RT-qPCR primers used in this study are outlined in table 4-1 and the sequencing primers are described in table 4-2 (Callahan et al., 2002).

The synthesized cDNA was amplified using a multiplex qRT-PCR assay. The qRT-PCR assay was performed using the TaqMan® Fast Virus Kit (Life Technologies) and kit manual. Two microlitres of eluate extracted in the manual extraction section were added to a Master Mix containing nuclease-free water, 4X TaqMan buffer, primers and TaqMan probe. The thermal cycler (BioRad, Hercules, California) was programmed as follows; reverse transcription at 50°C for 5 minutes; polymerase activation and DNA denaturation at 95°C for 20 seconds; two-step amplification for 40 cycles with denaturation at 95°C for 3 seconds; annealing and plate read at 60°C for 30 seconds.

The C_T values for the qRT-PCR were determined in order to establish the positive FMDV samples. Reactions were considered positive if the amplification was detected before 32.0 cycles (Shaw et al., 2007).

The 2022 DNA amplification was performed using the Luna® Universal qPCR Master Mix using primers targeting the VP1 protein on a BioRad CFX96 machine. Based on the epidemiological background of the region, primers specific for serotype SAT1, SAT2, A and O were used. The primers were synthesized at the Macrogen (Korea) and shipped in lyophilized form (Appendix 3). The PCR amplification was as follows: initial denaturation at 95°C for 60

seconds; two-step amplification for 45 cycles with denaturation at 95°C for 15 seconds and an extension at 60°C for 30 seconds with a plate read. The melting curve was set at 70°C.

Before sequencing, the primers and deoxynucleoside were removed by enzymatically adding the ExoSAP-IT PCR Product Cleanup Reagent consisting of 0.5µl of Exo and 2µl of SAP to 10µl of the qRT-PCR amplicons. The mix was incubated at 37°C for 15 minutes and later 85°C for 15 minutes to inactivate Exo and SAP respectively, lastly, the product was held at 4°C.

Table 4-1: List of primers used for an RT-qPCR for the detection of FMDV (all serotypes) in this study.

Primer/probe	Oligo name	Primer sequence 5'-3'	Genome direction	Working concentration
Forward primer	3DF	ACTGGGTTTTACAAACCTGTGA	Forward	10 pmol/µl
Reverse primer	3DR	GCGAGTCCTGCCACGGA	Reverse	10 pmol/µl
Taqman probe	3DP	TCCTTTGCACGCCGTGGGAC	Probe	5 pmol/µl

Table 4-2: List of oligonucleotide primers used for SAT 2 VP1 sequencing.

Primer name	Sequence (5′ – 3′)	Genome direction	Gene
SAT2-D	GGTGCGCCGTTGGGTTGCCA	Reverse	VP1
SAT2–1D209aF	CCACTTACTACTTTTGTGACCTTGA	Forward	
SAT2–1D209bF	CCACCTACTACTTTTGTGACCTTGA		
SAT2–1D209cF	CCACCTACTATTTCTGTGACCTGGA		
SAT2–1D209dF	CCACGTACTACTTCTGTGACCTGGA		

4.2.4. Amplification of cDNAs by RT-LAMP

Amplification was performed at 65°C for 30 minutes using the Axxin T8-ISO instrument as illustrated in figure 4-2. Primers and probes targeting VP1 proteins were obtained from Tokabio (Pty) Ltd, Johannesburg, South Africa. Briefly a total volume of 25 μL of reaction mixture consisting of 18.5 μL of isothermal master mix and 6.5 μL of cDNA were used for the amplification. Universal primers targeting various FMDV serotypes were used for the amplification. The amplified products were analyzed by using the real-time fluorescence detection method and the bands were evaluated by gel electrophoresis employing 2% agarose. The electrophoresis products were stained with ethidium bromide and visualized using the U.V. transilluminator (MRC laboratory-instruments).

Figure 4-2: The Axxin T8-ISO instrument used for isothermal amplification.

4.2.5. Sequencing and Phylogenetic analysis

The Whole Genome analysis of the African buffaloes' derived cDNAs was conducted without necessarily running a genome-specific PCR amplification. The buffalo samples were processed with the PureLink® Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA) and treated them with the Turbo DNA-free™ Kit (Ambion, TX, USA) to remove any residual host DNA. Thereafter, the Superscript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) was used to generate cDNA from the isolated RNA.

Next-Generation Sequencing cDNA libraries were prepared from the samples with the Ion Xpress™ Plus Fragment Library Builder Kit on the AB Library Builder system (Life Technologies). Each sample was uniquely barcoded during library preparation using the Ion Xpress Barcode Adapters 1–16 Kit. Template preparation was done with the Ion PGM Template OT2 200 Kit and the OneTouch™ 2 instrument, and the samples were sequenced on the Ion PGM next-generation sequencer using the Ion PGM Sequencing 200 Kit. All reagents and instruments were purchased from Thermo Fisher Scientific, Johannesburg, South Africa. Data were analysed with CLC Genomics Workbench Software v.10 (Qiagen Bioinformatics, Redwood City, CA, USA) and the NCBI database with the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis of FMDV SAT2 sequences was done using the NGphylogeny.fr platform. To perform the analysis, an initial alignment of the sequences was done using MAFFT version 7 (Katoh & Standley, 2013) and cleaned by Gblocks (Castresana, 2000; Talavera & Castresana, 2007). After that the phylogenetic trees were built using the MrBayes with the GTR model (Huelsenbeck & Ronquist, 2001) and visualised by Newick display (Lemoine et al., 2019).

4.3. Results

4.3.1. Serological results

The overall prevalence was 9.36% (CI $_{95\%:}$ 7.5%-11.6%) in cattle and 2.65% (CI $_{95\%:}$ 0.9%-6.1%) in goats. The FMD seroprevalence in cattle was as follows; 8.6% in Bugesera and 11.96% in Nyagatare. In goats, the seroprevalence was at 2.77% in Bugesera and 2.27% in Kayonza. The raw data presenting optical density to support these results are available at https://figshare.com/s/dbc28201eacecf5f2183 (Appendix 4).

4.3.2. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The 2017 FMD outbreak samples from Eastern Rwanda were analysed by RT-PCR. The one-step RT-PCR assays performed on the samples collected from the field samples showed that 6 out of 9 were FMDV positive. Selected RT-qPCR positive samples are displayed in figure 4-3 and an increase in fluorescence intensity measured as Relative Fluorescence Units was detected before the threshold of 32.0 cycles of amplification. The figure 4-4 illustrates the triplicates results of select samples (sample 8 and sample 26).

The 2022 FMD outbreak samples from the Nyagatare district were also analyzed. Reactions containing primers specific to serotype SAT 1, O and A did not amplify the target gene hence indicating that the strain(s) that caused the outbreak were different. A qRT-PCR analyses targeting SAT1 was positive for the FMDV but with inconclusive melting peaks. Primers targeting SAT2 revealed positive results also as shown in figure 4-5. The negative results for other three serotypes are presented in appendix 5.

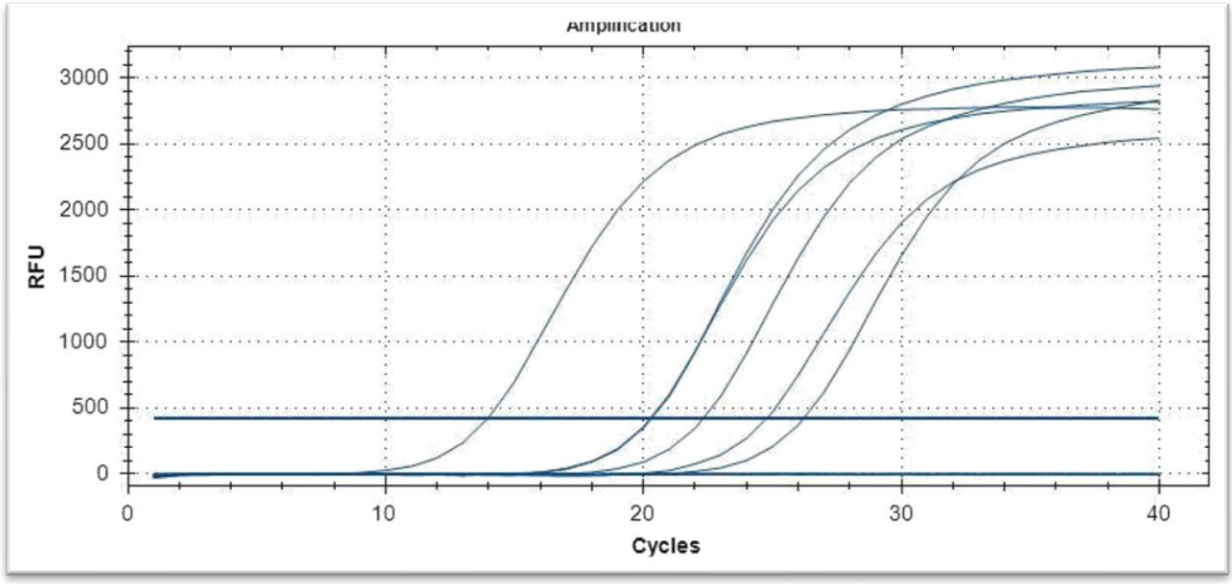

Figure 4-3: One-Step RT-PCR analysis. Amplification curves illustrate some of the selected positive samples.

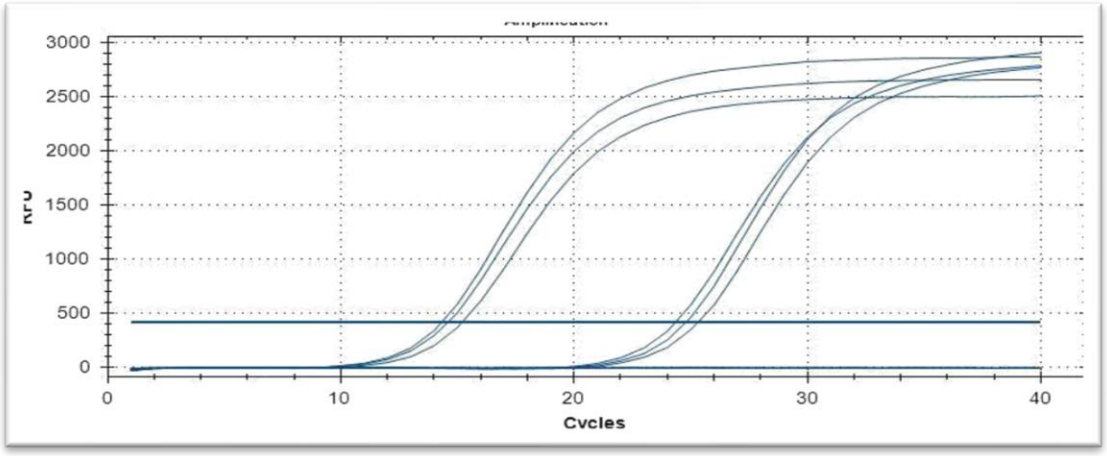

Figure 4-4: Early triplicates of sample 8 (from Gatsibo) and sample 26 (from Nyagatare).

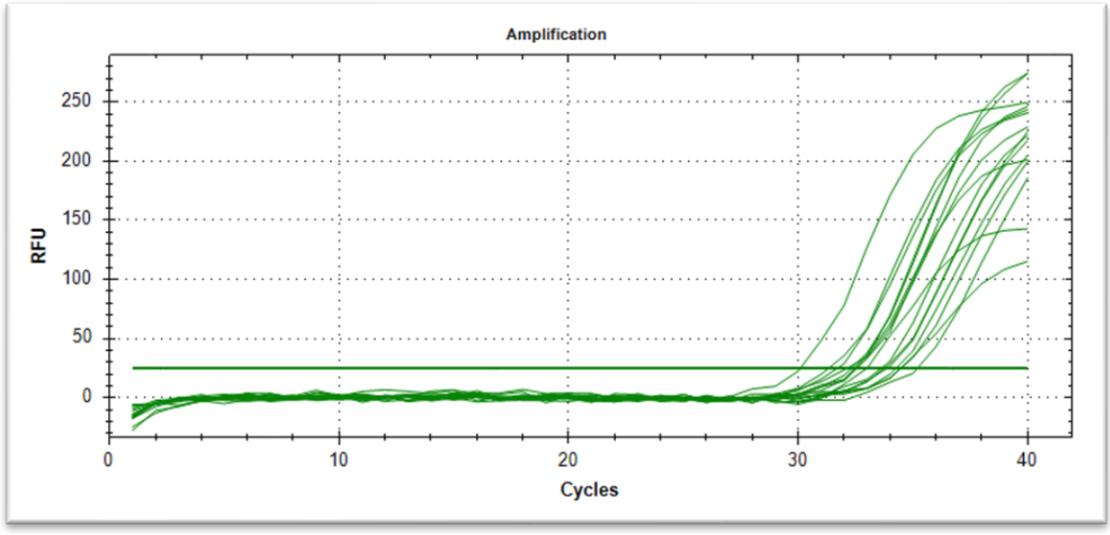

Figure 4-5: Real-time VP1 amplification of the cattle blood samples during the 2022 FMD SAT 2 outbreak in Nyagatare.

4.3.3. Reverse transcription loop-mediated isothermal amplification (RT-LAMP)

The RT-LAMP analysis revealed presence of FMDV in the samples consistent with results revealed by RT- qPCR from the samples collected in the field. The fluorescence detection over time is shown in figure 4-6 and the gel-based detection in figure 4-7. The LAMP products analyzed by electrophoresis revealed a typical array of bands with different sizes. The successful amplification is indicated by the bands becoming darker in the later stages of the gel running.

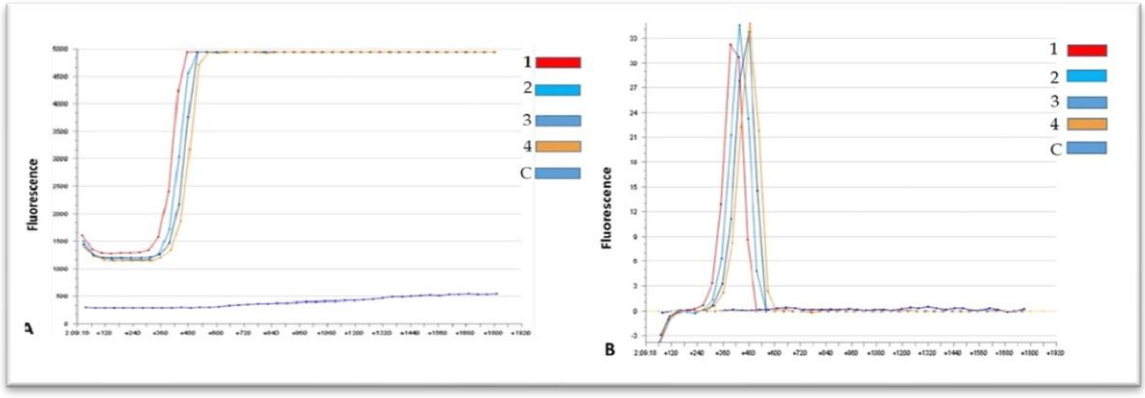

Figure 4-6: RT-LAMP results. Fig. 3.a: trial time of fluorescence detection and fig. 3.b: the second derivative.

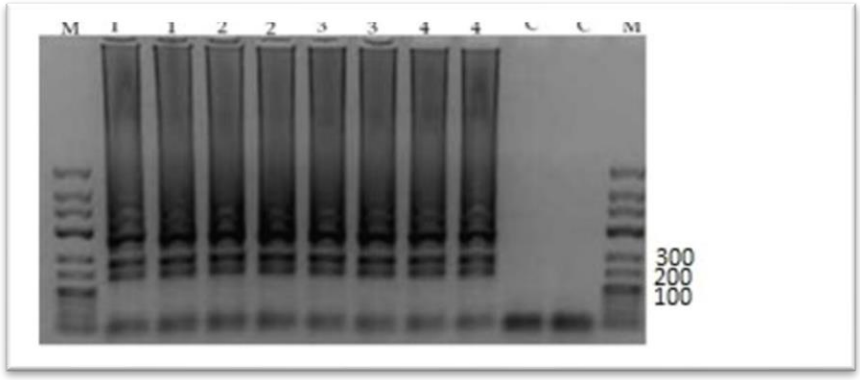

Figure 4-7: RT-LAMP cropped gel detection of sample duplicates. Lane M, DNA marker, 1-4, select positive samples.

4.3.4. Sequences and phylogenetic analysis results

The OPF sampled from African buffaloes were analyzed by next-generation sequencing and the raw data files are available at the Sequence Read Archive (SRA), NCBI and data information can be found at the BioProject (PRJNA865910).

All contigs analyzed by BLAST search did not reveal sequences identical to FMD sequences However, many of the contigs showed significant alignment to sequences of other pathogens and commensals as illustrated in figure 4-8, suggesting that African buffaloes may be reservoirs of other infectious diseases of interest that can infect domestic ungulates in Rwanda.

Bibersteinia trehalosi [Accession No. SAMN30146891] was identified in 1/7 African Buffaloes. Another isolated bacterium from 3/7 buffaloes was *Moraxella bovoculi* [Accession No. SAMN30146892 and SAMN30146888], responsible for infectious bovine kerato-conjunctivitis (Dickey et al., 2016). *Mannheimia varigena* [Accession No. SAMN30146890] found in 1/7 buffaloes.

Haemophilus sp. [Accession No. SAMN30146889] was also isolated in 1/7 of the sampled buffaloes. Since this parasite was identified with NGS, clear differentiation was possible from *H. similis*, which is difficult to distinguish morphologically. In addition to the three pathogens and two parasites detected, various commensals were also identified, with the most predominant being *Prevotella ruminicola* [Accession No. SAMN30146884], followed by *Eudiplodinium maggii* [Accession No. SAMN30146886]. No previous study has reported commensals in African buffaloes (*Syncerus caffer caffer*) of Rwanda or other wild ruminants. The presence of commensals such as enterococci in African buffaloes might play a considerable role in assessing antimicrobial resistance both in wild and domestic animals (Shin et al., 2017), future investigations need to explore it. The commensals and parasites identified in this study most likely originated from different water sources in the park as it has been suggested in other studies (VanderWaal et al., 2014a; VanderWaal et al., 2014b). These

watering points may be shared with livestock and humans and an infection from one species to another is plausible. In addition, *Treponema sp.* [Accession: SAMN30146887] and *Bacteriodes sp.* [Accession number: SAMN30146885] were also found.

Some of the sequence reads were 95% identical to Orf virus sequences and the reads were *De Novo* assembled. A phylogenetic analysis based on the B2L gene which is less variable across the genomes of different strains showed that it was related to geographically distant strains (figure 4-9).

FMDV whole genome was sequenced from OPF clinical samples collected from cattle.

As shown in figure 4-10, the VP1 sequences of SAT2 virus belonged to the same clade as that of SAT2 isolated in Zimbabwe in 1948 [Accession number: AY593847]. The same SAT2 virus isolated in Rwanda clustered in the same clade as isolates from Kenya [Accession number: AY344505] and Ethiopia [Accession number: AY343935].

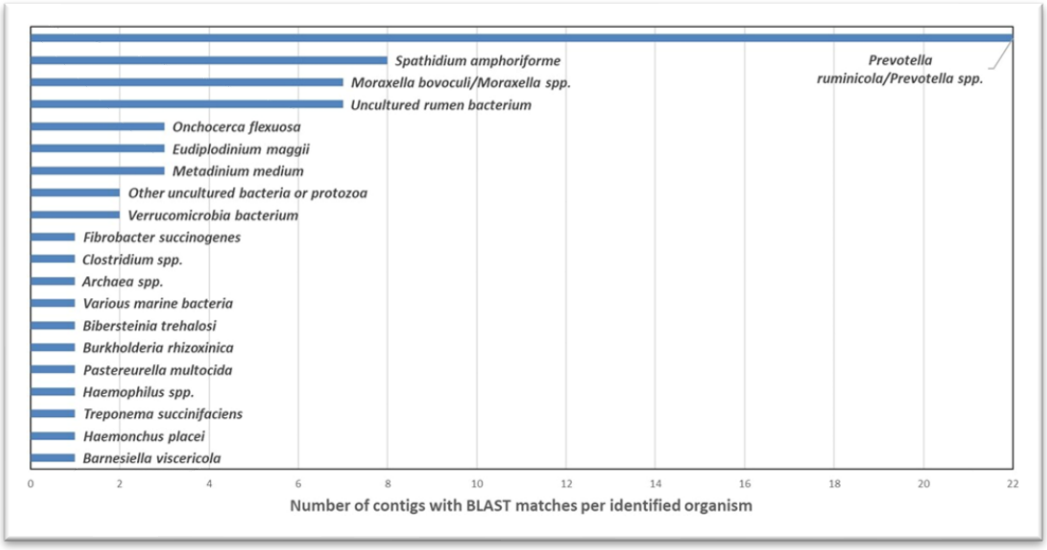

Figure 4-8: Sequenced contigs in A. buffaloes based on the top-scoring BLAST match for each contig.

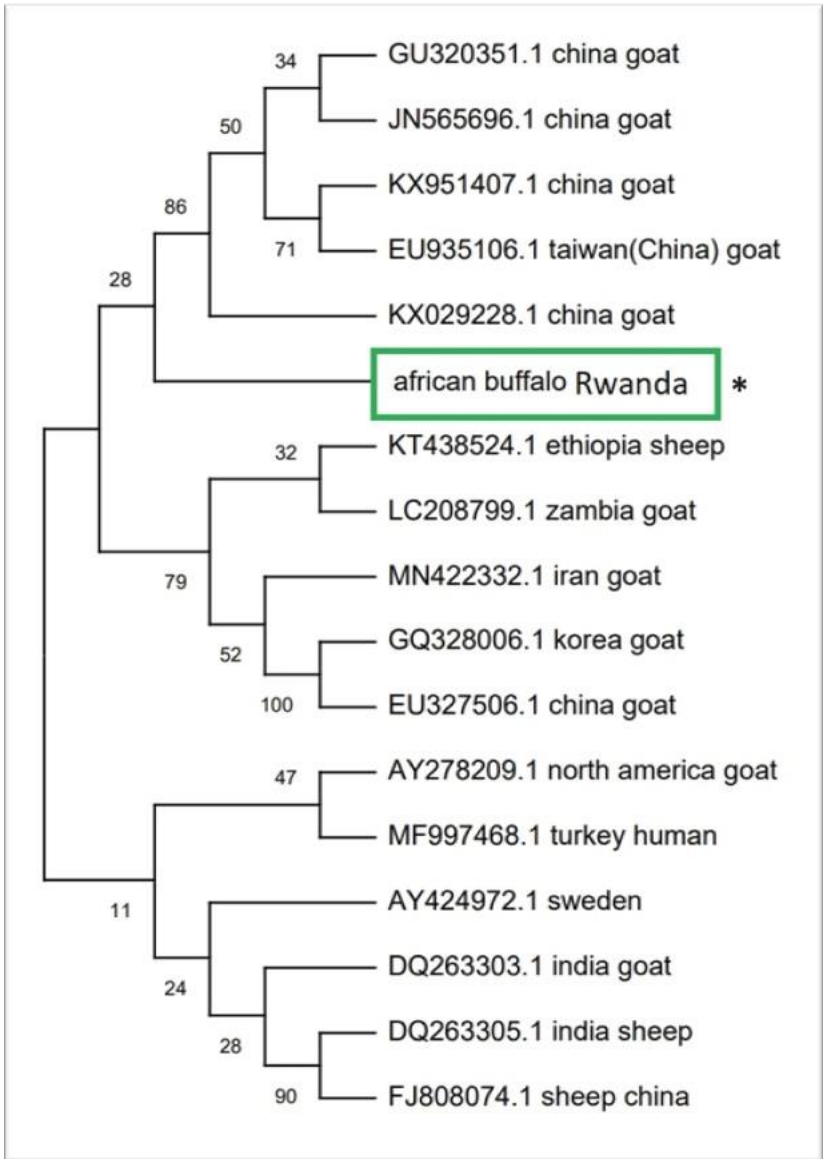

Figure 4-9: Phylogenetic tree of the VP1 protein sequences of Orf viruses isolated from African buffaloes (marked in a box). The buffalo isolatesbelonged to a separate clade as compared to the goat isolate from China [clade marked in *].

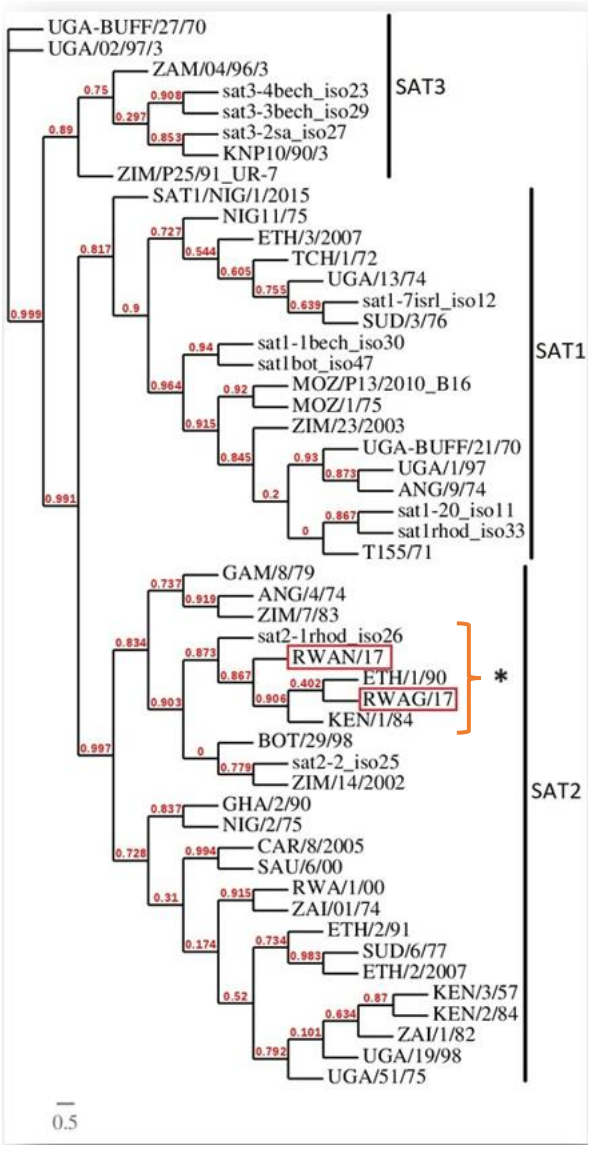

Figure 4-10: Phylogenetic tree of the VP1 protein sequences of FMDV-SAT1, SAT2 and SAT3. The Rwandan isolates designated as RWAN/17 and RWAG/17 (in boxes) shares the same clades as the Kenyan, Ethiopian and Zimbabwean (clade marked in *).

4.4. Discussion

Regions in Eastern Rwanda, at the border between Uganda and Tanzania, are at risk of transnational or transboundary circulation of the FMD virus. There was a gap in understanding both the seroprevalence of FMDV in susceptible animals and the circulating strains. Previously, there was a gap of data on FMDV seroprevalence and molecular epidemiology in Rwanda. The serotype responsible for the 2017 and 2022 FMD outbreaks in Eastern Rwanda was identified as SAT 2. Serotype SAT 2, together with other serotypes, is present in countries neighbouring Rwanda, including Uganda and Tanzania, and it appears to be the most predominant serotype in Sub-Saharan Africa (Doel, 2003; Kotecha et al., 2015).

4.4.1. Serological results

The detection of antibodies to NSP such as the highly conserved 3ABC is widely used to determine the FMDV seroprevalence (K. Sørensen et al., 1998). In a 2020 study it was not possible to get information on seroprevalence to investigate FMD risk factors in Eastern Rwanda (Udahemuka et al., 2020), therefore this study sets a baseline to get first-hand FMD sero-epidemiological information in cattle and small ruminants in Eastern Rwanda. Using the DIVA testing, the FMDV seroprevalence was at 9.36% and 2.65% in cattle and in goats respectively. This disparity was also observed elsewhere (Mwiine et al., 2019; Rout et al., 2014) It was observed that in the visited farms in Bugesera there were many on-site water resources and fodder banks that decreased the risk of animals roaming outside the farms. This could have contributed to the lower seroprevalence in Bugesera as compared to Nyagatare.

4.4.2. Molecular detection

4.4.2.1. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Amplification using specific primers targeting FMDV SAT 2 serotype revealed the presence of serotype SAT 2 in the OP samples. Although FMDV SAT 2 appears to be the most predominant in East Africa in general (Kerfua et al., 2018), the majority of outbreaks in Kenya

have been caused by serotypes O and SAT 2 (Omondi et al., 2019), in Uganda by serotype O and SAT 2 while in Rwanda the last reported outbreak in 2004 belonged to serotype O (WRLFMD, 2020). The current finding shows that SAT 2 was responsible for the 2017 FMD outbreak. In the previous year, SAT 2 and serotype A, were responsible for FMD outbreaks in the neighbouring Burundi (Garcia et al., 2022).

4.4.2.2. Reverse transcription loop-mediated isothermal amplification (RT-LAMP)

This method relies on auto-cycling strand displacement DNA synthesis that is performed by DNA polymerase with high strand displacement activity and a set of two specially designed inner and two outer primers (Notomi, 2000).

FMDV constant molecular diagnostics allow a better understanding of circulating strains for a smarter vaccination. This study confirmed what other studies have established that LAMP technology can be an alternative to traditional PCR since it is very portable and can yield reliable results. The LAMP results have shown that SAT 2 serotype was responsible for the 2017 outbreak, this in concordance with PCR results. This assay has been previously used in Southern Africa successfully on FMDV SATs serotypes (Bhoora et al., 2014).

The LAMP technology can be very useful for a point-of-care detection during an active outbreak to detect FMDV while sampling is still going on. The Axxin T8 being a portable device with batteries make it suitable to be used in mobile laboratories and since RT-LAMP can skip the cDNA synthesis step and the extraction step for some samples such as blood (Kaneko et al., 2007; Poon et al., 2006), it is much more fit for Low-and-Middle Income Countries and decreases the risk of contamination (Blomström et al., 2008). RT-LAMP has proved to detect positive samples and within 30 minutes it was possible to molecularly diagnose FMDV which would take a much longer time with laborious manipulations when diagnosing with RT-PCR. Also, RT-LAMP is better for blood samples because it is not hindered by inhibitory substances (Blomström et al., 2008).

4.4.3. Sequencing and Phylogenetic inference

FMDV was not detected in the seven samples of the African buffaloes randomly selected from the Nyamirama area inside the ANP in Rwanda by total RNA sequencing without prior amplification and *De Novo* sequence assembly. In South Africa, a pattern of infection from wildlife animals considered as natural reservoirs to livestock has been established, particularly African buffaloes (*Syncerus caffer*) (Hedger, 1976; Thomson et al., 1992). However, in Uganda Dhikusooka *et al.* were not able to confirm this in cases of SAT 3 and SAT 1 (Dhikusooka et al., 2015). In South Africa, physical separation consisting of electro-fencing of parks and movement restriction combined with effective vaccination campaigns (Thomson, 1995; Thomson et al., 2000) have assisted in reducing outbreaks from cross-infection and FMD control. However, in East Africa, few studies have been carried out on the circulating strains of FMDV, which constitutes a knowledge gap that prevents any conclusions that such measures have been similarly effective in this region. In 1979, SAT 3 was detected in African buffaloes in Queen Elizabeth National Park and in 2013 a healthy long-horned calf that grazed near this park (Dhikusooka et al., 2015). But even then, the finding could not prove the possibility of cross infection between buffaloes and cattle that intermingled in Uganda, an area that is very close to Rwanda.

Several reports have demonstrated that African buffaloes play an important role in the epidemiology of the SAT serotypes of FMDV (Anderson et al., 1979; Ayebazibwe et al., 2010; Di Nardo et al., 2015). In the present study, the sampled buffaloes had been enclosed inside the park and separated from farm access with an electrical fence since 2013 (Bariyanga et al., 2016). Although there is a need for a greater number of buffalo samples to be analysed, the results of this study suggest that the series of outbreaks observed in Eastern Rwanda between 2017 and 2022 may be arising from different sources, probably from transboundary and intra-national livestock movements as well as proximity with unvaccinated small ruminants although

this is not proved yet. These factors would contribute to virus persistence in the area because FMDV can be recovered in small ruminants up to 9 months post-infection (Moonen & Schrijver, 2000).

4.5. Conclusion

In conclusion, this study reports a plethora of commensals and pathogens, including the zoonotic Orf Virus, isolated in African buffaloes inside the Akagera National Park. However, the study was not able to establish a relationship of cross-infection between African buffaloes and cattle. Identifying and monitoring these pathogens in buffalo populations is essential for early detection and mitigation of disease outbreaks.

This study also concludes by suggesting the introduction of topotype II of serotype SAT 2 in Rwanda from Southern Africa, revealing how the dynamics of the disease are linked to cattle movement patterns such as the East African cattle corridor. Furthermore, the identified strains in Rwanda have similar variability with other strains identified in East Africa.

Chapter 5 : *IN SILICO* EPITOPE PREDICTION AND *IN VITRO* ANALYSIS OF FMD VACCINE.

5.1. Introduction

FMD is an infectious disease caused by the FMDV of the family of picornaviridae (Stanway et al., 2000). FMD is endemic in Africa and South-Eastern Asia with sporadic outbreaks in other countries (Brito et al., 2017; Jamal & Belsham, 2013; Sumption et al., 2008). Seven distinct serotypes have been identified and geographically localized with seven pools (Brito et al., 2017) and SAT 2 has been responsible for several outbreaks in Eastern Rwanda (Udahemuka et al., 2020). Vaccination against one serotype does not confer immunity against other serotypes (Rowlands et al., 1983). Therefore, high potency multivalent vaccines have been developed (Waters et al., 2018) and a continuous study of molecular epidemiology at the regional and national levels is of paramount importance to have regional-tailored vaccines. Poor understanding of circulating strains leads to limited vaccine matching and vaccine failure (FAO/EuFMD, 2020). The peptide-based vaccines paradigm has been proposed as an alternative to the currently used inactivated vaccines (Defaus et al., 2020; Forner et al., 2021; C. Wang et al., 2002). This chapter is exploring the variability of FMDV SAT 2 VP1 protein in EAC and *in silico* predicting highly potential epitopes to be targeted for epitope-based vaccines. Furthermore, the prediction has been complemented with a three-dimension (3D) presentation of the FMDV capsid using a close model available on the Protein Database Bank platform. The epitopes predicted in this study can be used to guide in choosing strains to include in vaccines or designing a peptide-based vaccine. In this chapter, the epitopes have been modeled and analyzed using *in silico* methods in order to improve on vaccine development.

5.2. Materials and methods

5.2.1. Selection of FMDV SAT 2 VP1 sequences

In this study, the field sequences were analyzed with other sequences available online. For the latter, the online search was carried out on three repositories namely; National Centre for Biotechnology Information (NCBI), European Bioinformatics Institute (EBI) and DNA Data Bank of Japan (DDBJ). We selected VP1 sequences identified as SAT2, collected after 2010 in Burundi, Kenya, Rwanda, Tanzania and Uganda, with a sequence length between 212 and 216 amino acids. The latter criteria were not followed for samples collected in Burundi, DRC and Rwanda because of the scarcity of available sequences. The selected sequences following the above criteria are available online at 10.6084/m9.figshare.14979594.

5.2.2. VP1 sequence variability in Great Lakes cluster of FMDV pool IV

A Python 3 notebook was used to create a dictionary that can transcribe the bases of the raw field sequences into amino acids. The EBI ClustalW was used to run a multiple sequence alignment to obtain a consensus sequence. The VP1 consensus sequence was used to search for a three-dimensional (3D) model of the structure that is more likely to be found in the region of East Africa. The protein variability server (PVS) tool was used to calculate and represent a Wu-Kabat coefficient for variability following the formula below:

$$\text{Variability}=N*k/n \text{ (Garcia-Boronat et al., 2008; Kabat et al., 1977)}$$

With N being the number of sequences in the alignment, k is the number of different amino acids at each position and n is the number of times for the most common amino acids per position. The Wu-Kabat coefficient was used to compare the variability of the predicted epitopes.

5.2.3. Epitope prediction and 3D modelling

The SWISS-MODEL was used to select the fittest online available model and run its evaluation. The stereochemistry of the structure was evaluated using the Ramachandran plot to study the acceptability of the model (Ramachandran et al., 1963).

The BepiPred-2.0 server was used to predict sequential B-cell epitopes with an epitope threshold set at 0.5 (Jespersen et al., 2017). Likewise, the MHC-I binding predictions were made on 7/28/2021 using the IEDB analysis resource NetMHCpan (ver. 4.1) tool (Reynisson et al., 2020). For the protein digestion, the IEDB recommended 2020.09 (NetMHCpan EL 4.1) settings were selected, digesting the protein in peptides ranging from 8 to 14-mers. The Bovine Leukocyte Antigen (BoLA) alleles available on the platform were all selected as ligands with default parameters. The peptides scoring more than 0.5 were considered for further analysis. The PyMol 2.5 (Schrödinger, 2021) was used for the visualization of the selected model and to map the identified epitopes on the FMDV VP1 in 3-D presentation.

5.2.4. In vitro analysis of FMD vaccine

Viral particles were derived from inactivated, purified vaccine against FMDV serotypes including A, O, SAT 1 and SAT 2 adjuvanted with Aluminium and saponin stored at +4°C. To extract the RNA, the vaccine was frozen at -70°C for 24 hours after which was performed the following steps: The vaccine was thawed at room temperature for 30 minutes and centrifuged at 3000G for 10 minutes, and finally the aqua and turbid phases were collected (Je et al., 2018).

The total RNA was extracted using the RNeasy Mini Kit (QiaGen) and cDNA synthesis performed using the Superscript^{TM} Double-stranded cDNA synthesis kit (Invitrogen) following the manufacturer's instructions.

Using the Taq PCR Master Mix Kit (Qiagen), four sets of primers (Table 4-1) corresponding to four serotypes (O, A, SAT 1 and SAT 2) were used to amplify the cDNA. The thermocycler was set to run for an initial denaturation at 95C°C for 60seconds; two-step amplification for 45

cycles with denaturation at 95°C for 15 seconds and an extension at 60°C for 30 seconds with a plate read. The melt curve was set at 70°C. The 2% agarose gel stained with ethidium bromide was run at 75V for 1 hour and read under U.V. light.

5.3. Results

5.3.1. VP1 sequence variability in Great Lakes cluster of FMDV pool IV

Python 3 was used to transcribe field sequence bases into amino acids and aligned field sequences with retrieved sequences. The VP1 sequences alignment using the EBI ClustalW platform's Gonnet matrix is presented in figure 5-1.

The protein variability plot was obtained using the Protein Variability Server (PVS) by calculating the Wu-Kabat coefficient as illustrated in figure 5-2. At least two regions (residue 45-60 and residue 138-140) that are highly variable across the selected sequences and position 58 being the most variable were identified.

The residue 58-60 and at positions 85, 108 were variable in a way that Nyagatare isolate was observed to be identical at residues 58-60, positions 85 and 108 to the isolates QDC11914, QDC11915 and QDC11965 isolated in cattle (Bos taurus) in Kenya at the livestock-wildlife interface between 2015 and 2016. Likewise, at the same positions, the Gatsibo isolate was identical to AJI77552 (cattle, Kenya, 2011), AJI77553 (cattle, Kenya, 2012), AJM93656 (cattle, Uganda, 2013), AXI68799 (cattle, Tanzania, 2011), AXI68832 (cattle, Tanzania, 2012) and QBY97746 (cattle, Tanzania, 2016).

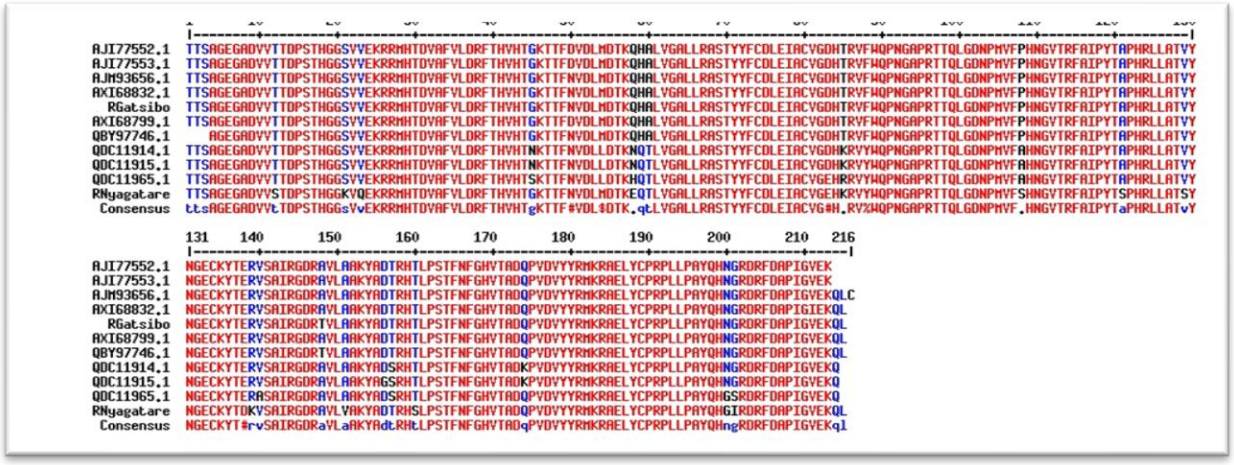

Figure 5-1: Similarity alignment of putative eleven VP1 proteins of Foot and Mouth Disease serotype SAT 2 in East Africa.

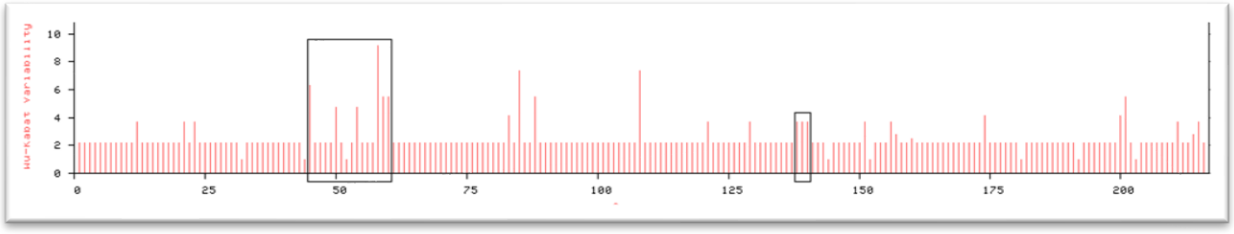

Figure 5-2: Wu-Kabat plot displaying the variability of VP1 proteins of FMDV SAT 2 isolates from East Africa. The black rectangles highlight variable motifs with at least 3 variable positions. The plot was constructed using the Protein Variability Server (Garcia-Boronat et al., 2008).

5.3.2. Epitope prediction and 3D modelling

The multiple sequence alignment of the 11 FMD VP1 sequences was obtained using the EBI online platform. Using the EBI's EMBOSS program, the resulting consensus sequence was obtained. A model close to the consensus sequence with available structures in the protein data bank was selected (with an 80.84 identity score and a 0.56 sequence similarity score). A Ramachandran plot, as illustrated in figure 5-3, showed that the structure can be accepted and considered for 3D structure presentation.

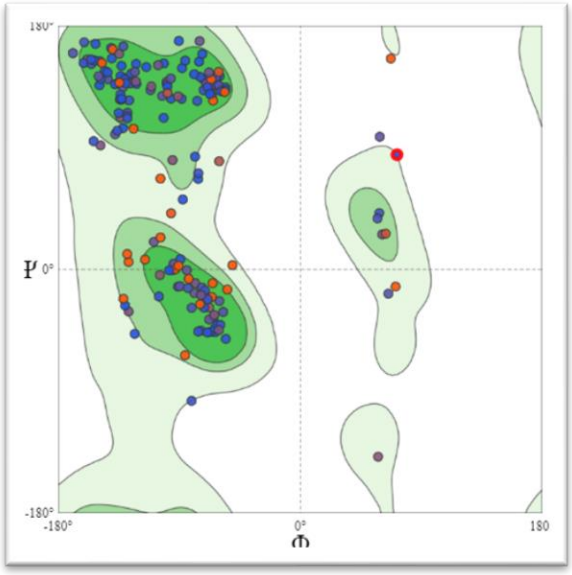

Figure 5-3: A Ramachandran plot for the selected 3D structure model shows the different amino acids' positions.

5.3.2.1. B-cell epitopes

The mapping of epitopes in figure 5-4 was obtained with the consensus sequence to predict the sequential B-cell epitopes DTU online platform (BepiPred-2.0) (Galgonek et al., 2017) and table 5.1 describes the predicted peptides with their positions.

The 5ACA VP1 based model was selected for a 3D presentation and the consensus sequence aligned to the 5ACA VP1 sequence to study the variability results are presented in table 5.1 with the predicted B-cell epitopes.

Discontinuous B-cell epitopes were determined using the IEDB online platform by setting the threshold at 0.5. The resulting seven linear and discontinuous epitopes are represented in table 5-1 and table 5-2 consecutively.

Figure 5-4: Representation of sequential B-cell epitopes prediction. The epitopes

Table 5-1: Predicted linear epitopes found on capsid proteins.

No	Start	End	Peptide	Number of residues	Score
1	198	213	PGYDHADRDRFDSPIG	16	0.816
2	1	30	TTSSGEGADVVTTDPSTHGGAVTEKKRVHT	30	0.798
3	129	168	RYNGECKYTQKLPSTF	16	0.67
4	92	114	NGAPRTTTLRDNPMVFSHNNVTR	23	0.64
5	81	88	LGEHERVW	8	0.614
6	175	178	ADKP	4	0.612
7	121	125	APHRL	5	0.539

Table 5-2: Predicted Discontinuous Epitopes

No	Residues	Number of residues	Score
1	T136, T137, Q138	3	0.929
2	T1, T2, S3, S4, G5, E6, G7, A8, D9, V10, V11, T12, T13, D14, P15, S16, T17, H18, G19, G20, A21, V22, T23, E24	24	0.854
3	P198, G199, Y200, D201, H202, A203, D204, R205, D206, R207, F208, D209, S210, P211, I212, G213	16	0.816
4	Y130, N131, G132, E133, C134, K135	6	0.806
5	T44, N45, T47, L81, G82, E83, H84, E85, R86, W88, T97, T98, T99, L100, R101, D102, N103, P104, M105, V106, F107, S108, H109, N110, N111, V112, A175, D176, K177, P178	30	0.645
6	P91, N92, G93, A94, P95, R96	6	0.562

Using the PyMol software, the model (PDB ID:5aca) was loaded in the software and mapped both linear and discontinuous epitopes as illustrated in figure 5-5. For visibility, only epitopes scoring ≥ 8 are presented.

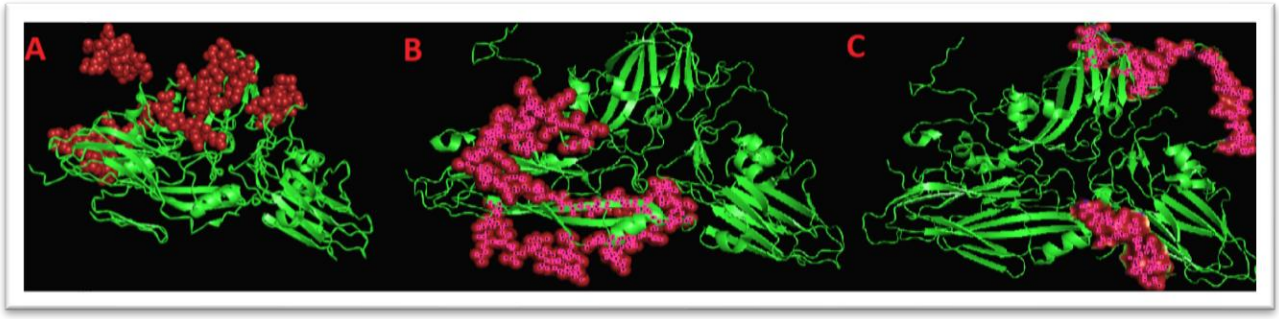

Figure 5-5: Humoral linear epitope prediction on the FMDV SAT 2 capsid surface proteins: VP1 (A), VP2 (B) and VP3 (C). Visualization using the PyMol v2.5 on the 5ACA protein model.

5.3.2.2. T-cell epitopes

The following putative Bovine Leukocyte Antigen (BoLA) system alleles were selected: BoLA-6:01301, BoLA-6:01302, BoLA-6:04101, BoLA-amani.1 and BoLA-gb1.7 to analyze their affinity to different peptide sizes ranging from 8 to 12 amino acids. Epitopes of the capsid surface proteins (VP1, VP2 and VP3) were predicted and those scoring ≥ 0.5 in the NetMHCpan tool as strong binders were considered (Table 5-3).

After merging the overlapping peptides as one residue, the resulting epitopes were mapped to the 3D model as illustrated in figure 5-6.

Table 5-3: Different combinations of BoLA alleles with several peptides recognized as strong binders.

	VP1		VP2		VP3	
Allele	*Position*	*Peptide*	*Position*	*Peptide*	*Position*	*Peptide*
BoLA-6:01301	89	WQPNGAPRTTTL	102	STQFNGGSLL	98	AQYQGSLNYHF
	184	RAELYCPRPLL	119	SLKDREEFQLSL		
			102	TQFNGGSLL		
BoLA-6:01302	184	RAELYCPRPLL	119	SLKDREEFQL	98	AQYQGSLNYHFM
			46	AERFFKEKL		
			101	STQFNGGSLL		
BoLA-6:04101	185	AELYCPRPLL	46	AERFFKEKL	147	SEWDTGLNSQF
	56	TNEKTLVGALL	44	EQAERFFKEKL	197	AEAAVVVSV
			46	AERFFKEKLF	48	AEACPTFLNF
			45	QAERFFKEKL		
			102	TQFNGGSLL		
			124	EEFQLSLYPHQF		
BoLA-amani.1	107	FSHNNVTRF	86	YTYMRNGW	20	KTADPIYGYVY
			16	SSVGITYGY	141	AAHCYHSEW
			142	TTAHIQVPY	102	GSLNYHFMY
					181	TTNGWVAVF
BoLA-gb1.7	92	NGAPRTTTL	140	TNTTAHIQVPYL	55	LNFDGKPYV
	107	FSHNNVTRF	58	TSDKPFGTL	195	HSAEAAVVV
	117	VPYTAPHRL			207	AGPDLEFRF
	157	TKHKLPSTF				

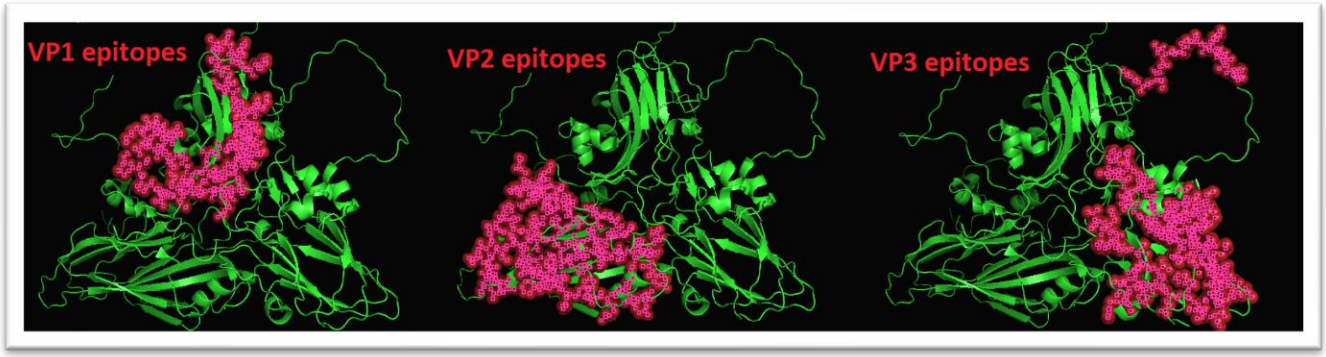

Figure 5-6: Three-dimensional mapping on the selected model (PDB ID: 5aca) of the predicted BoLA T-Cell epitopes scoring ≥0.5 for the capsid surface proteins (VP1, VP2 and VP3).

5.3.3. In vitro analysis of an FMD vaccine

The visualisation of amplicons on a 2% agarose gel did not show bands for serotypes O and A (not shown here) and for serotype SAT 1. Serotype SAT 2 showed faint bands, as illustrated in figure 5-7, indicating that the method was able to amplify nucleic material related to serotype SAT 2.

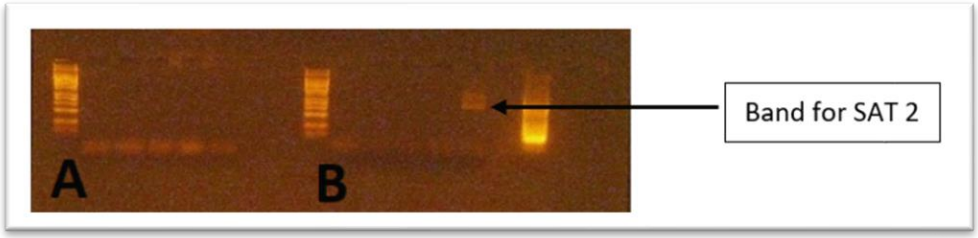

Figure 5-7: Gel-based analysis of amplicons resulting from A: SAT 1 specific amplification and B: SAT 2 specific amplification.

5.4. Discussion

The present study proposes peptides with a higher probability to induce B and T cell immune responses in cattle and therefore can be considered as good candidates for vaccine design. Using the Wu-Kabat variability index available at the protein variability server (Garcia-Boronat et al., 2008), the obtained variability plot with SAT 2 was less variable compared to the Asia1 serotype variability obtained elsewhere (Sultana et al., 2013). This study emphasizes a regional intratypic variability found at least in three positions. Another study described variable sites (positions 46-51, 109-114, 134-147 and 152-157) in SAT 1 that are approximately equivalent to the ones observed with this study's SAT 2 sequences (positions 45-50, 107-111, 135-141 and 151-160) with the RGD site (149-151) and C- terminus (202-217) being relatively conserved for both serotypes (Sahle et al., 2007).

Though *in silico* prediction of epitopes is very important, it has to be coupled with thermo-stabilization of the identified epitopes because of the thermal lability of SAT serotypes (Doel & Baccarini, 1981). The identified epitopes can be used to design a polytope-based plasmid DNA vaccine using linkers and adjuvant (Michel-Todó et al., 2020).

The analyzed vaccine demonstrated the presence of nucleic material corresponding to SAT 2 but there was no other serotype detected in the vaccine. This may be due to the used methodology. The inefficacy of the methodology can be from the used primers and/or the PCR settings as well. More experiments can improve the assay.

5.5. Conclusion

Experimentations using the predicted peptides can lead to region-tailored DNA vaccines development. Special focus should be put on residues that showed low variability and to induce B-cell immunity and high affinity to BoLA alleles for *in vitro* and *in vivo* testing. A deeper understanding of molecular epidemiology in East Africa can improve the effectiveness of the predicted epitopes. In Conclusion, the predicted epitopes for SAT 2 are similar to epitopes

predicted for SAT 1 elsewhere, this shows that these sites are highly antigenic. However, a in vivo study to confirm the antigenicity of these epitopes is highly needed.

Chapter 6 : GENERAL DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

6.1. General discussion

It is important to clearly state with a data-backed decision at what age vaccination would not interfere with maternal antibodies and with a mature immune system to optimally respond to vaccination. Following the vaccine manufacturer's instructions would have made this risk factor less confounding. Therefore, it is recommendable to conduct more vaccinations a year as per the manufacturer's instructions and regular vaccine matching studies. Furthermore, a sensitization to farmers to include animals of all ages in vaccination and at least two vaccination campaigns a year with booster doses, would also have a positive impact.

Rearing of cattle together with sheep and goat in the same farm has been documented to be one of the risk factors responsible for FMD outbreak and transmission (Wungak et al., 2016). There are reports that breeding methods using AI and natural breeding is responsible for the spread of FMD during outbreaks (Callis, 1996; Paton et al., 2018), and these results show the same trend. Most of the farmers interviewed reported the likeliness of more outbreaks during the dry season than in the wet season. Indeed, this finding is consistent with official records of the Rwanda Agriculture and Animal Resources Development Board (Personal communication). In addition to OIE reports (MINAGRI, 2015; J. Weaver et al., 2019), other studies, in other places, have also reported more outbreaks of the disease during the dry season as opposed to the wet season (Tekleghiorghis et al., 2014; Waret-Szkuta et al., 2011; Wungak et al., 2016). FMD is usually spread by contact and to some extent by airborne means. It is more likely that Eastern Rwanda would experience less FMD outbreaks if the physical contact of animals of different farms was controlled. The role of African buffaloes in epidemiology of FMD has been established elsewhere (Condy et al., 1985; Paton et al., 2018; Thomson et al., 1992, 2013; Thomson & Vosloo, 2004; Vosloo et al., 1996), but this study did not report the same situation in Eastern Rwanda.

It is probably possible that the uncontrolled transboundary livestock movements, porous borders and mass movements in this region could be contributing to the presence of the current circulating FMDV strains that are identified in this area.

The higher prevalence in cattle, compared to the small ruminants, may be explained by the fact that viral replication is expected to be more luxuriant and more persisting in cattle than in small ruminants (Rout et al., 2014). A sero-surveillance study in wild FMD-susceptible animals would be valuable to understand the role played by the wild animals.

The used RT-PCR detection assay revealed that SAT 2 serotypes was responsible for FMD outbreaks in Rwanda in concordance with other outbreaks in the region. This calls for the need to incorporate appropriate SAT 2 virus strains in vaccines that may be used in Rwanda areas if a regionally coordinated vaccination campaign is to be carried out. Recently, Mahapatra *et al.* have demonstrated that LAMP's concordance with RT-qPCR reaches 100% (Mahapatra et al., 2019). This pen-side, rapid and cost-effective technology can be of great importance to quickly identify an outbreak and quarantine the area for further investigation. The democratisation of such technologies would fill the gap of lack of proper knowledge on circulating strains in Eastern Rwanda and other countries with similar challenges.

The phylogenetic results suggest that the isolated virus in Rwanda may have evolved and circulated from Zimbabwe to East Africa (Kenya) and spread in two branches Northward to Ethiopia and Southward to Rwanda. The 2017 sequences are quite different from the sequences previously characterized in Rwanda (isolate RWA/1/00) and in the neighbouring Democratic Republic of Congo (isolate ZAI/01/74). This shows the complexity of circulating strains in this region and considering that many outbreaks are unnoticed and or unreported, more strains might be circulating.

6.2. Conclusion

- Vaccinating calves under 12 months would protect the herds from the incursion of FMD in Eastern Rwanda. The presence of FMDV- antibodies in cattle and goats indicate previous exposure to the virus.
- The buffaloes appeared not to be infected with FMDV but they had other viruses and bacteria.
- Serotype SAT 2 topotype VIII is circulating in Rwanda suggesting that there is a dynamic of strains between Southern Africa pool and Eastern Africa pool.
- Potent epitopes against circulating strains in Rwanda and the region were identified and nucleic material corresponding to SAT2 were extracted from the used vaccine.

6.3. Recommendations

- Vaccination programs should target both older and younger cattle and consider the identified risk factors when developing the risk-based strategic plan (RBSP).
- Further studies on the incursion of FMD in the area should focus on investigating the role played by the domestic-wildlife interaction.
- The control actions should be coordinated regionally.
- More studies on improving the assay on detecting serotype-nucleic material that are incorporated in vaccine formulas are needed.
- Further studies to include the identified epitopes in vaccines and assess the indirect relationships (r-1 value) between field strains and potential vaccine strains based on antibody responses.

6.4. Limitations of the study

The study was affected by different factors, including the limitation of funds, the occurring of the SARS-CoV-2 pandemic. If more funds were available, more samples from A. buffaloes

could have been collected for a wider picture of the pathogens infecting buffaloes and a more robust conclusion of whether they have FMDV or not. During the pandemic (2021), there was an FMD outbreak but due to travel restrictions it was not possible to timely be on the field to collect appropriate samples that would enrich the phylogenetic analysis.

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APPENDICES

Appendix 1: A ten-year retrospective summary of FMDV strains circulating in FMD Pool 4 adapted from (OIE and FAO World Reference Laboratory for Foot-and-Mouth Disease, 2022)

WRLFMD Batch No.	**Date received**	**Location**	**Serotype**	**Topotype**	**Lineage**
WRLMEG/2019/00030	19/04/2019	Comoros	O	EA-2	
WRLFMD/2021/00011	19/07/2021	Congo (DR)	O	EA-2	
WRLFMD/2012/00029	01/08/2012	Eritrea	O	EA-3	
WRLFMD/2012/00006	03/02/2012	Ethiopia	O	EA-3	
WRLFMD/2012/00024	29/06/2012	Kenya	O	EA-2	
			SAT 1	I (NWZ)	
			SAT 2	IV	
WRLFMD/2013/00019	25/09/2013	Kenya	A	AFRICA	G-I
			SAT 1	I (NWZ)	
			SAT 2	IV	
WRLFMD/2017/00031	15/12/2017	Kenya	O	EA-2	
			SAT 1	I (NWZ)	
WRLFMD/2018/00023	29/08/2018	Kenya	O	EA-2	
			A	AFRICA	G-I
			SAT 1	I (NWZ)	
			SAT 2	IV	

WRLFMD/2018/00024	29/08/2018	South Sudan	O	EA-3	
WRLFMD/2012/00002	13/01/2012	Tanzania	SAT 2	IV	
WRLFMD/2012/00031	08/08/2012	Tanzania	O	EA-2	
			A	AFRICA	G-I
			SAT 1	I (NWZ)	
			SAT 2	IV	
WRLFMD/2013/00005	13/03/2013	Tanzania	SAT 1	I (NWZ)	
			SAT 2	IV	
WRLFMD/2014/00002	27/01/2014	Tanzania	SAT 1	I (NWZ)	
			A	G-I	
WRLFMD/2015/00008	30/04/2015	Tanzania	O	EA-2	
			SAT 1	I (NWZ)	
WRLFMD/2012/00028	20/07/2012	Sudan	O	EA-3	
			A	AFRICA	
			SAT 2	VII	
WRLFMD/2015/00038	03/12/2015	Sudan	O	EA-3	
			A	AFRICA	G-IV
			SAT 2	VII	Alx-12
WRLFMD/2018/00020	20/07/2018	Sudan	O	EA-3	
			A	AFRICA	G-IV
			SAT 2	VII	Alx-12
WRLMEG/2015/00001	21/01/2015	Uganda	SAT 3	V	
WRLFMD/2017/00027	19/10/2017		O	EA-2	

WRLFMD/2019/00009	12/02/2019		O	EA-2	
			A	AFRICA	G-I
WRLMEG/2020/00003	18/01/2020	Uganda	O	EA-2	
			SAT 1	I (NWZ)	
			SAT 2	VII	
				IV	
WRLFMD/2021/00014	11/10/2021		O	EA-2	

Appendix 2: QUESTIONNAIRE FORM FOR CATTLE FARMERS IN EASTERN RWANDA

This questionnaire is for identification of risk factors of Foot and Mouth Disease Virus outbreaks in Eastern Rwanda. The interview has no intention to exploit the obtained data in any purpose other than research.

Interviewer: **UDAHEMUKA Jean Claude**

Date: ……./……./2018

Form number: ……

Farm geographical coordinates: ……

Farmer general information

1. Names: …… ……
2. Farm code …….
3. Sex: a. Male

   b. Female
4. Age: ……. years old
5. Marital status: a. single d. Divorced

   b. Married e. Widowed

   c. Engaged

6. District……....
7. Sector……
8. Cell ……...

9. Village (Umudugudu) ……...

10. Educational status:

a) Never attended formal education

b) Primary school

c) Secondary

d) University

e) Other (specify)

11. How many people do you have in your family? ……

12. What is your social class? ……..[1]

Animal general information

13. How many cows do you have? ……...

14. Which breed do you keep:

    a) Local (Ankole)

    b) Exotic

    c) Cross

    d) More than one breed

15. How are your cows acquired?

    1. Donation

    2. Buying

3. Inherited

4. Given by an NGO

5. Government

6. Other (Specify)

16. How many cows did you purchase / acquire in the last five years?.....

17. How many calves younger than 6 months are in the herd?.....

18. How many calves are aged between 6 and 12 months? .....

19. How many bulls do you have? .....

20. What size is your farm?: ha ……

21. What type of farming do you practice?

    a. Strict Zero grazing
    b. Zero grazing with cattle walking for water
    c. Free ranging

22. Is your farm adjacent to another farm?

    a. Yes
    b. No

23. Do you have an off-farm job? (Is farming your only source of income?)

    a. Yes
    b. No

24. If yes, how would you rate the part played by your farm activities with your overall income? If no, how do your farming activities contribute to the overall income?

    a. Animal farm activities generate more than half of the income
    b. Animal farm activities generate half of the income
    c. Animal farm activities generate less than half of the income

25. Do you or any of your close neighbours have any of these animals: goat, sheep, pigs?

    a. Yes
    b. No

26. If there are pigs in the neighbourhood, are they swill fed?

    a. Yes
    b. No

27. When was the last FMD outbreak (Year and season of year)?

……./……

28. How long did that outbreak last (in months)?.....

29. In case of an outbreak, are samples being taken by animal health specialists?

    a. Yes

    b. No

30. If they collect samples, after how many days do they come prior to an outbreak?.....

31. How often are you likely to face an outbreak of FMD?

    a. Several times a year

b. Once in a year

c. Once in two years

d. Once in more than two years

32. According to your experience, at what period of year is FMD outbreak likely to surface (rain or dry season)?

33. What control measures are taken in case of an outbreak?

a. Nothing

b. Vaccination

c. Quarantine

d. Pre-emptive culling

e. Depopulate after an outbreak

34. Did you face one or more outbreaks in the last five years?

35. Have you been facing an FMD outbreak annually for the last five years?

36. At the time of the most recent outbreak, did FMD occur in the radius of up to 5km?

37. How far is your usual cattle market (in Km)?.....

38. Is your farm adjacent to the park?

a. Yes

b. No

39. Is your herd being systematically vaccinated?

a. Yes

b. No

40. If yes, how often?

a. Once a year

b. Twice a year

c. Other (specify)……

41. If no, what is/are reason(s)?

a. The authorities don’t propose it

b. You don’t have enough means for it

c. You don’t trust the vaccines

d. Other (specify)……

42. Do you vaccinate your calves younger than 12 months?

a. Yes

b. No

43. Is the time elapsed from the last vaccination of adults >6 months?

a. Yes

b. No

44. What was the morbidity rate during the last outbreak?.....

45. What was the mortality during the last outbreak?.....

46. How do you breed your cows?

a. Artificial insemination (AI)

b. Natural methods

c. A combination of the two alternatives

47. If you use the natural method, do you use your own bulls or you borrow a bull from your neighbours?

48. Is your farm fenced?

a. Yes

b. No

49. Is there any restriction to a stranger to access your farm?

a. Yes

b. No

50. Do you have disinfectant bath at the entrance of your farm?

a. Yes

b. No

51. Where do you sell the milk?

a. The nearest Milk Collection Centre (MCC)

b. The nearest dairy

c. Informal selling

d. Exclusively consumed by family members

Notes:

1: Answers will be according to social classification levels (Ubudehe) by the Ministry of Local Affairs of the Republic of Rwanda.

Appendix 3: Primers targeting serotypes SAT1, SAT2, A and O.

macrogen

Macrogen Europe
(Jean Claude Udahemuka)
KG 691 St, No 82, Kigali Rwanda
KK 18 Ave, Kigali
5016

HO00013538
Order Date :2020-04-30
Packing date :
Page :1/2

Oligo	SAT1 - 1C559F					
SEQ	5'-GTGTATCAGATCACAGACACACA-3' (23mer)					
GC%	MW		Yield		scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
43	7025.6		6.7	25	0.025	60.9
vol. for 100pmol/ul	Purification		Modification			
250	Desalt					

Oligo	SAT - 2B208R					
SEQ	5'-ACAGCGGCCATGCACGACAG-3' (20mer)					
GC%	MW		Yield		scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
65	6121	6122.6	2	9	0.05	63.9
vol. for 100pmol/ul	Purification		Modification			
90	PAGE					

Oligo	SAT2-_1C445F					
SEQ	5'-TGGGACACMGGIYTGAACTC-3' (20mer)					
GC%	MW		Yield		scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
50	6147.3	6188	6.6	30	0.05	57.9
vol. for 100pmol/ul	Purification		Modification			
300	MOPC					

Oligo	A-1C562F					
SEQ	5'-TACCAAATTACACACGGGAA-3' (20mer)					
GC%	MW		Yield		scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
40	6103		5.9	25	0.025	53.9
vol. for 100pmol/ul	Purification		Modification			
250	Desalt					

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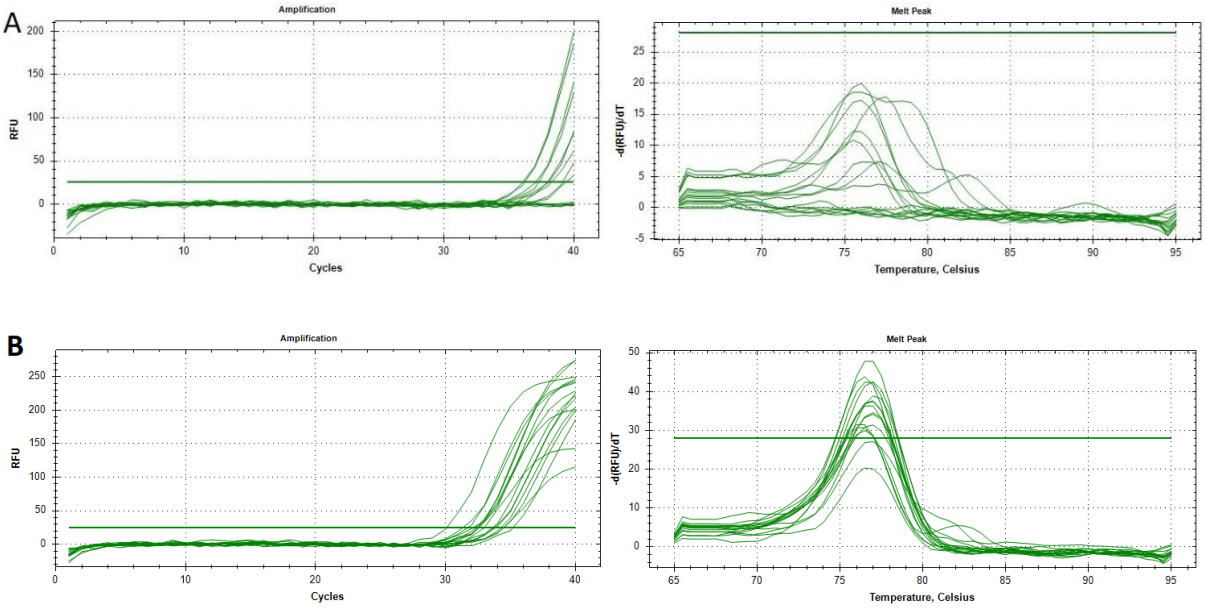
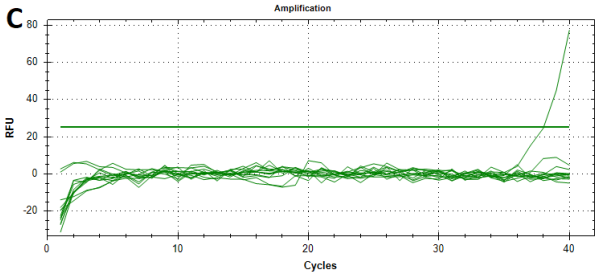
Appendix 4: Calculations of the confidence intervals at 95% for the seroprevalence.

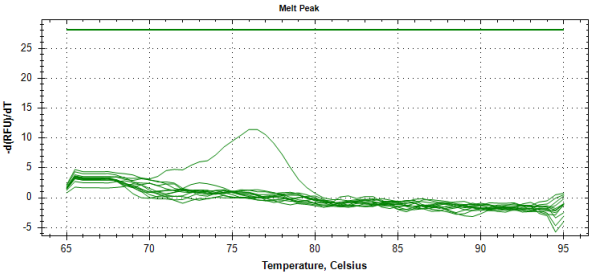
The dataset showing the calculations of the confidence intervals for FMD seroprevalence is found at: 10.6084/m9.figshare.20731066

Appendix 5: Results of the 2022 FMD outbreak in Nyagatare Eastern Province, Rwanda); detection (amplification and melt peak) of the VP1 for A: serotype SAT1, B: serotype SAT2, C: serotype O and D: serotype A.

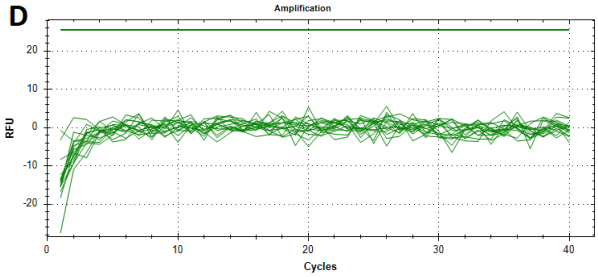


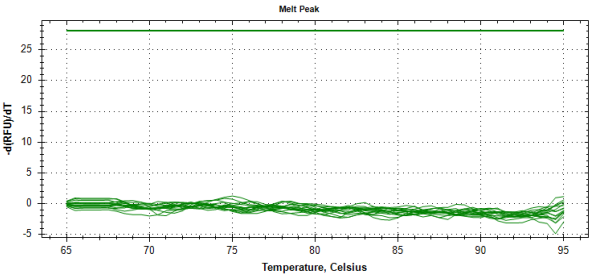
C


Amplification
RFU
Cycles


Melt Peak
-d(RFU)/dT
Temperature, Celsius

D


Amplification
RFU
Cycles


Melt Peak
-d(RFU)/dT
Temperature, Celsius