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Sudan University of Science and Technology

College of Graduate Studies

Epidemiological Studies of Foot-and-Mouth Disease in Northern Sudan

دراسات وبائية عن مرض الحمي القلاعية في شمال السودان

A thesis submitted to the College of Graduate Studies in fulfillment of the requirements for the degree of Master of Veterinary Medicine (M.V.M.) in Microbiology (Virology)

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Declaration of the Status of Thesis

By Student

The work described in this master degree thesis was carried out at Foot and Mouth Disease Unit, Central Veterinary Research Laboratories (CVRL), Soba and the Department of Pathology, Parasitology and Microbiology at the College of Veterinary Medicine, Sudan University of Science and Technology from February 2017 to April 2022 under the supervision of Dr. Nussieba Ahmed Osman Elhag and co-supervision of Dr. Yazeed A/Raouf Hussien.

The experimental work is original and the thesis has not been submitted partially or fully to any other University.

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April, 2022

Dedication

With the gratitude and warm interest

I dedicate this work to my parents, who are the source of my inspiration and without them I would not have been here

To my brothers and sisters

To my dear soulmate who supports and encourages me to move forward despite the difficulties

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Abstract

The Nile basin north to Khartoum encompasses two administrative States: the Northern and the River Nile States. The area represents a geographical cluster characterized by an exclusive desert and semi-desert ecosystems and low animal density. Northern Sudan is an important corridor cluster between pools of foot-and-mouth disease virus (FMDV) in East and North Africa. It involves almost the whole border area with Egypt; an important trade partner of the country, and represents a considerable part of a projected disease-free zone in Sudan. The study monitored FMD infection between 2016 and 2018 in Northern Sudan. Passive and active surveillance of clinical FMD in cattle were, all, utilized. A total of 184 bovine sera proved to be positive to nonstructural proteins (NSPs) of FMD virus during the programme "surveillance of trade sensitive diseases" in 2016 were examined for antibodies against serotype O, A and SAT2 using virus neutralization test (VNT). These sera had originated from the River Nile (143) and the Northern State (41).

Results largely confirmed previous reports that have described the relatively lower circulation of FMDV in the area than in other parts of the country. Clinical FMD was confirmed once in the three years period. Only serotype O of an unnamed lineage within the topotype East Africa 3 (O-EA3), like all other Sudanese O viruses, was typed during the study period in 2016 in cattle smuggled to Egypt and in resident cattle. It was closely related to Egyptian and Sudanese isolates of 2017 (phylogenetic identity ≥ 99.4%) rather than the Sudanese viruses that had been detected in the Northern State in 2012. These sequences formed a large temporal cluster that included in addition Israeli, Ethiopian and Palestinian isolates from 2017. Obviously, these sequences, like the sequences in 2012, were of transboundary nature.

Antibodies against the structural proteins (SPs) of the 3 serotypes of FMDV in Sudan; O, A and SAT2, were detected. Consistently, sero-prevalence estimates were statistically significantly higher in the River Nile than in the Northern State. In the River Nile, the 95% C.I. for the estimates were 9.5%-16.4% for serotype O, 11.7%-19.1% for serotype A and 11.0%-18.2% for serotype SAT2. In the Northern State, corresponding estimates were 3.5%-9.3%, 4.3%-10.7% and 1.2%-5.6%. In the River Nile, sero-prevalence estimates were lowest in the most Northern district of Abu Hamad while in the Northern State the Western district of Al Goled proved to be negative (n = 64) for anti-NSPs activity. Results suggested a direction of infection from the South and East to

the North and West; South Eastern districts in the River Nile and Eastern districts in the Northern State were showing higher sero-prevalences and were likely crucial points of entry of the infection.

The serological study involved a serial testing approach i.e. sera positive by both test systems, ID Screen® FMD NSPs Competition ELISA and VNT, were considered positive. Sero-prevalence by the ID Screen® FMD NSPs Competition ELISA in the River Nile State was found to be statistically significantly higher (P = 0.000725) than that by the combined VNT (O, A and SAT2) but not in the Northern State (P = 0.106567). Earlier, it was expected that mild exposure (limited virus multiplication) to different serotypes could result in boosting immune response to NSPs but not to SPs and consequent difference in performance between NSPs and SPs serology. Mild exposure to different serotypes was clearly more expected in the River Nile than in the Northern State where very low levels of circulation of FMD virus prevail.

Epidemiological pattern of FMD infection in Northern Sudan as recognized by the study presented the lowest level of circulation of FMD viruses in the country. Concurrently, unlike other parts of Sudan, no predominance of serotype O antibody was detected.

It was concluded that low animal density and limited animal movement in Northern Sudan together with the high antibody levels against serotype O in immediately neighbouring States (Khartoum and Kassala) effectively decreased infiltration of endogenous O viruses.

ملخص البحث

يتكون حوض النيل شمال الخرطوم من ولايتين إداريتين: الولاية الشمالية وولاية نهر النيل. تمثل المنطقة كتلة جغرافية تتميز بنظم بيئية صحراوية وشبه صحراوية وقلة الكثافة الحيوانية. شمال السودان عباره عن معبر هام بين تجمعات فيروس مرض الحمى القلاعية (FMDV) في شرق وشمال إفريقيا. وتشمل كل المنطقة الحدودية مع مصر ؛ وهي شريك تجاري مهم للبلد ، وتمثل جزء معتبر من المنطقة الخالية من الأمراض في السودان.

رصدت الدراسة الإصابة بمرض الحمى القلاعية في شمال السودان خلال الأعوام 2016م و2018م. وقد تم إستخدام جميع طرق التقصي السلبية والفعالة لمرض الحمى القلاعية في الماشية. ثبت أن عدد 184 عينة مصل بقري كانت إيجابية للبروتينات غير التركيبية (NSPs) لفيروس مرض الحمى القلاعية وذلك خلال برنامج "مراقبة الأمراض الحساسة للتجارة" في عام 2016 حيث تم فحص الأجسام المضادة ضد الأنماط المصلية O و A و SAT2 باستخدام إختبار معادلة الفيروس (VNT). كانت هذه الامصال من ولايتي نهر النيل (143) والولاية الشمالية (41).

أكدت النتائج إلى حد كبير التقارير السابقة التي وصفت الإنخفاض النسبي في تداول مرض الحمى القلاعية في المنطقة مقارنة باجزاء اخرى من البلاد. تم تأكيد مرض الحمى القلاعية السريرية مرة واحدة خلال فترة الثلاث سنوات. تم تحديد نوع النمط المصلي O فقط من سلالة غير مسمية ضمن النوع العلوي O-EA3 وذلك مثل جميع فيروسات O السودانية الأخرى وذلك خلال فترة الدراسة في عام 2016م وتم ذلك في الماشية المهربة إلى مصر وايضاً في الماشية المقيمة. وإتضح أنه ذو علاقة اقرب للفيروسات المعزولة المصرية والسودانية لعام 2017م (هوية النشوء والتطور كانت 99.4٪) اكثر من الفيروسات السودانية التي تم إكتشافها في الولاية الشمالية في عام 2012م. شكلت هذه التسلسلات كتلة زمنية كبيرة شملت بالإضافة إلى ذلك الفيروسات المعزولة من إسرائيل وإثيوبيا وفلسطين خلال عام 2017م. بالتأكيد فإن هذه التسلسلات ، كتلك التسلسلات من عام 2012م ، لها طبيعة عابرة للحدود.

تم رصد الأجسام المضادة ضد البروتينات الهيكلية (SPs) للأنماط المصلية الثلاثة لفيروس مرض الحمى القلاعية في السودان O و A وSAT2. بشكل ثابت ، كانت تقديرات الإنتشار المصلي أعلى من الناحية الإحصائية في ولاية نهر النيل اكثر منها في الولاية الشمالية. في نهر النيل ، كانت التقديرات لل .C.I 95% هي 9.5٪ -16.4٪ للنمط المصلي O ، 11.7٪ -19.1٪ للنمط المصلي A و 11.0٪-18.2٪ للنمط المصلي SAT2. في الولاية الشمالية ، كانت التقديرات المقابلة لها هي 3.5٪-9.3٪ ، 4.3٪-10.7٪ و 1.2٪-5.6٪. في نهر النيل كانت تقديرات الإنتشار المصلي الادنى في اقصي منطقة شمالية للولاية وهي ابو حمد بينما في الولاية الشمالية كانت تقديرات الإنتشار المصلي سلبية في المنطقة الغربية وهي القولد (ن = 64) للنشاط المناهض للبروتينات غير التركيبية للفيروس NSPs. اشارت النتائج الي إتجاه العدوى من الجنوب والشرق إلى الشمال والغرب ، كما اظهرت المناطق الجنوبية الشرقية من نهر النيل والمناطق الشرقية من الولاية الشمالية اعلي معدل للإنتشار المصلي وكانت على الأرجح نقاطاً حاسمة لدخول العدوى.

تضمنت الدراسة المصلية نهج إختبار تسلسلي ، اي أن الأمصال الإيجابية من خلال كلا نظامي الإختبار ، الاليزا ID Screen® FMD NSP Competition ELISA ومعادلة الفيروس VNT ، اعتبرت ايجابية. معدل الإنتشار المصلي بواسطة اختبار الاليزا في ولاية نهر النيل كان اعلى من الناحية الإحصائية (P = 0.000725)

مقارنة مع معدل الإنتشار المصلي بواسطة اختبار معادلة الفيروس المتكامل (O, A and SAT2) ولكن ليس في الولاية الشمالية (P = 0.106567). في وقت سابق ، كان من المتوقع أن يؤدي التعرض الخفيف (تكاثر محدود للفيروس) لعدة انماط مصلية الى تعزيز الإستجابة المناعية للبروتينات غير التركيبية للفيروس NSPs ولكن ليس للبروتينات التركيبية للفيروس SPs وما يترتب على ذلك من إختلاف في الأداء بين البروتينات المذكورة من الناحية المصلية. كان من الواضح أن التعرض الخفيف للأنماط المصلية المختلفة كان متوقعاً في ولاية نهر النيل اكثر منه في الولاية الشمالية حيث تسود مستويات منخفضة للغاية بالنسبة لإنتشار فيروس الحمى القلاعية.

تم التعرف علي النمط الوبائي لعدوى مرض الحمى القلاعية في شمال السودان عبر الدراسة والتي وضحت ادنى مستوى لإنتشار فيروسات الحمى القلاعية في البلد. في نفس الوقت لم يتم الكشف عن غلبة النمط المصلي O للفيروس وذلك علي عكس الأجزاء الأخري من السودان.

يمكن الإستنتاج أن إنخفاض كثافة الحيوانات ومحدودية حركتها في شمال السودان مع إرتفاع مستويات الأجسام المضادة ضد النمط المصلي O في الولايات المجاورة (الخرطوم وكسلا) قللت بشكل فعال من تسلل فيروسات O الذاتية.

Introduction

Foot-and-mouth disease (FMD) is an important transboundary and an economically significant viral infection of domestic and wild ruminants. It reduces animal productivity and forces severe restrictions on trade of animals and animal byproducts (Alexandersen *et al.,* 2003a). It is No. 1 in the World Organization for Animal Health (OIE) list of infectious diseases and ranked by some workers (Domenech *et al.,* 2006) as the first and foremost priority animal disease.

Loeffler and Frosch (1897; 1898) were the first to demonstrate that the cause of FMD is a filterable agent (i.e. virus). Foot-and-mouth disease virus (FMDV) is a member of the *Aphthovirus* genus of the *Picornaviridae* family. it is with seven immunologically distinct serotypes: O, A, SAT1-3, C and Asia 1 (Murphy *et al.,* 1999). All the seven serotypes cause clinically similar disease characterized by fever and vesicular lesions mainly in the mouth, snout, udder and feet (MacLachlan and Dubovi, 2011).

Foot-and-mouth disease is endemic in large areas of Africa, Asia and South America (Knowles *et al*., 2001; Department of FMD Report, 2013). In Sudan, the first record of FMD was in 1903 (Eisa and Rweyemamu, 1977). Historically four serotypes of FMDV had been reported in the country: O, A, SAT1 and SAT2 (Abu Elzein, 1983). SAT1 serotype has not been reported in Sudan since 1976 whereas the maintained activity of the other three serotypes; O, A, and SAT2 has been currently and repeatedly confirmed by disease and serological surveillances (Raouf *et al*., 2009; 2010; 2016; Habiela *et al,* 2010a; 2010b; http://www.wrlfmd.org/). In Sudan, clinical FMD is seen in cattle only which is the main target species, while domestic small ruminants (sheep and goats) undergo largely silent infection and play minor role in the epidemiology of FMD (Raouf *et al*., 2017; Raouf, 2020). In spite of the long history of FMD in Sudan and the little efforts of control practiced, different levels of FMD infection were recognized in different geographical areas of the country (Raouf *et al*., 2011; 2016; 2017; Department of FMD Report, 2016; Saeed, 2019; Saeed and Raouf, 2020).

Saeed (2019) and Saeed and Raouf (2020) studied the problem of FMD, between 2011 and 2013, in the uppermost area of Northern Sudan; the Northern State. Modest circulation of FMDV was described there (Saeed, 2019; Saeed and Raouf, 2020). Similarly, indices of prevalence of FMD infection in Northern Sudan as indicated by seroprevalence of antibodies against nonstructural proteins (NSPs) of FMDV, studied during the programme "Surveillance of Trade Sensitive Diseases" in 2015 and 2016,

were low or relatively low (Department of FMD Report, 2016). Seroprevalence of anti-NSPs antibodies was around 15% in the Northern State and 40% in the River Nile State. The present work meant to continue the study of FMD in Northern Sudan extend the time-scale and geographical area of investigation and confirm the suggested mild circulation of FMD there. The Northern and River Nile States represent the geographical cluster of the Nile valley North to Khartoum. Epidemiologically, the Nile valley North to Khartoum is distinguished by a desert and semi-desert ecosystem, low animal density and limited animal movement. It is part of the projected disease free zone and involve almost the whole border area with Egypt what add a further dimension to the significance of FMD infection. Many African countries are currently conducting studies to delimit candidate FMD free areas (Picado *et al.,* 2011). In the present study, to define entry and circulation of FMDV (O, A and SAT2) in Northern Sudan, active and passive disease surveillance were utilized and bovine sera positive to FMD anti-NSPs antibodies were serotyped to determine prevalence of serotype specific antibodies. The serological study involved serial testing approach i.e. only sera positive in the two test systems were considered positive. The approach is advantageous in decreasing test workload and raise specificity but decrease sensitivity. Of particular interest, is to examine its performance at the expected low levels of infection in Northern Sudan.

Objectives:

This study was designed to achieve the following objectives:

1. To monitor FMD infection in the whole area of Northern Sudan (the Northern and the River Nile States) between 2016 and 2018.
2. To perform clinical investigation, serotyping and genotyping of FMD outbreaks in Northern Sudan.
3. To determine FMD structural proteins (SPs) serology on cattle sera from Northern Sudan.
4. To expand the geographical and 90-time scale of the study of FMD infection in Northern Sudan to avoid biased impressions on disease situation and epidemiology.

Chapter I

Literature Review

1.1. Importance of foot-and-mouth disease (FMD):

Foot-and-mouth disease (FMD) is a severe, clinically acute, vesicular disease of cloven-hoofed animals including domesticated ruminants and pigs and more than 70 wildlife species (Thomson, 1994). The clinical disease is characterized by fever and development of vesicular lesions on the feet and the interdigital spaces, the mouth, snout, prepuce, vulva, and the mammary glands "udder and teats" in females. Fatalities may occur, especially in young animals (Alexandersen *et al*, 2003a; OIE Manual, 2021). FMD causes severe economic losses in susceptible animals. High morbidity, low mortality and complex epidemiology are hall marks of the infection.

1.2. History of the disease:

The first written description of FMD probably occurred in 1514 when a similar disease of cattle was described in Italy (Francastorius *et al*, 1546). Later, almost in 1897, Loeffler and Frosch (1897; 1898) demonstrated that a filterable infectious agent smaller than bacteria causes FMD what was considered by many as the first description of an animal viral disease.

1.3. Geographical distribution:

The seven FMDV serotypes are not uniformly distributed in the regions of the world where the disease still occurs (Knowles and Samuel, 2003). Serotype O shows the world widest distribution; SAT serotypes are unique to the African continent, mainly sub-Saharan Africa; serotype Asia1 confined mostly to Asia (Knowles and Samuel, 2003) and serotype C is perhaps the first extinct serotype (Paton *et al.*, 2021). Currently, North America, Central America, Australia, New Zealand, islands of Oceania and many parts of Europe are considered free of FMD (Samuel and Knowles, 2001; Sammin *et al*., 2007; Rweyemamu *et al*., 2008). In the last 15 years, Africa showed the incidence of five serotypes (O, A, SAT1, SAT2 and SAT3), in Asia three serotypes (O, A, Asia-1), and in South America only two serotypes (O and A) had been reported (http://www.wrlfmd.org/).

1.4. Etiology:

The etiological agent, foot-and-mouth disease virus (FMDV) is classified within the *Aphthovirs* genus as a member of the *Picornaviridae* family, being a non-enveloped, icosahedral virus, 26 nm in diameter, containing positive sense RNA of around 8.4 Kb (Belsham, 1993).

Seven distinct serotypes of FMDV, with indistinguishable clinical effects, have been defined, namely types O, A, C, Southern African Territories (SAT) 1-3, and Asia 1 (Murphy *et al.,* 1999). Recovery from infection, or protective vaccination, with one serotype will not protect against subsequent infection with another. Each serotype has been further subtyped on the basis of quantitative differences in cross protection and serological test. Moreover, within a serotype a wide range of strains may occur, some of which may be sufficiently divergent to reduce the efficacy of existing vaccines (Kitching *et al.,* 1989; Kitching, 1998).

1.4.1. Structure of FMD virus:

The virion is a 140s particle consisting of a single-stranded RNA genome and 60 copies each of four structural proteins (VP [ID), VP2 [IB], VP3 [IC), and VP4 [IA]) (Maclachlan and Dubovi, 2011). The FMDV genome has a basic organization similar to those of other members of the Picornaviridae family. The nomenclature of the viral proteins was established by Ruecket and Wimmer (1984).

By electron microscopy, the FMD virion appears to be a round particle with a smooth surface and diameter of about 25 nm (Bachrach, 1968). The fine structure of the viral capsid has been determined for a number of serotypes by virus x ray crystallographic techniques. Unlike those of other picornaviruses, the FMDV capsid is dissociated, at pH of below 6.5, into 125 pentameric subunits (Brown and Cartwright, 1961).

1.4.2. Virus multiplication:

FMDV, like other members of the *Picornaviridae* family, has a relatively short infectious cycle in cultured cells. According to the multiplicity of infection (MOI), newly formed infectious virions begin to appear at between 4 and 6 hours after infection (Grubman and Baxt, 2004). The virus is cytocidal causing cytopathic effects (CPE) characterized by cell rounding and inhibition of host translation and transcription (Maclachlan and Dubovi, 2011).

The virus multiplication cycle begins by attachment and adsorption. Foot-and-mouth disease virus, mainly, utilizes integrins and heparin sulphate molecules to attach to cells though other type of cell receptors are also suggested. The main mechanism of uncoating involves receptor-mediated endocytosis in which the virus capsids associate and traffic with endosomes to allow the low pH of the endosomes to trigger uncoating by dissociation of the capsid resulting in the release of the RNA genome (Grubman and Baxt, 2004; Ruiz-Sáenz *et al.,* 2009). Once the viral RNA is released into the cytoplasm of the host cell, it begins a round of viral translation before any transcriptional step. A single polypeptide, that undergoes a series of cleavages reactions (apart from a maturation cleavage) to produce structural and non-structural proteins, is synthesized (Gao *et al.,* 2016). Replication of the RNA is a function of the RNA-dependent RNA polymerase; earlier known as FMD virus infection associated antigen (FMD-VIAA). Transcription and replication of FMD viral RNA has not been well studied but it is likely similar to models of other picornaviruses and poliovirus (Mason *et al.,* 2003; Grubman and Baxt, 2004).

The final steps in the replication cycle are encapsidation and maturation. The so called maturation cleavage to form the mature virion occurs after packaging of RNA into a mature virion (encapsidation) (Grubman and Baxt, 2004; Gao *et al.,* 2016).

1.4.3. Genetic and antigenic variation:

The presence of seven serotypes and multiple and various subtypes has added to the difficulty of the laboratory diagnosis and control of FMD but facilitates tracing of FMDV strains isolated from outbreaks (Haydon *et al.,* 2001; Sobrino *et al.,* 2001; Knowles and Samuel, 2003). Mutational changes and the rise of new variants are inevitably caused by continued circulation of the virus in the field and the quasispecies nature of the RNA genome. The quasispecies concept was developed to explain the effects of errors in replication and on the evolution of replicating RNA molecules (Domingo *et al.,* 2003). In an initial work (Eigen, 1971; Eigen and Schuster, 1979), quasi-species were defined as stationary (equilibrium) mutant distributions of infinite size, centered around one or several master sequences (Domingo and Perales, 2019). In its simplest term, the concept envisions that within any population of different viral genome sequences, selection occurs at the population level rather than at the individual level. Thus, there is not a “wild type” as such but rather an observed “average”

phenotype which has adapted to and replicates "best" within any given environment. The environment can be in either tissue culture or particular host species, and in either situation, immunologic pressure or physical conditions such as temperature or pH are influential (Domingo *et al.,* 2003).

Antigenic variation in the field increases with time and most probably results from immunologic pressure placed on the virus by either the infected or vaccinated host species (Haydon *et al.,* 2001). Currently, neutralization reaction is used to assess this intratypic antigenic differences and define protection (Rweyemamu *et al.,* 1977; Rweyemamu, 1984) and in somewhat similar manner phylogenetic analysis of the VP1 gene is used to measure genetic diversity and specify epidemiological links (Samuel and Knowles, 2001; Knowles and Samuel, 2003). However, the impact of specific amino acid changes of the nucleic acid on antigenicity are not well determined and extrapolating between nucleotide differences and antigenic homology is not recommended (Paton *et al.,* 2005). The two-dimensional microneutralization test is used to quantify antigenic differences and define protection; values of r1 greater or equal to 0.3 indicate close antigenic relationship and likely cross protection while values less than 0.3 indicate poor antigenic relationship and unlikely cross protection (OIE Manual, 2021). The classification of new field isolates into subtypes (Brooksby, 1968) was abandoned in favor of a pragmatic approach where new strains are compared using r1 value with reference vaccine strains or established vaccine strains of commercial producers.

On the other hand, phylogenetic analysis of the VP1 gene results into grouping FMD viruses into genotypes (less than 15% nucleotide difference) that circulate into a geographical boundary and used the term topotype to describe them (Samuel and Knowles, 2001). Knowles and Samuel (2003) described 10 topotypes for serotype O, 8 for serotype C and one for serotype Asia1. Tosh *et al.* (2002) described 10 major genotypes (I-IX) of serotype A. Vosloo *et al.* (2004) classified serotype SAT1 into eight topotypes, SAT-2 into fourteen topotypes and serotype SAT-3 into six topotypes.

1.4.4. Survival of FMDV:

In general, most strains are strongly stable at pH 7.2-7.6, and at pH 7.0-8.5, especially at lower temperature, but increasingly labile at pH values outside that range (Bachrach *et al.,* 1957; Bachrach, 1968). The acidity produced in carcass meat during rigor mortis in

cattle will inactivate virus. Furthermore, the pH in bone marrow, lymph nodes and certain organs and offal does not decline during rigor mortis; virus can therefore be found in such material (especially if refrigerated or frozen) for an extended period of time, and may cause new outbreaks if feed to livestock as unheated waste food (Donaldson, 1987).

Foot-and-mouth disease virus is resistant to some detergents and organic solvents such as ether and chloroform (Donaldson, 1987). It is affected by sodium hypochlorite, potassium hypochlorite and sodium hydroxide (Calvarin and Gayot, 1978).

Airborne virus is stable at humidity above 55-60% and drying will inactivate most of the virus. Survival of the virus in an environment will depend on the nature of the material, the initial concentration of the virus in the material, the strain of the virus, the humidity, the pH and the temperature (Alexandersen *et al.,* 2003a).

1.5. The disease:

1.5.1. Host range of FMD:

The main domestic species susceptible to FMD are cattle, sheep, goats, water buffalo, and pigs (Thomson, 1994; Murphy *et al.,* 1999; Maclachlan and Dubovi, 2011).

Alongside these key domestic species, over 70 other species are known to be susceptible. Wildlife species including African buffalos, wild pigs, antelope and yaks (*Bos grunniens*) can become infected, although infections in many species are usually subclinical. Camelids show different susceptibility to FMDV (Wernery and Kinne, 2012). Bactrian camels (two-humped camels) (Larska *et al.,* 2009; OIE Manual, 2021) and the domesticated New World Camels (OIE Manual, 2021) [llama (*Lama glama*) and the alpaca (*Lama pacos*)] can contract the disease while dromedary camels are not (Wernery and Kinne, 2012). No information is available about the Wild New World Camels species [guanaco (*Lama guanacoe*) and vicuña (*Vicugna vicugna*)] (Wernery and Kinne, 2012).

1.5.2. Pathogenesis:

Most information about pathogenesis is available from studies on cattle and pigs. Comprehensive reviews of the subject are available (Alexandersen *et al.,* 2003a; Grubman and Baxt, 2004; Arzt *et al.,* 2011a; 2011b).

1.5.2.1. Routes of entry:

The most common mechanism of spread of FMD is by direct contact between infected and susceptible animals. Indirect contact also may occur and takes the form of mechanical transfer of the virus to susceptible animals. The virus gains entry through cuts or abrasions in the animal skin or mucosae, or by the deposition of droplets or droplet-nuclei (aerosols) in the respiratory tract of recipient animals (Alexandersen and Donaldson, 2002; Alexandersen *et al.,* 2002a). Ruminants are highly susceptible to infection via the respiratory tract while pigs are relatively resistant to infection by inhaled FMDV and are generally infected via the oral route (Alexandersen *et al.,* 2003a) Nonetheless, feeding cattle thorny materials (which penetrate the tissues of the mouth) and teat abrasions of dairy cows by milking machines predispose infection through mucosa and skin. Mechanical transfer of the virus from infected to susceptible animals may occur via contaminated personnel, vehicles and all classes of fomites (Alexandersen *et al.,* 2003a).

1.5.2.2. Minimum infective dose:

Sellers (1971) reviewed the minimum infective dose of FMDV and concluded that exposure to even high levels of the virus does not necessarily result in infection. The minimal infective dose of a particular virus strain required to infect a susceptible animal varies for the different animal species and for the different routes of infection.

Cattle injected in the tongue epithelium with only 1 IU may become infected, while a higher dose of 10-100 IU is required for aerosol exposure (Sellers, 1971; Sutmoller *et al.,* 2003).

Pigs need only a very small amount of FMDV (1-10 IU) when inoculated into the skin of the bulb of the heel to set up infection (Burrows, 1966), however, a pig would require 1000 or more IU to become infected by the intranasal route. Sheep requires 10000 IU by the intra-nasal and intra-tracheal routes for successful infection (Mc Vicar and Sutmoller, 1968).

1.5.2.3. Primary and secondary sites of virus multiplication:

When virus gains entry through damaged skin or mucosa in the inter-digital space or the tongue (the predilection sites) or when it is inoculated directly into the circulation, the primary site of virus multiplication is the predilection sites (tongue and epithelium of

the inter-digital space). Using RT-PCR, one day after infection, the virus was found to be 10 to 1000 times greater in the tongue and the inter-digital space than in other tissues and organs (Zhang and Alexandersen, 2004).

Lack of consensus ensues with respect to the primary replication site, when animals were infected through the respiratory tract. Some reports suggested the pharynx (nasopharynx) and not the lungs as the primary site of virus replication (Burrows *et al.,* 1981; Zhang and Kitching, 2001; Alexandersen *et al.,* 2003a; Stenfeldt *et al.,* 2016a). Brown *et al.* (1996) suggested the lungs as the primary site of virus replication. Other recent studies (Pacheco *et al.,* 2010; Arzt *et al.,* 2010) described a particular dynamic of infection in cattle respiratory tract characterized by increasing of the infectious viral titer in lung tissues and its decreasing in the pharyngeal tissues as viraemia approach. In cattle, generally, the virus spread from the primary site of virus multiplication through regional lymph nodes and enters the circulation to other organs and tissues before the onset of clinical signs. Viraemia lasts for 4 to 5 days. Further seeding of virus in the cornified epithelia of skin, tongue and mouth, and further virus amplification occurs (Hess *et al.,* 1960; Alexandersen *et al.,* 2001; Alexandersen *et al.,* 2003a; Sutmoller *et al.,* 2003). Alexandersen and Gloster (2004) emphasized the importance of cornified, stratified squamous epithelium of the tongue and skin for virus amplification. Alexandersen and Mowat (2005) explained that the particular significance of the pharyngeal region lies in its special non-cornified stratified squamous epithelia while most of the oral cavity is covered by cornified/keratinized (dead cell) stratified squamous epithelium. In comparison, apparently normal skin, hair and hairless, unlike lymph nodes, lymphocytes and macrophages were found to contain significant amount of the virus (Alexandersen *et al.,* 2001; Rigden *et al.,* 2002).

1.5.3. Incubation period:

The incubation period for FMD depends on the strain and dose of the virus, and the route of transmission. At the farm level, the incubation period is generally 2-14 days, but may be as short as 24 h especially in pigs and under very high challenge conditions. At the animal level, the typical incubation period is 2-6 days, although, as mentioned above, under certain condition it may be as short as 1 day or as long as 14 days (Alexandersen *et al.,* 2003b; 2003c).

1.5.4. Clinical signs and post-mortem lesions:

FMD is characterized by an acute febrile reaction and the formation of vesicles in and around the mouth and on the feet. Pain causes lameness, foot "flicking", a tucked up stance (stuck posture) and reluctance to stand or walk, as well as inappetence (Alexandersen *et al.,* 2003a). Heat and pain may be detected in the feet for 1-2 days before vesicular lesions appear. Lesions appear first as blanched areas, which subsequently developed into vesicles. Vesicles may be seen on the snout or muzzle, teats, mammary gland, prepuce, vulva and other sites of the skin but most consistently, however, in and around the mouth and on the feet (Kitching 2002; OIE Manual, 2021). At postmortem examination lesions may also be seen on the ruminal pillars. Lameness, especially in sheep, may not be a consistent finding in all animals (Alexandersen *et al.,* 2003a). However, careful clinical inspection including examination of the feet is indicated in an infected flock of sheep (Hughes *et al.,* 2002). Only foot lesions were seen in infected sheep following experimental infection with the UK 2001 strain of FMDV (Alexandersen *et al.,* 2002a). In another experiment, Hughes *et al.* (2002) reported that 93% of the lesions detected in 79 experimentally infected sheep were on the feet.

Clinical FMD is usually described as severe in pigs, obvious in cattle, and blurred in sheep and goats. In pigs, the early signs include acute lameness, reluctance to stand, adaption of a dog-sitting posture, depression, loss of appetite and fever. In cattle, the obvious clinical signs include the drooling of saliva and mouth lesions that are often typical and rather sever, and sometimes lesions of the feet. In small ruminants experimental investigations have shown that FMD may be clinically inapparent in a significant proportion of animals (Kitching, 2002; Kitching and Hughes, 2002; Donaldson and Sellers, 2007; OIE Manual, 2021).

Mortality in adult animals is generally low, but it may be high in young animals, including calves and especially lambs and piglets, due to acute myocarditis (Alexandersen *et al.,* 2003a). FMD may also cause abortion in pregnant animals; however, the precipitating factors for the various livestock species have not been determined (Murphy *et al.,* 1999).

1.6. Epidemiology:

1.6.1. Transmission:

Transmission most commonly occurs by direct contact (Thomson, 1994; Sutmoller *et al*., 2003), although the virus can be mechanically disseminated by animals, animal products, farmers, farming equipment, and during animal transport (Sobrino *et al*., 2001; Saiz *et al*., 2002). Contact transmission between susceptible animals may occur during transport, in markets, shows and fairs (Donaldson, 1979).

1.6.1.1. Transmission by the oral route:

Several outbreaks have been linked to feeding animals virus contaminated material. For example, the South Africa 2000 and UK 2001 epidemics have been attributed to the feeding of unheated waste food to pigs, and the Japan 2000 epidemic to the feeding of contaminated fodder (Knowles *et al*., 2001). It should be noted that animals are relatively insensitive to experimental infection by the oral route for example the dose for pigs being about 10^4-10^5 $TCID_{50}$ (Sellers, 1971). However, animals with abrasions of the epithelium in and around the mouth may be infected by smaller doses (Donaldson, 1987). Sharp objects, such as pieces of bone, may therefore facilitate infection by contaminated waste food (Alexandersen *et al*, 2003a).

1.6.1.2. Airborne transmission:

Airborne transmission requires favorable topographical and meteorological conditions (Alexandersen *et al.,* 2003a). It is especially significant when pigs are the source of infection, because they liberate the largest quantities of airborne virus (Donaldson and Ferris, 1980; Donaldson *et al.,* 1982; Alexandersen and Donaldson, 2002), meanwhile, ruminants are highly susceptible to infection by respiratory rout (Gibson and Donaldson, 1986; Donaldson and Alexandersen, 2001). Accordingly, likely pattern of airborne FMD spread is from pigs to cattle and sheep. Favorable conditions for long-range airborne transmission include cold weather (Donaldson, 1972), relative humidity 55% or more, minimal mixing of air by turbulence (Alexandersen *et al.*, 2003a), flat terrain and low precipitation (Sorensen *et al.*, 2000; 2001). Consequently, this mode of transmission is uncommon in temperate zones and extremely unlikely in hot and arid conditions (Paton *et al.*, 2018). In addition, FMD isolates differ widely in their ability to

spread via air. Type (O) UK 2001 strain, was unlikely to spread more than 20 Km by the wind (Alexandersen and Donaldson, 2002; Donaldson and Alexandersen, 2002), while C Noville strain has the potential to spread up to about 300 Km by the wind (Sorensen *et al.,* 2000; 2001).

1.6.1.3. Role of wild-life in transmission:

Although FMD can infect wide variety of wildlife, the risk of spread of the infection by wildlife in domestic stock is controversial (Thomson *et al.,* 2003). In general, transmission of FMDV among animals of different species requires further studies (Fukai *et al.,* 2020). Wildlife, apart from African buffalo, has not been shown to be able to maintain FMD viruses independently for more than few months (OIE Manual, 2021). Currently, transmission from African buffalo is considered to occur rarely, but can be of great significance (Tenzin *et al.,* 2008; Nelson *et al.,* 2017; Paton *et al.,* 2018).

1.6.1.4. Dissemination by people:

Any person whom had been exposed to FMD infected animals might become a source of infection, and had to stay away from ruminants for 3-5 days (Sutmoller *et al*., 2003). Exceptionally and for a short period, FMDV can be carried in the nose and throat of human being which could be transmitted to animals (Sellers *et al*., 1971). Nasal swab of human who had been in contact with diseased animals proved to contain 100-10,000 IU (Alexandersen *et al.,* 2003a). Shower and changing cloths reduced the amount of the virus from nasal swab by 100-folds. Moreover, wearing surgical or industrial gauze and cotton wool masks reduced the amount of the virus by nearly ten-fold, but paper masks had no effects (Sellers *et al.,* 1970). In the last 10-12 year, movement of people and commodities were often blamed for introduction of FMDV in Japan in 2010 (Muroga *et al.,* 2012) and some other countries in South-East Asia in 2015 (Qiu *et al.,* 2017).

1.6.2. Carrier state (persistent infection state):

Van Bekkum *et al*. (1959a; 1959b) showed the continuing presence of infectious virus in the oesophageal pharyngeal [OP] fluid of a proportion of convalescent cattle for many weeks after infection. In general, this is found to be true for all the seven serotypes of FMDV (Thomson, 1996).

The infectivity titre of the virus in OP samples from carriers is usually low 10-100 $TCID_{50}$/ml, excretion is also intermittent and the titre declines over time (Rossi *et al.,* 1988; Donaldson and Kitching, 1989; Alexandersen *et al.,* 2003a). Both the animal species and strain of the virus appear to be determinants in the development and persistence of the carrier state (Barnett and Cox, 1999; Alexandersen *et al.,* 2003a).

1.6.2.1. Definition of carrier state:

Carrier animals are defined as those from which live-virus can be isolated from oesophageal-pharyngeal region for more than four weeks after infection (Sutmoller *et al.,* 1968; Salt, 1993; OIE Manual, 2021). Bronsvoort *et al.* (2016) observed that though this definition has been a useful construct for experimental designs but it fail to capture the dynamics of either persistence of the virus or the uncertainty in transmission from such animals.

1.6.2.2. Mechanism of persistent infection:

The mechanisms involved in virus persistence within carrier ruminants are not fully understood. Determinants of FMDV carrier state are generally thought of as factors related to hosts' immunological responses or intrinsic viral factors that permit the virus to evade the immune response and persist instead of get cleared. Accumulating evidences suggested that they are mainly host rather than viral factors (Stenfeldt and Arzt, 2020). For host factors, a number of studies suggested determinants that fall into the general categories of innate factors, humoral immunity, and cell-mediated immunity (Stenfeldt and Arzt, 2020). At the cellular level, transcriptomics/host transcriptome analysis of tissue samples (the study of the transcriptome is the study of complete set of RNA transcripts that are produced by the genome, under specific circumstances or in a specific cell using high-throughput methods, such as microarray analysis) reported significant negative correlations between detected quantities of FMDV RNA and some antiviral host factors (IFN-ω -λ CXCL10, and IRF-7 mRNA) in samples of micro-dissected nasopharyngeal epithelium of FMDV carriers (Stenfeldt *et al.,* 2016a). At the level of the host, Bronsvoort *et al.* (2016) found that younger animals are more likely to be carriers.

For viral factors, no specific mutations in the virus genome were found to be consistently associated with carrier state (Parthiban *et al.,* 2015; Arzt *et al.,* 2019). Also,

though some field studies have confirmed that changes to the FMDV genome, potentially affecting viral antigenicity, continued to occur during persistent infection (Bertram *et al.,* 2018; Biswal *et al.,* 2019); no evidence of such processes leading to the emergence of novel viral lineages that having contributory effects on the establishment and maintenance of FMDV persistence (Cortey *et al.,* 2019). On the other hand, co-infection studies in African buffalo identified infectious virus and viral genomes in lymphoid tissues of the head and neck, mainly in germinal centres, and found correlation between persistence and in vitro cell-killing capacity of different virus isolates (Maree *et al.,* 2016). These results concluded that FMDV persistence occurs in the germinal centers of lymphoid tissue and that the duration of persistence is related to virus replication and cell-killing capacity.

1.6.2.3. Carrier state in different animal species:

In African buffalo (*Syncerus caffer*), it was found that SAT-type viruses can persist in an individual animal for up to 5 years and within a herd for 24 years or longer (Hedger and Condy, 1985; OIE Manual, 2021). In the Kruger National Park, one of the largest game reserve in Africa and the world, in South Africa, more than 98% of buffaloes develop antibodies to all three SAT serotypes by the age of 2 years (Thomson *et al.,* 1992; Keet *et al.,* 1996). The individual buffalo may be persistently infected with more than one SAT serotype (Hedger, 1972; Anderson *et al.,* 1979) but SAT1 is the serotype most frequently isolated (Maree *et al.,* 2016).

Around 15-50% of infected cattle can become carriers for 3.5 years (Alexandersen *et al.,* 2002b; 2003b). In general, sheep and goats are less frequently become carriers and often for shorter periods than cattle (Burrows, 1968; McVicar and Sutmoller, 1969). The maximum reported duration of carrier state in sheep and goats is 9 and 4 months, respectively (Alexandersen *et al.,* 2003b). Pigs usually clear FMD infection in 3-4 weeks and so do not become carriers (Alexabdersen *et al.,* 2002b). Though FMDV RNA (but not live virus) was detected in pigs after 28 days from infection (Orsel *et al.,* 2008; Zhang and Bashiruddin, 2009), a recent study (Stenfeldt *et al.,* 2016b) stressed that pigs are not carriers of FMDV infection.

Viral persistence in antelope has been reported in the kudu (*Tragelaphus strepticeros*) and in sable antelope (*Hippotragus niger*) (Ferris *et al.,* 1989). Other cloven-hoofed wildlife species including deer and impala, which may become acutely infected, are

either do not become carriers or do so for only a relatively short period constituting no significant carrier problem (Thomson, 1997; Bastos *et al.*, 2000).

1.6.2.4. Significance of carrier state:

The role of carrier animals in the spread of FMDV in the field was described as controversial (Grubman and Baxt, 2004) and remains so to date. No unequivocal evidence of transmission from carrier animals, apart from African buffalo to susceptible host has been demonstrated; neither experimentally nor in the field. Transmission of SAT-type viruses from persistently infected African buffalo to cattle under experimental and natural conditions has been unequivocally demonstrated (Dawe *et al.*, 1994a; 1994b; Vosloo *et al.*, 1996; Bastos *et al.*, 2000). Nevertheless, old (Condy and Hedger, 1974) and recent (Maree *et al.*, 2016) attempts to effect transmission from carrier buffaloes have also failed.

1.7. Diagnosis:

1.7.1. Clinical diagnosis:

FMD might be diagnosed initially on observation of clinical signs with or without a history of contact of infected herd or evidence of FMD in the area (Kitching, 2002)
Field diagnosis of FMD may be confounded by similar livestock viral diseases, by mild clinical signs in enzootic areas and fall short of serotyping the disease incidence. Differential diagnosis of FMD may include bovine popular stomatitis, bovine herpes mammilitis, infectious bovine rhinotracheitis, bovine mucosal disease, malignant catarrhal fever, bluetongue, parapox-virus, peste des petits ruminants and foot-root in sheep. Laboratory confirmation must be undertaken and is recommended (Remond *et al.*, 2002; OIE, 2021).

1.7.2. Laboratory diagnosis:

1.7.2.1. Samples required for diagnosis:

Laboratory diagnosis of FMD depends on virus isolation or demonstration of viral antigen or nucleic acid in samples of morbid tissues or fluids. Diagnosis based on serological response could be performed by detection of virus specific-antibodies in disease-free areas and in the absence of vaccination (OIE Manual, 2021). The sample of

choice is the epithelium or vesicular fluid collected from an unruptured or recently ruptured vesicles from the tongue, buccal mucosa or feet (Shaw *et al.,* 2004; OIE Manual, 2021). Samples should be obtained from recent cases showing typical signs of the disease. At least two animals should be sampled. Enough material "equivalent to 1 gram" of the epithelium tissues should be collected (OIE Manual, 2021). From dead animals, samples from the heart, lymph nodes and thyroid may be collected (Maclachlan and Dubovi, 2011). OP fluid from the proximal part of the oesophagus and pharynx should be collected in absence of epithelium tissue (OIE Manual, 2021).

1.7.2.2. Virus isolation and identification:

1.7.2.2.1. Virus isolation:

Virus isolation is considered more sensitive than ELISA for antigen detection. FMDV can be isolated by inoculation into cell culture (primary bovine thyroid cells and primary pig, calf or lamb kidney cells, cell lines such as (baby hamster kidney) BHK's and IB-RS-2 may also be used but are generally less sensitive for detecting low amounts of the virus (OIE Manual, 2021). Cells are examined for cytopathic effect (CPE) after 24 hours, primary bovine thyroid cell after 48 h (Alexandersen *et al.,* 2003a).

Depending upon the amount of the virus present, two passages of 48 h each test inoculums may be required before a final result can be declared (Clavijo and Kitching, 2003; Alexandersen *et al.,* 2003a).

1.7.2.2.2. Antigen detection ELISA:

ELISA is the preferred procedure for the detection of FMD viral antigen and identification of the viral serotype (Roeder and Le Blanc Smith, 1987; Ferris and Donaldson, 1992; OIE Manual, 2021). It is performed on epithelial suspension and sometimes on tissue culture supernatants showing cytopathic effect (Alexandersen *et al.,* 2000; Alexandersen *et al.,* 2003a). ELISA has replaced complement fixation (CF) in most laboratories (OIE Manual, 2021).

ELISA results can be obtained in 3-4 h after the sample is received by the laboratory (Grubman and Baxt, 2004). If the sample is inadequate or the diagnosis remains uncertain, sample material can be tested by RT-PCR and/or virus isolation using susceptible cell cultures or 2-7 day old unweaned mice. Once amplification or

cytopathic effect (CPE) is completed, material can be retested using ELISA (OIE Manual, 2021).

1.7.2.2.3. Reverse-transcription polymerase chain reaction (RT-PCR):

It is particularly suitable for the direct examination of OP samples and serum. Agarose gel-based RT-PCR (Reid *et al.*, 2000) or real time RT-PCR (Reid *et al.*, 2003) are both described in the OIE Manual (2021). It is generally used in parallel with conventional assays (Marquardt *et al.*, 1995; Callens and De Clercq, 1997; Callens *et al.*, 1998). Real-time RT-PCR is as sensitive as virus isolation and in addition liable to automated procedures (Reid *et al.*, 2003). Simplified RT-PCR systems for potential field-use are under development (Callahan *et al.*, 2002).

Molecular diagnosis permits tracing of FMD outbreaks with accuracy hitherto unknown. Polymerase chain reaction and nucleotide sequencing of the VP1 region (one of the three major capsid-coding genes) disclosed the origin of serotype O and A viruses in Europe over 20 years period (Beck and Strohmaier, 1987) and traced origins of FMD outbreaks on a global scale (Samuel and Knowles, 2001). Polymerase chain reaction and nucleotide sequencing of the whole genome [(near) full-length FMDV genome sequence (NGS)] provide enough resolution for within-epidemic tracing (Cottam *et al.*, 2008a; 2008b)

1.7.2.3. Serological diagnosis:

Serological tests for FMD are either detecting antibodies to structural proteins (SPs) or to non-structural proteins (NSPs) of FMDV. The SPs tests are serotype-specific and detect antibodies elicited by infection or vaccination while the NSPs tests are not serotype-specific and detect antibodies elicited by infection (OIE, 2021). Repeated vaccination with non-purified FMD vaccines has been reported to induce antibodies to NSPs (Pinto and Garland, 1979). However, NSPs tests are essentially developed for the main purpose of the so called differentiation of infection from vaccination (DIV) (Berger *et al.*, 1990; Bergmann *et al.*, 1993; De Digo *et al.*, 1997; Sørensen *et al.*, 1998). Currently, reactivity to NSPs is largely acceptable as an indication of the degree of circulation of FMDV in animal herds. In general terms, serological tests for FMD are applied to confirm suspected cases, to substantiate absence of infection, to certify

individual animals prior to import or export and to evaluate the immune response following vaccination (OIE, 2021).

Examples of the SPs tests are VNT (Golding *et al*., 1976), the liquid-phase blocking ELISA (LPBE) (Hamblin *et al*., 1986) and the solid-phase competition ELISA (SPCE) (Brocchi *et al*., 1990; Mackay *et al*., 2001; Paiba *et al*., 2004). The NSPs tests include the OIE index screening method used at Panaftosa (Brocchi *et al*., 2006; OIE, 2021) and commercial kits, validated for detection of antibodies against FMDV NSPs in different animal species, like Chekit, Priocheck NS ELISAs (Brocchi *et al*., 2006) and the ID Screen® FMD NSP Competition ELISA (Roche *et al.*, 2014).

1.8. Control of FMD:

Foot-and-mouth disease is expensive to control and eradicate (Alexandersen *et al.*, 2003b). It is notifiable disease in most countries and must be reported immediately to the appropriate government authority (Murphy *et al.*, 1999). Countries that are free of FMD usually employ control policies (stamping out) including slaughter of all clinically infected and in-contact susceptible animals together with inhibition of animal movement (Kitching, 2002; Grubman and Baxt, 2004). Disease-free countries also, to retain this status, adopt restrictions on importation of susceptible livestock and animal products from countries where FMD was present (Bachrach, 1968). Due to frequent introduction in disease-free countries and the high cost of the stamping out policy, the later gave way to a vaccinate-to-live policy (Sutmoller *et al.*, 2003).

In endemic regions vaccination, restriction of animal movement and biosecurity measures are logical approaches for control (Pereira, 1978; Kitching, 2002). Biosecurity measures include safely disposal of cadavers and through cleaning and disinfection of contaminated premises, trucks and other equipment (Sutmoller *et al.*, 2003). Any persons who had contact with infected animals or carcasses must take strict bio-safety measures and avoid contact with susceptible animals for at least 3-5 days. Protective clothes and gloves must be worn handling contaminated materials, particularly infected animals and cadavers (Sutmoller *et al.*, 2003).

1.8.1. FMD vaccines:

FMD vaccines contain defined amounts of one or more chemically inactivated cell-culture-derived preparations of a seed virus strain blended with a suitable adjuvants and

excipients (Kitching and Hutber, 2002). Oil adjuvants are preferred in swine, but can also be used in ruminants, and may have advantages from less interference with maternal antibody and longer duration of immunity. FMD vaccines may be classified as either 'standard' or 'higher' potency vaccines (Sutmoller *et al*, 2003; Hutber *et al*, 2011).

Live-attenuated vaccines are not acceptable due to danger of reversion to virulence (Gurbman and Baxt, 2004; OIE Manual, 2021). Inactivated vaccine have been extremely successful, however, they have a number of concerns and limitations (Grubman and Baxt, 2004). The current FMD vaccine is an inactivated whole-virus preparation formulated with adjuvant prior to use in the field. Because of the presence of multi-serotype of the virus, it is common to prepare vaccines from many different serotypes (OIE Manual, 2021).

1.9. Economic importance of FMD:

FMD has great potential for causing severe economic loss in susceptible cloven-hoofed animals (OIE Manual, 2021). Direct losses include decrease of milk production, weight loss, mortality in young animals and loss of draught power (Gribman and Baxt, 2004). The loss of milk production extend to the whole of the lactation period and mastitis caused by FMD result in a permanent loss of more than 25% of milk production (Murphy *et al* ,1999). The disease losses also include abortion of pregnant animals (Alexandersen *et al.*, 2003a), and yearling cattle may fail to fully recover their production potential (Kitching, 1992). Indirect losses include cost of control and its drastic effects on trade (Barnett and Cox, 1999; Mahul and Durand, 2000).

FMD is considered as the major constraint to international trade in livestock and animal products (Alexandersen *et al.*, 2002b) and a curse haunting the livestock industry around the world (Randolph *et al.*, 2002). The Office International des Epizooties (OIE) has recognized FMD as the most important constraint to international trade in animals and animal by products (Leforban, 1999).

The direct and indirect losses of the 2001 UK FMD outbreaks were estimated to have cost £8 billion (Thompson *et al.*, 2002; Alexandersen *et al.*, 2003b). In the Netherlands, the economic damage was estimated to be around 1 billion Euros (Huirne *et al.*, 2002). The direct cost for controlling FMD in Taipei China in 1997-1998 was estimated at approximately USS400 million, while the direct cost was about USS1.6 billion per year

(Yang *et al.*, 1999) and the overall economic impact was estimated to be as high as USS6.9 billion (Wilson and Tuszynski, 1997).

1.10. FMD in Sudan:

Historically, four serotypes; A, O, SAT1 and SAT2; of FMDV had been isolated from cattle in Sudan (Abu Elzein, 1983). The first record of clinical FMD in the country was in 1903 (Eisa and Rweyemamu, 1977; Abu Elzein, 1983), of serotype O was in 1952, of serotype SAT1 was in 1952, of serotype A was in 1957 (http://www.wrlfmd.org/) and of serotype SAT2 was in 1977 (Abu Elzein and Crowther, 1979).

Sudan is part of East Africa which is enclosed entirely in pool 4 of FMDV; one, and perhaps the most active, of the 3 FMDV pools that cover the African continent (Paton *et al.*, 2009) Serotypes, topotypes and variants circulating in pool 4 are expected to be circulating in the country (Habiela *et al.*, 2010a; Raouf *et al.*, 2022). Nevertheless, of the 4 serotypes (O, A, SAT1 and SAT2) historically known in Sudan and frequently reported in Africa, serotype SAT1 has not been reported in the country since 1976 (Raouf *et al.*, 2009; 2010; 2016; 2017; 2022; Habiela *et al.*, 2010a; 2010b; http://www.wrlfmd.org/).

Of the massive and diverse population of domestic and wild livestock, clinical FMD was confirmed in one species only; cattle (Abu Elzein, 1987; Habiela *et al.*, 2009; 2010a; 2010b; Raouf *et al.*, 2010; 2022; http://www.wrlfmd.org/). Sheep and goats undergoes silent infection and are expected to play an insignificant role in the epidemiology of the disease in Sudan (Habiela *et al.*, 2010a; Raouf *et al.*, 2012; 2017; Raouf, 2020) whereas camels are free of infection (Habiela *et al.*, 2010a).

Serotype O was the most predominant and most widespread, followed by A then SAT2 (Abu Elzein, 1987; Raouf *et al.*, 2016). All Sudanese serotype O viruses were of one topotype; East Africa-3 topotype (EA-3). Sudanese serotype A viruses were also of one topotype, toptype Africa, but Sudanese SAT2 isolates were of two topotypes, XIII and VII (Habiela *et al.*, 2010a; Raouf *et al.*, 2022; http://www.wrlfmd.org/). The geographical distribution of FMD was described as penetrating along the Nile basin up to Khartoum state, particularly of serotype O and A, but more favorable in Western, Eastern and Northern Sudan (Raouf *et al.*, 2016; Department of FMD Report, 2016; Saeed and Raouf, 2020; Alfouz *et al.*, 2021).

A working hypothesis of how FMDV is introduced and circulated in Sudan is emerging. Serotype O circulates intensely along the Nile basin and spread from there to Western, Eastern and Northern Sudan (Raouf *et al.,* 2016). Epidemiological links with O isolates from East Africa, particularly Ethiopia, was also highlighted (Raouf *et al.,* 2022). Circulation of serotype A across the Eastern border from Wad El Helew in Kassala to Aljabalein in the White Nile state is perhaps more significant or as important as its circulation along the Nile basin (Raouf *et al.,* 2016; Raouf *et al.,* 2022) while across border circulation of serotype SAT2 was perhaps the most important mechanism for maintenance of SAT2 infection in Sudan (Raouf *et al.,* 2016).

Chapter II
Materials and Methods

2.1. Materials:

2.1.1. Viruses:

Three serotypes of FMDV were used throughout this work; O, A and SAT2. All of them were isolated locally at the department of FMD-CVRL in BK or BHK cells from FMD events in cattle. Disease events had been typed using reference antigen detection ELISAs (Pirbright and IZSLER). All viral materials were adapted (through 16-22 passages) to grow in BHK cells, typed and retyped several times using reference ELISAs. They were designated according to their serotype, geographical origin within Sudan, year and order of isolation from that origin. Four isolates were used in this work; two of serotype SAT2 isolated from Khartoum in 2008 (SAT2-Kh 1/08) (Raouf *et al.,* 2010), and from North Kordofan in 2010 (SAT2-NK 1/010) (Department of FMD Report, 2010), one of serotype A isolated from Khartoum in 2011 (A-Kh 2/011) **(**Raouf *et al.,* 2016) and one of serotype O isolated from Khartoum in 2015 (O-Kh 1/015) (Department of FMD Report, 2015). At the commencement of the study, all viral materials were retyped using IZSLER antigen detection ELISA (Grazioli *et al.,* 2012).

2.1.2. Control sera:

Control sera were known positive bovine field sera for either O, A and SAT2 serotypes (Raouf *et al.,* 2016) and fetal calf serum (FCS) (Sigma) free from antibodies against FMDV was used as the negative control serum.

2.1.3. Samples:

2.1.3.1. Serum samples:

Serum samples were discriminated as positive or negative to anti-NSPs antibodies of FMDV using the ID Screen® FMD NSP Competition ELISA (Roche *et al.,* 2014) during the programme STSD (Department of FMD Report, 2016). A total of 184 bovine sera have proven positive to anti-NSPs antibodies of FMDV constituted the serum samples of the study. Of these, 41 sera have originated from the Northern State and 143 from the River Nile State. These samples had originated from 343 and 409 serum samples

collected in 2016 from the Northern and the River Nile States, during the programme STSD. Serum samples were collected from apparently healthy cattle, one year old or above with no history of vaccination against FMD (Department of FMD Report, 2017). Relevant data of NSPs serology and the origin of positive sera within each State were shown in Table 1.

2.1.3.2. Tissue samples:

Between 2016 and 2018, suspicion of FMD had arisen at first by the end of 2016 (November and December). Vesicular lesions were seen in cattle smuggled to Egypt and in resident cattle in Dongola district in the Northern State. The smuggled cattle were in confinement by the Border Control Department at Dongola. The veterinary authority was notified.

Tissue samples included eleven tongue epithelium tissues were collected in December 2016 from suspected FMD events in the Northern State. Six epithelium samples were collected from resident cattle and five from the smuggled animals. Epithelium samples were collected in viral transport medium composed of equal amounts of glycerol and 0.04 M phosphate buffer, 0.001% phenol red, antibiotics and antimycotics (pH 7.2-7.6). Samples were collected by Dongola regional laboratory and transferred refrigerated to the Department of FMD at CVRL, Soba, Khartoum, and they were kept at –20ºC.

Table 1. Numbers and origin of anti-NSPs positive sera.

States data of NSPs serology				Districts data of NSPs serology				
State	No.* of sera tested	No of positive sera	Sero-prevalence	District	No.** of sera tested	No.** of positive sera	Sero-prevalence	No.***of sera tested by VNT
Northern State	343	53	15.45%	Marawi	66	17	25.76%	15
				Dongola	65	21	32.31%	16
				Al-Dabbah	70	8	11.43%	6
				Al Goled	64	0	Nil	-
				Al Burgaig	63	5	7.94%	4
Totals					328	51		41
River Nile State	409	161	39.36%	Shendi and El Matamma	137	58	42.34%	53
				Ed-Damar	69	31	44.93%	27
				Atbara	68	31	45.59%	28
				Berber	67	22	32.84%	20
				Abu Hamad	68	19	27.94%	15
Totals					409	161		143

*Out of 350 collected sera in the Northern State 7 sera were lost.

**Fifteen sera from the Northern State were with unidentified district origin including two +ve sera to NSPs serology.

***10 (Northern State) and 18 (River Nile State) sera +ve for NSPs serology were lost before performing the VNT.

2.1.4. Cells, cell culture media and reagents:

2.1.4.1. Cells:

Baby Hamster Kidney-21 (BHK-21) clone 13 originated from Foot-and-Mouth Disease Research Institute (ŞAP Enstitüsü Müdürlüğü), Ankara, Turkey was used throughout the study.

2.1.4.2. Cell culture media and reagents:

Medium	Company
Glasgow Minimum Essential Medium (GMEM)	Sigma Aldrich, United Kingdom
Fetal Calf Serum (FCS)	Sigma Aldrich, USA
Trypsin 1:250	GIBCO
Versene (EDTA)	Sigma Aldrich, USA

2.1.5. Antibiotics and Antimycotics:

Name	Company
Benzylpenicillin Sodium	NCPC, China
Streptomycin Sulphate	NCPC, China
Amphotericin B solution	Sigma Aldrich, USA
Gentamycin	J.B. Chemicals & Pharmaceuticals Ltd., India

2.1.6. Chemicals:

Chemical Name	Company
Sodium Chloride	Sigma Aldrich, USA
Sodium Hydroxide	Applichem Biochemica
Di-Sodium Hydrogen Phosphate-anhydrous	Sigma Aldrich, USA
Potassium Chloride	Applichem Biochemica
Sodium Dihydrogen Phosphate Monohydrate	Sigma Aldrich, USA
Tris	Sigma Aldrich, USA
Sodium bicarbonate	Sigma Aldrich, USA
Tryptosephosphate broth	Sigma Aldrich, USA
Formalin	Sigma Aldrich, Germany

Crystal Violate ($C_{25}H_{30}CIN_3$)	Riedel-Dehaënag Seelze, Germany
HCl	Surechem Products Ltd., England
Disinfectants:	
Dettol	-
70% Ethanol	-

2.1.7. Equipment and apparatus:

Name	**Company**
Laminar Flow Cabinet class II	Labcaire, SC-R, UK
Inverted microscope (Olympus CK×31)	Krüss, Germany
BIO-TEK (ELISA reader)	BIO-TEK Instrument, INC. USA
Water bath for heat inactivation (56°C)	Lauda, Germany
Mini orbital shaker	Stuart®, UK
Multichannel micropipette	Socorex, Swiss made
Single channel micropipette	Socorex, Swiss made
Vortex mixer	Appleon Woods, UK
Incubator	Scott Science, UK
Autoclave	Vertical Pressure Steam Sterilizer, UK
Oven	Scott Science, UK
Sensitive Balance	Ohaus Corporation, USA
Thermo Centrifuge	Thermo Electron LED GmbH, Germany
pH meter Jenway 3510	Bibby Scientific Ltd., UK
Refrigerator (+1°C and +8°C)	Liebherr & LG
Deep Freezer (-20°C)	Liebherr

2.1.8. Disposables, glassware and plastic ware:

Name	**Company**
Eppendorf tubes	-
Tissue culture Micro-plates 96-well	Corning Incorporation, USA

Tips (Yellow, White)	-
Plastic reservoir sterilized	-
Seals sterile, loose fitting lids (adhesive tape) approx 18x133 mm	-
Glass Pipettes	-
Glass Bottles	-
Glass Beakers	-
Cotton	-

2.1.9. ELISA kit for FMD antigen detection and serotyping (FMDV O, A, C, Asia1, SAT1, SAT2) (Brescia, ltaly):

2.1.9.1. Kit components:

1. ELISA Microplates, ready to use, pre-coated with anti-FMDV monoclonal antibodies (MAbs); type-specific MAbs and a pan-FMDV MAb (catching antibodies), and with positive inactivated and negative controls.
2. Conjugate A: apan-FMDV MAb for detection of serotype O, A, C, Asia1 (a detector conjugate) conjugated with horseradish peroxidase (HPRO).
3. Conjugate B: pool of SAT1 and SAT2 (MAbs) for detection of these two SATs serotypes conjugated with HPRO (a detector conjugate).
4. ELISA diluent buffer for samples and conjugate, ready to use.
5. Washing solution (PBS-Tween): 10X concentrated.
6. Substrate/Chromogen solution (TMB/ Tetramethylbenzidine), ready to use.
7. Stop solution (H_2SO_4-0.6N) ready to use.

2.2. Methods:

2.2.1. Study area:

The study area falls between 16-22 ºN and 22-32 ºE and include two States: the Northern and the River Nile States (Figure 1). It covers an area of around 458,697 Km^2 of a desert and semi-desert traversed by the River Nile. Of particular significance, that it is part of the projected disease-free area and constitutes nearly the whole of Sudan Northern border with Egypt. It represents the whole of Northern Sudan and the Nile valley North to Khartoum. Northern Sudan is distinguished from other geographical

clusters in Sudan; Eastern, Western and the rest of the Nile valley by an exclusive desert and semi-desert ecosystem, low animal density and limited animal movement. Animal density usually reaches 5 cattle/sq km in the desert and semi-desert ecosystem (FAO, 2005) but it is higher beside the River Nile and irrigation canals.

The prevailing animal production systems are the urban and the peri-urban production systems. No or very little pastoralism is practiced in Northern Sudan and animal movement is limited to that related to trade. Recently, the River Nile state is crossed by a national road from Central Sudan to the country seaport, Port-Sudan, which intensified livestock movement related to international trade. Foot-and-mouth disease susceptible species ranges from 2,429,144 in the River Nile State (105,148 head of cattle and 2,323,996 head of small ruminants) to 2,473,964 in the Northern State (262,871 head of cattle and 2,211,093 head of small ruminants) according to the Data Centre of the Ministry of Animal Resources, Sudan. Reared cattle are usually cross breeds or milking cows of local breeds (Butana and Kenana). In geography, apart from Northern Sudan which forms one cluster, three geographical clusters include Western, Eastern and the South Eastern cluster (Figure 1), were described in Sudan (Raouf *et al.*, 2016). Northern Sudan cluster includes the Nile valley North to Khartoum enclosed in two administrative States, the River Nile and the Northern States. Northern Sudan is distinguished by an exclusive desert and semi-desert ecosystem unlike all other three clusters which are traversed by the low rainfall savannah belt. The geographical distribution of FMD was described as penetrating along the South Eastern cluster up to Khartoum State but less prevailing in Eastern, Western and Northern Sudan (Raouf *et al.,* 2016). The Southern regions of the Nile Valley together with Western and Eastern Sudan are mainly animal breeding areas while Central and Northern parts of the Nile valley are animal marketing or trade routes areas. In general, the relatively lower levels of FMD infection are important and encouraging for control efforts, yet FMD infection in Northern Sudan, in particular, though low, could be crucial for virus spill from the country. Northern Sudan is part of a projected disease-free area broadly demarcated by the government of Sudan since 1970s. Additionally, Northern Sudan involves almost the whole border area with Egypt where cross-border trade of livestock through official and unofficial channels is known. Northern Sudan with the River Nile crossing it to Southern Egypt is a rare junction between sub-Saharan and North Africa. Cross-border trade at this junction represents an extra-regional trade i.e. that involved two epidemiological clusters as described by Di Nardo *et al.* (2011). Increasingly, viruses

belonging to pool 4 of FMDV, known in the epidemiological cluster of East Africa (Di Nardo *et al.*, 2011), were revealed in Egypt in North Africa (Jamal and Belsham, 2013).

2.2.2. Clinical disease investigation:

2.2.2.1. Active surveillance:

The investigation was carried out in the River Nile State in March 2018 (7-17/3/2018) and involved many locations and small dairy farms in four districts; Atabara, Ad-Damar, Shendi and Berber. Visited animal holdings included small dairy holdings (15) and small dairy farms (4). In the small holdings herd size was around 20, and between 40 and 60 in the dairy farms. Case definition is "an animal possessing vesicular lesions (oral or foot)". Of note, no affected animals were seen.

2.2.2.2. Passive surveillance:

Eleven epithelium samples that have been collected by Dongla regional laboratory were included in the study. Regional laboratories were supplied with transport media and enrolled in FMD passive surveillance efforts carried out by the Department of FMD-CVRL. Samples had been collected from suspected FMD events in cattle smuggled to Egypt and in cattle in the Northern State late in 2016 (25/12/2016).

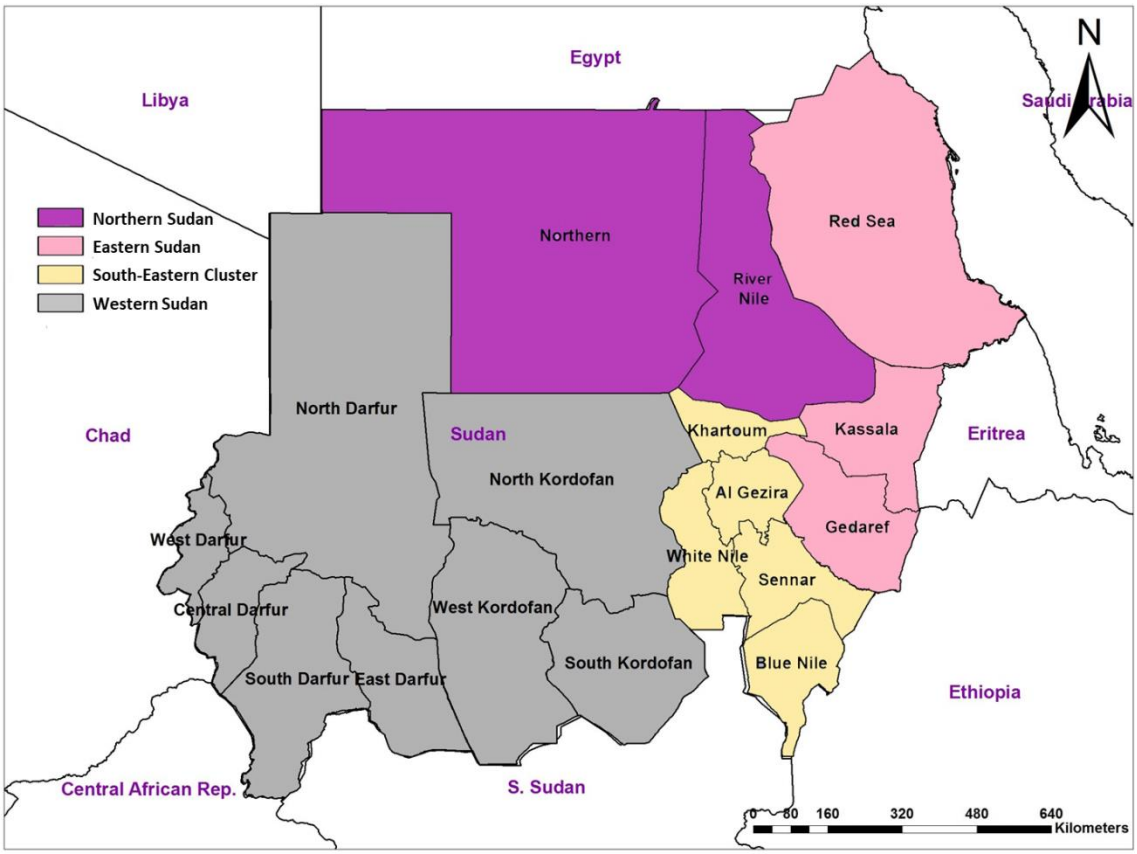

Figure 1. Map of the Sudan showing the study area "Northern and River Nile States" (Violet). The four geographical clusters of the country, the Northern Cluster (Violet), Eastern Sudan (Pink), South-Eastern Cluster (Yellow) and Western Sudan (Grey), were presented.

2.2.3. Preparation of samples:

2.2.3.1. Serum samples:

Serum samples (positive to NSPs antibodies) were separated from serum lots collected from the River Nile and the Northern States. Serum samples were thawed at room temperature and heat inactivated by placing in a water bath at 56°C for 30 minutes. Sera were allowed to cool down, received 6 µl of antibiotics (Penicillin/Streptomycin mixture) then stored at -20°C till used.

2.2.3.2. Tissue samples:

The glycerinated epithelium samples were blotted dry on absorbent filter paper. A10% suspension (w/v) was prepared in GMEM (containing double fold of antibiotics and antimycotics) by grinding using pestle, mortar and sterile sand. The suspension was clarified by centrifugation at 2000 rpm for 10 minutes, divided into two aliquots and stored in liquid nitrogen vapor till used.

2.2.4. Virus neutralization test (VNT) for detection of antibodies against SPs of FMDV:

Sera were tested using a screening format (Raouf *et al.*, 2012) of virus neutralization test (VNT) against serotype O, A and SAT2 viruses throughout the study. The procedure of the test was similar to the standard procedure of VNT (OIE Manual, 2021) except that sera were tested at merely two dilutions; 1/32 and 1/64, rather than several dilutions to decrease the test workload and span the standard cut-off of 1/45 ($10^{1.65}$) described for the purpose of serosurveillances by the OIE Manual (2021). To increase further the sensitivity of the assay, the cut-off is lowered to 1/32 ($10^{1.5}$) which is usually considered retest (doubtful) in case of individual serum screening (OIE Manual, 2021). The format was carried out into Baby Hamster Kidney-21 (BHK-21) clone 13 cell line and employed locally isolated FMD viruses, detects serum titres as low as 1.5 $\log_{10}$ and quantifies positive titers as high as $\geq$ 1.95 $\log_{10}$.

2.2.4.1. Preparation of viral materials for VNT:

The FMD viruses used in VNT were adapted (through 16-22 passages) to grow into BHK-21 cell cultures. Cells and supernatant viruses were harvested at 24 hours post-

infection (p.i.) by freezing of the whole culture. The viral material was thawed, clarified by centrifugation at 2000 rpm for 10 minutes in refrigerated centrifuge, distributed in 2 ml aliquots and stored in liquid nitrogen vapor. Subsequently, viruses were titrated using microtiter system (Raouf *et al.*, 2010). In brief, 10-fold serial dilutions of the virus were prepared in complete GMEM [GMEM containing 10% TPB (v/v) and 10% Tris buffer (v/v)] then distributed in 50 µl volumes in flat-bottomed Coaster plate employing 8 wells per dilution. Immediately, each well of the plate received 100 µl of BHK-21 cell suspension in outgrowth media supplemented with 10% newborn calf serum (v/v). Each virus titration plate encompassed cell control wells. Plates were sealed and incubated at 37°C in a humidified incubator for 3 days. Examination for CPE was carried out daily using an inverted light microscope. Virus titers were calculated according to the method of Kärber (1931).

2.2.4.2. The test proper:

Serum diluents, composed of complete GMEM with 10% Tris buffer 0.05 M, was distributed in all wells of the plate as follows: 94 µl in each well of rows A, C, E and G and 50 µl in each well of rows B, D, F and H. Six µl of tested sera were distributed in rows A, C, E and G according to the plan layout (Figure 2). Columns 11 and 12 were used as controls as shown in Figure 2. Virus and cell control wells were devoid of sera and received instead 6 µl of diluent while –ve and +ve serum controls wells received 6 µl of known –ve and known +ve control sera. Serum dilution was performed using multi-channel micropipette by mixing and transferring 50 µl to the second row then mixing and discarding 50 µl. For each 2 rows a new set of tips was used.

The pretitrated virus preparation was diluted to contain 100 $TCID_{50}$/50 µl. Each well apart from the cell control wells received 50 µl of the virus preparation. The plate was shaked lightly and kept at room temperature for 1 hour. At the end of incubation time, each well received 50 µl of BHK-21 cell suspension adjusted to produce confluent or semi-confluent monolayer 24 hours later. The test plate was incubated at 37°C with a source of humidity for 3 days. Microscopic examination was carried out daily. Negative serum showed CPE in all 4 wells and positive serum shows intact sheet in one or more well. On the third day, the plate was stained with 0.1% crystal violet stain in 10% formal saline. Cytopathic effects appear as empty wells or focal areas devoid of cells.

		Serum samples (Columns 1-10)										Controls (Columns 11-12)	
Serum dilutions		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1/16	**A**	*S1*	*S1*	*S2*	*S2*	*S3*	*S3*	*S4*	*S4*	*S5*	*S5*	-ve Control	
1/32	**B**	*S1*	*S1*	*S2*	*S2*	*S3*	*S3*	*S4*	*S4*	*S5*	*S5*		
1/16	**C**	*S6*	*S6*	*S7*	*S7*	*S8*	*S8*	*S9*	*S9*	*S10*	*S10*	+ve Control	
1/32	**D**	*S6*	*S6*	*S7*	*S7*	*S8*	*S8*	*S9*	*S9*	*S10*	*S10*		
1/16	**E**	*S11*	*S11*	*S12*	*S12*	*S13*	*S13*	*S14*	*S14*	*S15*	*S15*	VC	
1/32	**F**	*S11*	*S11*	*S12*	*S12*	*S13*	*S13*	*S14*	*S14*	*S15*	*S15*		
1/16	**G**	*S16*	*S16*	*S17*	*S17*	*S18*	*S18*	*S19*	*S19*	*S20*	*S20*	CC	
1/32	**H**	*S16*	*S16*	*S17*	*S17*	*S18*	*S18*	*S19*	*S19*	*S20*	*S20*		

Figure 2. Layout of the VNT plate.

-ve control= Negative control serum (A11-12, B11-B12)

+ve control= Positive control serum (C11-12, D11-D12)

VC = Virus Control (E11-12, F11-F12)

CC = Cells control (G11-12, H11-H12)

Sn (S1 - S20) = Sample number

2.2.5. Statistical analysis:

The serological study employed serial testing approach i.e. only sera positive in two test systems were considered positive (Fletcher and Fletcher, 2005). Calculations for serial testing were performed according to standard procedure (Thrusfield, 2007). Prevalence was calculated as proportion positive to both tests; test A and test B. Test A is the ID Screen® FMD NSP Competition ELISA and test B is VNT. Accordingly:

Prevalence = proportion positive detected by test B x proportion positive detected by test A x 100.

Proportions positive by test A were provided by the STSD (Table 1). Proportions positive by test B (VNT) in each sub-population were determined by dividing the number of positive reactors identified by the VNT by the number of sera tested in that sub-population. For calculations based on combined VNT, sera eligible to the calculation included sera positive to one or more serotypes but negative to the three serotypes.

The prospective power analysis depended on the previous study and had been used in the STSD (Department of FMD Report, 2016) to estimate sufficient sample sizes (Table 1). In the present study, *post-hoc* analysis (retrospective power analysis) was conducted to drive 95% confidence interval (C.I.) measures and *p-values*. Sera were collected from a sampling frame of 6 (River Nile State) and 5 (Northern State) geographical districts (sampling units) (Table 1, Figure 3A and 3B) and five sampling epi-units (herds or collection sites) per each sampling unit. Therefore, a minimal number of 25 epi-units per State was achieved what conform to statistical theory regarding unbiased parameter estimates (Ferrari *et al.*, 2016). A sample size of 70 sera from each sampling unit (district) and 14 sera from each epi-unit (herds or collection sites) was collected using a simple random sampling (SRS) method and standard statistical procedure to determine the sample size (Department of FMD Report, 2016). Accordingly, at least 350 bovine sera had to be collected from each state (Table 1).

In the *post-hoc* analysis, prevalence rates were compared by deriving the 95% C.I. derived from a simple random sample, based on the Normal approximation to the binomial distribution, using the formula: $P \pm 1.96\sqrt{p(1-p)/n}$ (Thrusfield, 2007). Where P is the estimated prevalence, n is the number of samples tested and 1.96 is the appropriate multiplier for the selected level of confidence. When C.I. values did not overlap then the statistics will always be statistically significantly different (Knezevic,

2008). For overlapping C.I. values, *p-values* were calculated using chi-squared and Fisher Exact test available at the Statistical Packages for Social Sciences (SPSS) (www.sociostatistics.com); results were significantly different, if $p < 0.05$. The Fisher Exact test was used for smaller sample sizes.

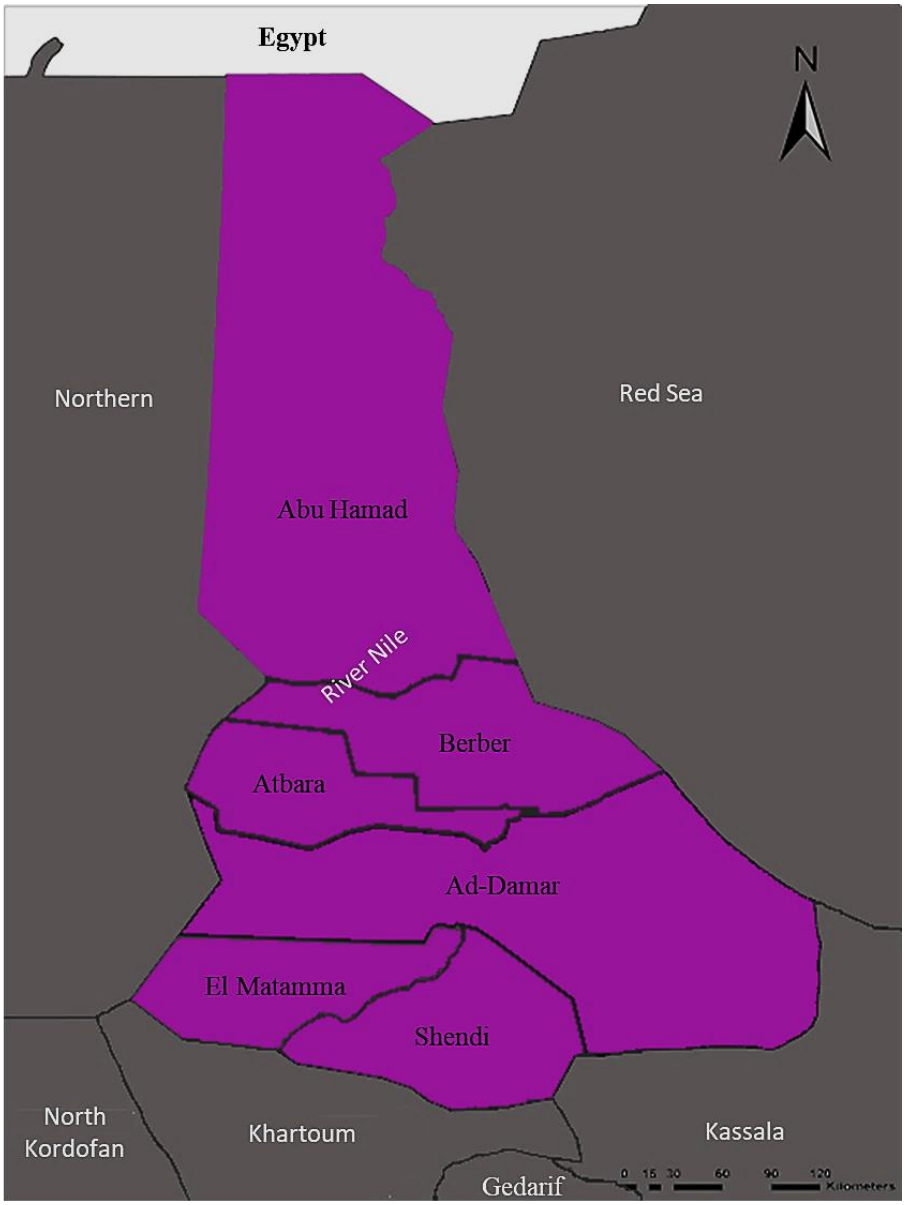

Figure 3A. Districts (localities) of serum collection in River Nile State.

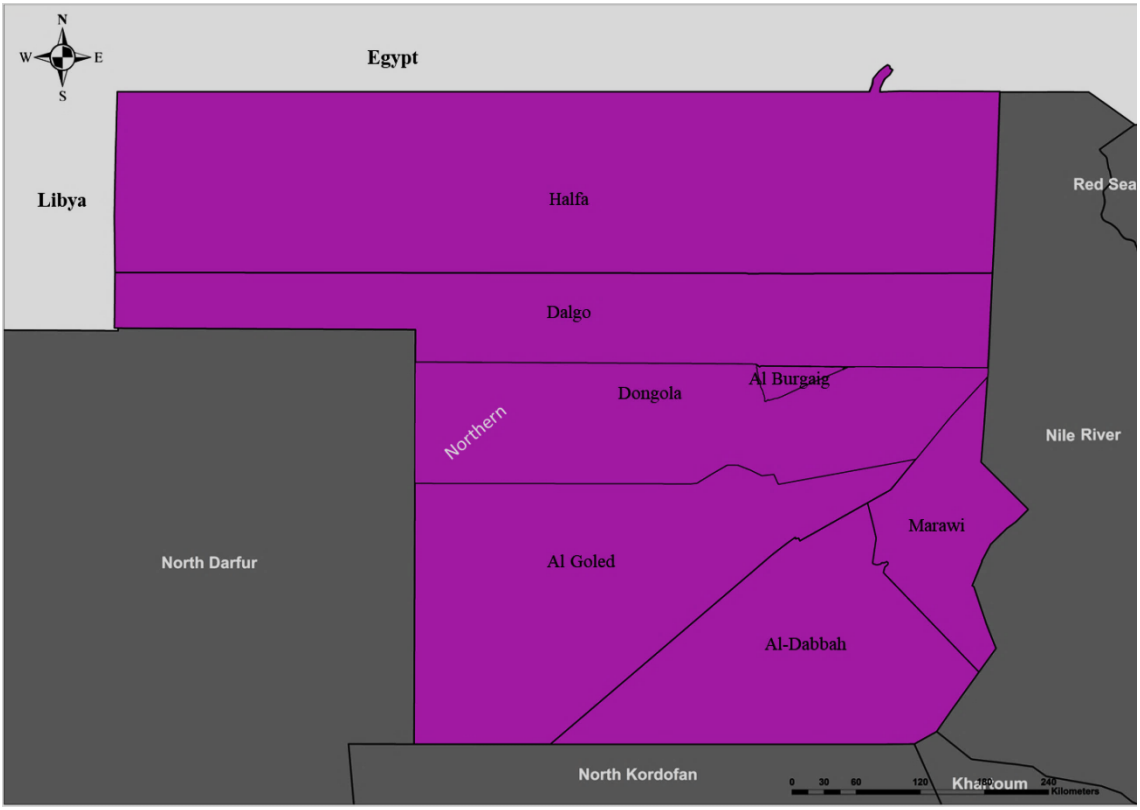

Figure 3B. Districts (localities) of serum collection in Northern State.

2.2.6. ELISA for FMD antigen detection (Grazioli *et al.*, 2012) (Bresia, Italy):

The ELISA plates were pre-coated with MAbs against FMDV serotypes (O, A, Asia1, C, Pan O-A-Asia1, SAT2 and SAT1) and with positive (inactivated) and negative controls according to the plate layout shown in Figure 4. Test samples were diluted 1:2 in ELISA diluent buffer and distributed in 50 µl/well, each in one column from columns 1to 10. Columns 11 and 12 received 50 µl/well of ELISA diluent buffer in place (Figure 4). The plate was covered and incubated for one hour at room temperature (18-30°C). ELISA plates were then washed 3 times with washing buffer (PBS-Tween 20). Washing cycles were performed by addition of 200 µl of the washing buffer to all wells of the ELISA plate, incubating for 3 minutes at room temperature, emptying and tapping hard onto an absorbent towel. Afterwards, 50 µl/well of appropriately diluted conjugates were distributed according to the plate layout (Figure 4). The plate was covered and incubated for one hour at room temperature. At the end of incubation time, the plate was washed 4 times as before but leaving the last wash for 5 minutes instead of 3 minutes. 50 µl of the Substrate/Chromogen solution (TMB) was distributed to all wells of the plate. The ELISA plate was covered and incubated at room temperature for 20 minutes in the dark. The reaction was stopped by addition of 50 µl/well of the stop solution ($H_2SO_4$0.6N). Thereafter, the plate was immediately read spectrophotometerically at 450 nm using ELISA reader (Elx 808).

For validation of results, positive control antigen should give values of 1.0 or above, the negative controls antigen usually gives values less than 0.1 (O, A, Asia1, C and pan-FMDV) or $\leq$ 0.2 (SAT1 and SAT2). Corrected sample OD value (obtained by subtracting the OD value of the negative control of the corresponding catching MAb) of 0.1 or greater considered positive.

Catching MAbs		*Sample 1*	*Sample 2*	*Sample 3*	*Sample 4*	*Sample 5*	*Sample 6*	*Sample 7*	*Sample 8*	*Sample 9*	*Sample10*	*+ Control*	*- Control*	Conjugate
		1	2	3	4	5	6	7	8	9	10	11	12	
Type O	A													Conjugate A (pan-O-A-C-Asia1 + some SATs)
Type A (1st MAb)	B													
Type A (2st MAb)	C													
Type Asia1	D													
Type C	E													
Pan-O-A-C-Asia1	F													
Type SAT2	G													Conjugate B (SAT1-SAT2)
Type SAT1	H													

Figure 4. Layout of ELISA plate for detection of FMDV antigen types (O, A, SAT1, SAT2).

2.2.7. Serotyping of FMDV:

Detection and serotyping of FMDV was carried out on the epithelial samples. The glycerinated epithelium samples were blotted dry on absorbent filter paper. A 10% suspension (w/v) was prepared in Glasgow minimum essential medium (GMEM) (containing double-fold of antibiotics and antimycotics) using pestle, mortar and sterile sand. The suspension was clarified by centrifugation at 2000 rpm for 10 min, divided into two aliquots and stored in liquid nitrogen vapor till used.

An antigen ELISA kit developed and distributed by the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Italy, was used for serotyping of FMDV according to the manufacturer instructions. The assay is a sandwich ELISA performed with selected combinations of anti-FMDV monoclonal antibodies (MAbs), used as coated and conjugated antibodies (Grazioli *et al.*, 2012).

2.2.7.1. Serotyping of FMDV at the World Reference Laboratory (WRL) for FMD:

Five samples out of the 11 collected samples were dispatched, under dry ice, to the World Reference Laboratory for foot-and-mouth disease (WRLFMD), the Pirbright Institute, UK, as dangerous biological substance category B UN 3373. Samples were originated from the smuggled (1) and the resident (4) cattle and kept unprocessed in the described transport medium at –20°C till dispatched to the WRL for FMD (July 2018). More information about the dispatched samples is available in Table 2.

Table 2. Epithilum samples collected from suspected cases of FMD in cattle in Northern Sudan between 2016 and 2018.

Serial No.	Sample identity (CVRL Reference*)	Sample Origin	Description of Sample
1	Ep-/2017 (1)	Northern State (Dongola), resident cattle	Cattle, epithelium, collected on 25/12/2016
2	Ep-/2017 (2)		
3	Ep-/2017 (3)		
4	Ep-/2017 (4)		
5	Ep-/2017 (border control-1)	Northern State (Dongola), Department of Border Control	Cattle, epithelium, collected on 25/12/2016
6	Ep-/2017 (border control-2)		
7	Ep-/2017 (border control-3)		
8	Ep-/2017 (border control-4)		

CVRL = Central Veterinary Research Laboratory.

Ep = Epithelium.

*Samples were collected late in 2016 and are included in the disease season of the following year.

2.2.7.2. FMD virus isolation and identification of isolates:

At the WRL for FMD, samples were passaged once or twice into IB-RS-2 and thyroid cell culture (De Castro, 1964) then subjected to antigen detection and serotyping by ELISA assay using the indirect sandwich ELISA kit (WRL for FMD) for detection of FMDV antigen (Roeder and Le Blanc Smith, 1987).

FMDV isolates were named according to its serotype, place of collection in Sudan, SUD "the three-letter country code", laboratory record number and year of isolation.

2.2.8. Molecular and genetic characterization of FMD viruses:

For determining the serotype and prototype of Sudanese FMD viruses, molecular and genetic characterization including amplification of the 639 nt partial FMDV serotype-O VP1 (1D) coding region using reverse-transcription polymerase chain reaction (RT-PCR) followed by gene sequencing were performed at the WRL for FMD, the Pirbright Institute, UK, following the protocol described previously by Knowles *et al.* (2016).

2.2.8.1. RNA extraction:

Total RNA was extracted from the epithelial samples using the RNeasy kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions as described by Knowles *et al.* (2016). Briefly, total RNA was extracted from 460 µl of the epithelial sample as described by the manufacturer. The purified RNA was eluted in 50 µl of nuclease-free water and placed on ice to perform the RT-PCR immediately, otherwise stored at –20°C.

2.2.8.2. Reverse transcription-polymerase chain reaction (RT-PCR):

Based on serotyping results confirmed by antigen-detection ELISA, FMDV serotype-O specific primer sets [O-1C244F (5′ GCAGCAAAACACATGTCAAACACCTT 3′) and O-1C272F (5′ TBGCRGGNCTYGCCCAGTACTAC 3′) forward primers to anneal with the VP3 and EUR-2B52R reverse primer (5′ GACATGTCCTCCTGCATCTGGTTGAT 3′) to anneal with the 2B coding region] were used for amplification of the full length FMDV VP1 coding sequence as described previously by Knowles *et al.* (2016). PCR primers were summarized in Table 3.

A one-step reverse-transcription polymerase chain reaction (RT-PCR) was carried out using QIAGEN One-Step RT-PCR kit (Qiagen, Germany) using the following RT-PCR cycling conditions: cDNA synthesis in one cycle of reverse-transcription performed at 50°C for 30 min, RT inactivation and initial polymerase activation at 95°C for 15 min followed by 35 cycles of cDNA amplification corresponding to denaturation at 95°C for 60 sec, annealing at 60°C for 60 sec, extension at 72°C for 120 sec, and finally 1 cycle for termination of the PCR reaction by heating at 72°C for 5 min. A thermal cycler (Bio-Rad, USA) was used for RT-PCR reaction.

The correct size of the amplicon was determined by analyzing the PCR product on 1.5% agarose-Tris-borate-EDTA gel containing 1× GelRed nucleic acid stain (Biotium Inc., USA) using a DNA size markers (GeneRuler 100 bp DNA Ladder Plus, Fermentas Inc., USA).

2.2.8.3. DNA sequencing, sequence and phylogenetic analysis:

Determining sequencing of the partial FMDV serotype-O VP1 (1D) coding region [639 nt] from the PCR product was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and FMDV serotype-O specific primer sets [FMD-3161F (5′ TCGCVCAGTACTACRCACAGT 3′) and FMD-4303R (5′ TGACGTCRGAGAAGAAGAARGG 3′)] (Dill *et al.*, 2017) were used for amplification of the FMDV VP1 coding sequence as described by Knowles *et al.* (2016). Sequencing primers were summarized in Table 3.

To determine the serotype and prototype of FMD viruses from Sudan, the yielded VP1 nucleotide sequences were assembled from multiple reads using SeqMan Pro (Lasergene package, DNAstar Inc., Madison, Wisconsin, USA). To determine the identity of the Sudanese FMDV isolates, the FMDV VP1 obtained sequence was compared with the respective gene sequences of other FMD virus isolates using BLAST Nucleotide (https://blast.ncbi.nlm.nih.gov/). Accordingly, alignment of FMDV VP1 nucleotide sequences (633 nt) of FMDV serotype-O retrieved from NCBI GenBank database was performed using BioEdit v7.2.5, which uses ClustalW multiple alignment program (http://www.ebi.ac.uk/Tools/msa/clust alw2/).

Neighbor-joining phylogenetic tree, employing the Kimura 2-parameter nucleotide substitution model for FMDV serotype-O with 1,000 bootstrap replicates, was

constructed using the MEGA7.0.26 (Molecular Evolutionary Genetics Analysis) program (http://www.megasoftware.net/mega.html).

Table 3. Oligonucleotide primers for PCR amplification and DNA sequencing of FMDV serotype O VP1 gene.

PCR Primers			
Primers Name	Sequence (5′ – 3′)	Gene	Product size (nt)
O-1C272F	TBGCRGGNCTYGCCCAGTACTAC	VP3	1,135
O-1C244F	GCAGCAAAACACATGTCAAACACCTT	VP3	1,165
EUR-2B52R	GACATGTCCTCCTGCATCTGGTTGAT	2B	
Sequencing Primers			
FMD-316IF	TCGCVCAGTACTACRCACAGT	VP1	639
FMD-4303R	TGACGTCRGAGAAGAAGAARGG		

Notes:

F = Forward

R = Reveres

Chapter III

Results

3.1. Index of sero-prevalence of FMD infection in Northern Sudan by the multiple testing approach:

In absence of vaccination both test systems, NSPs and SPs testing's, are indicative of previous infection of FMD. Using serial testing approach, only sera positive in both test systems are considered positive. Around 70% (130/184) of NSPs antibodies positive bovine sera in Northern Sudan have screened positive to antibodies to one or more of the three serotypes of FMDV; O, A or SAT2 (Table 4). Indices of prevalence of FMD infection in cattle in Northern Sudan as indicated by prevalence of antibodies to structural proteins (SPs) of FMDV was 27.82% in the River Nile State and 10.96% in the Northern State (Table 4; Figure 5). It was statistically similar to sero-prevalence of NSPs antibodies in the Northern State but significantly lower than estimates of NSPs serology in the River Nile State (Table 5). Around 30% of anti-NSPs positive sera in each State have failed typing by VNT (Table 4). These sera proved to be negative to the 3 serotypes; O, A and SAT2. However, indices by both test systems were statistically significantly higher in the River Nile State than in the Northern State.

Table 4. Typing of NSPs antibodies positive bovine sera by cVNT in Northern Sudan.

State	Sero-prevalence of anti-NSPs antibody	Typing of NSPs antibodies positive sera			Sero-prevalence of anti-SPs antibody (neutralizing antibodies)
		No. tested	No. positive*	% of typed sera	
River Nile State	39.4% (161/409)	143	101	70.62% (101/143)	27.82%
Northern State	15.5% (53/343)	41	29	70.73% (29/41)	10.96%

*Positive to one or more of the three serotypes of FMDV (O, A and SAT2)

Table 5. Comparison between indices of infection of FMD by SPs and NSPs serology in Northern Sudan.

State	Seroprevalence of anti-NSPs antibodies		Seroprevalence of anti-SPs antibodies		*P-value* (Chi squared test)
	Sero-prevalence	95% C.I.	Sero-prevalence	95% C.I.	
River Nile State	39.4%	34.6%-44.1%	27.82%	23.2%-32.4%	0.000725
Northern State	15.5%	11.6%-19.3%	10.96%	7.2%-14.7%	0.106567

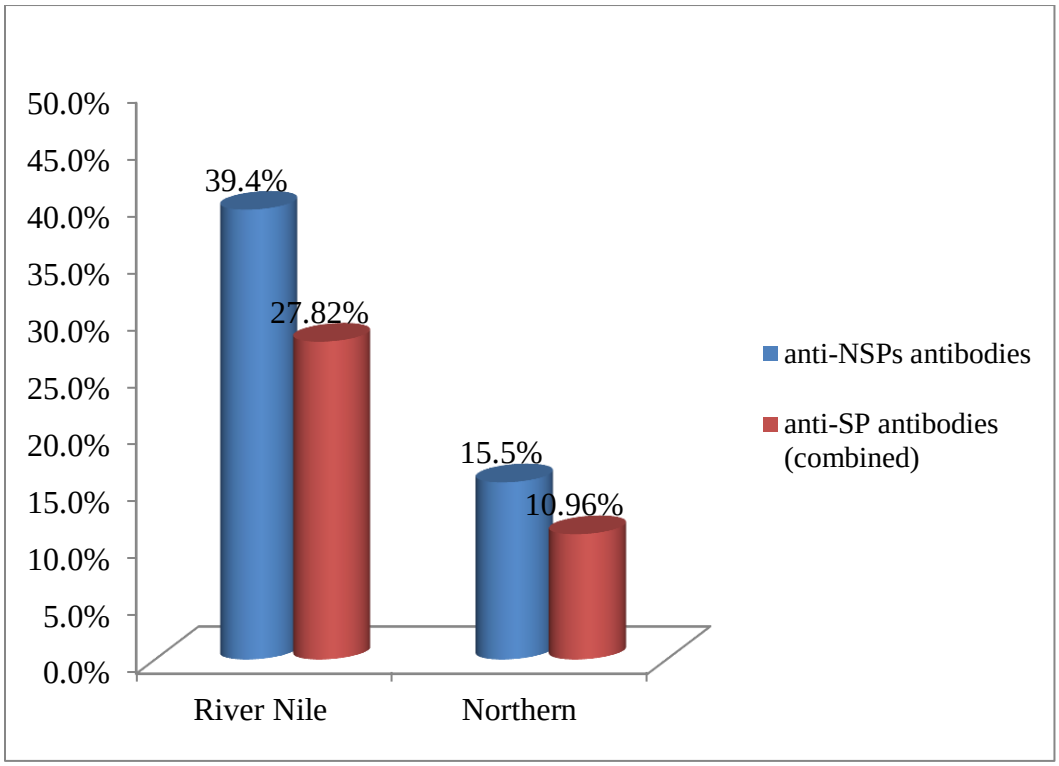

Figure 5. Indices of prevalence of FMD infection in cattle in Northern Sudan by NSPs and by multiple testing (NSPs and SP serology). In every case indices were higher in the River Nile than in the Northern State.

No predominance of antibodies to any serotype could be described in Northern Sudan. Sero-prevalence estimates of serotype-specific antibodies to the three serotypes in each State were similar; apart from that of SAT2 in the Northern State which was to some extent lower than that of the other serotypes (Table 6; Figure 6). On the other hand, sero-prevalence estimates were consistently statistically significantly higher in the River Nile than in the Northern State (Table 6; Figure 6).

Table 6. Sero-prevalence of FMDV serotype-specific antibodies in cattle in River Nile and Northern States.

	River Nile State			Northern State			
Serotype	% Positive in test sera	Estimated prevalence	95% C.I.	% Positive in test sera	Estimated prevalence	95% C.I.	*P-value* (chi-squared test)
O	32.9% (47/143)	12.9% (47/363)	9.5%-16.4%	41.5% (17/41)	6.4% (17/265)	3.5%-9.3%	0.00753
A	39.2% (56/143)	15.4% (56/363)	11.7%-19.1%	48.8% (20/41)	7.5% (20/265)	4.3%-10.7%	0.002788
SAT2	37.1% (53/143)	14.6% (53/363)	11.0%-18.2%	22.0% (9/41)	3.4% (9/265)	1.2%-5.6%	0.000003
P-value		0.604489			0.10665		

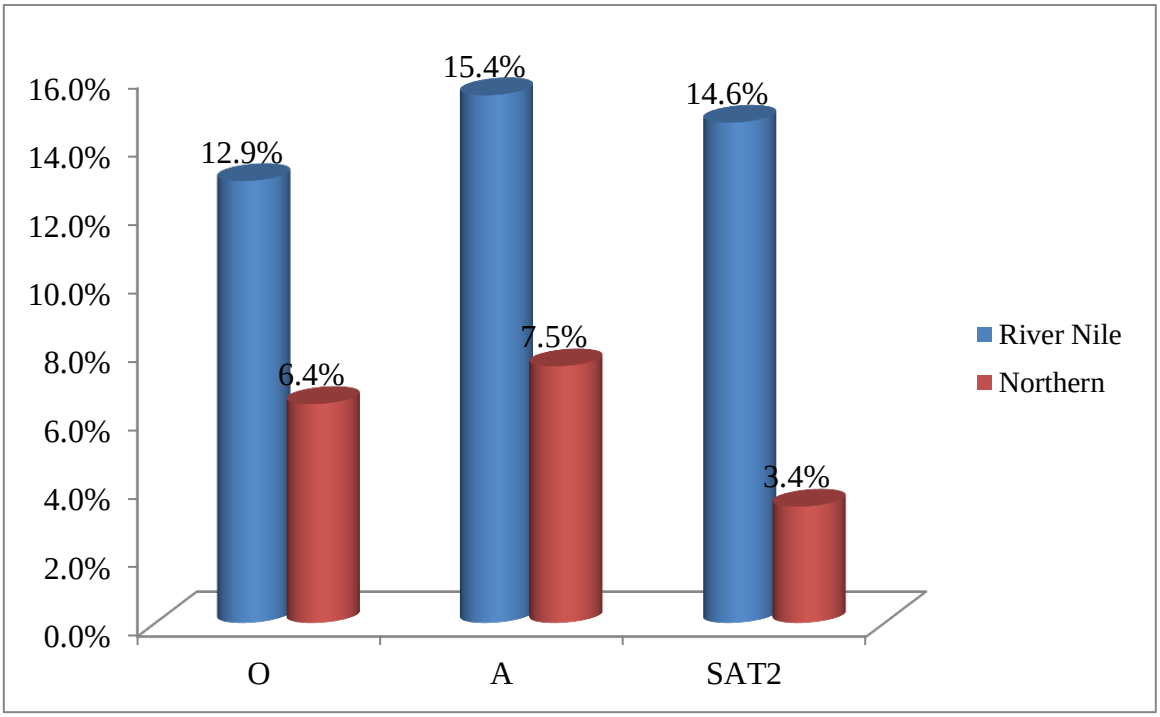

Figure 6. Sero-prevalence of FMDV serotype-specific antibodies in cattle in Northern Sudan. In each State estimated sero-prevalence's for the three serotypes were almost similar while sero-prevalence of serotype specific antibodies of each serotype was significantly higher in the River Nile than in the Northern State.

3.2. Prevalence of FMDV serotype-specific SPs antibodies in different localities in the River Nile State:

In the River Nile State, sero-prevalence's of serotype-specific antibodies at different districts have ranges from 5.5% (O at Abu Hamad) to 26.6% (SAT2 at Ad-Damar) (Table 7). Lowest sero-prevalence's were exclusively detected at the most Northern districts of Abu Hamad (Table 7; Figure 7). In general terms, the estimated sero-prevalence's could be described as highest at Ad-Damar at the center of the State, consistently relatively high at the Southern districts of Shendi and El Matamma, variable at Atbara and lowest at Abu Hamad and Berber (Table 7; Figure 7).

Sero-prevalence of serotype O antibodies, unlike that of serotypes A and SAT2, was consistently higher in the three Southern districts (El Matamma, Shendi and Ad-Damar) neighbouring Khartoum and Kassala States (Figure 7) than the Northern districts (Atbara, Berber and Abu Hamad) neighbouring the Red Sea and Northern State (Table 7 and 8).

Table 7. Prevalence of FMDV serotype-specific antibodies in cattle sera in different districts in the River Nile State.

		O		A		SAT2	
District	No. tested	Positive (%)	Sero-prevalence estimate	Positive (%)	Sero-prevalence estimate	Positive (%)	Sero-prevalence estimate
El Matamma	27	10/27 (37%)	15.8%	10/27 (37%)	15.8%	8/27 (29.6%)	12.6%
Shendi	26	10/26 (38.5%)	16.2%	8/26 (30.8%)	12.9%	10/26 (38.5%)	16.2%
Ad-Damar	27	12/27 (44.4%)	19.9%	11/27 (40.7%)	18.3%	16/27 (59.3%)	26.6%
Atbara	28	5/28 (17.9%)	8.1%	14/28 (50%)	22.8%	11/28 (39.3%)	17.9%
Berber	20	7/20 (35%)	11.5%	8/20 (40%)	13.1%	4/20 (20%)	6.6%
Abu Hamad	15	3/15 (20%)	5.5%	5/15 (33.3%)	9.2%	4/15 (26.7%)	7.3%

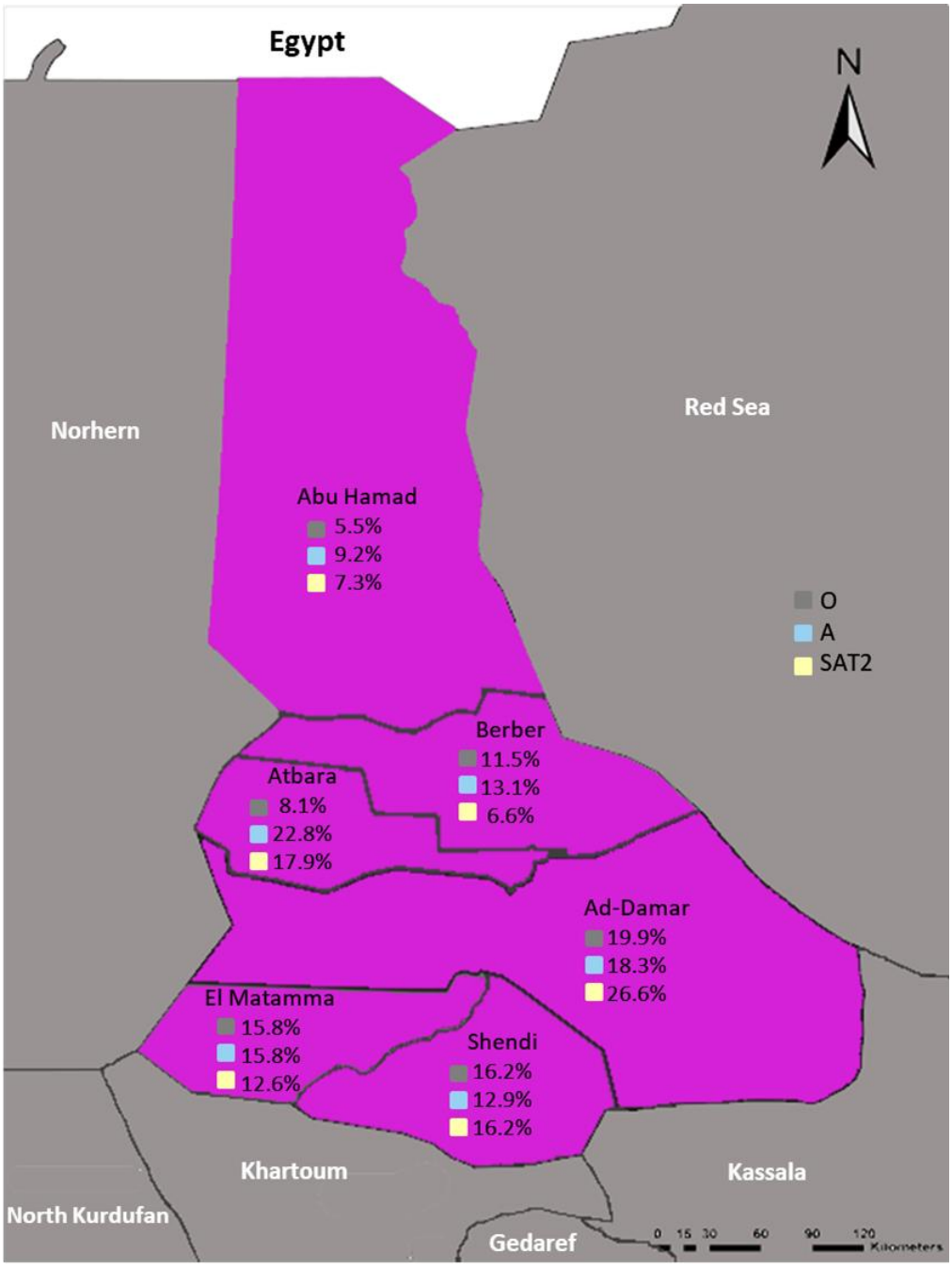

Figure 7. Prevalence of FMDV serotype-specific SPs antibodies in different localities in the River Nile State.

Table 8. Comparison between sero-prevalence estimates in Southern and Northern districts in the River Nile State.

	O		A		SAT2	
Districts	Positive (%)	Estimated prevalence	Positive (%)	Estimated prevalence	Positive (%)	Estimated prevalence
Southern districts*	32/80 (40%)	17.28%	29/80 (36.25%)	15.66%	34/80 (42.5%)	18.36%
Northern districts**	15/63 (23.8%)	8.4%	27/63 (42.85%)	15.12%	19/63 (30.15%)	10.64%
P-value Fisher exact test	0.0492	0.0126	0.4909	1.0	0.1633	0.0525

*El Matamma, Shendi and Ad-Damar.

**Atbara, Berber and Abu Hamad.

3.3. Prevalence of FMDV serotype-specific SPs antibodies in different localities in the Northern State:

Four out of the seven districts in the Northern State were included in this study; Marawi, Dongola, Al-Dabbah and Al Burgaig (Table 9; Figure 8). Two districts in the uppermost North, Halfa and Dalgo, were not studied for anti-NSPs activity and cattle from a third Western district, Al Goled (n = 64) were all negative for anti-NSPs activity (Table 1). Sero-prevalences detected to NSPs and SPs (Table 1 and 9; Figure 8) in Al Burgaig, (7.9%, 3.9%, 3.9% and 1.9%) in the North and in Al-Dabbah (11.4%, 5.7%, 3.8% and 0%) in the South West were also insignificant. In spite of the small numbers of reactors (4, 6, 15 and 16) in different districts to NSPs and SPs serology, serotype O and A antibodies were detected in all four surveyed districts while SAT2 antibodies were not. Trends in distribution of serotype-specific antibodies in the Northern State are shown in Figure 8.

In the Northern State, sero-prevalences of serotype-specific antibodies in different districts could be described as significant at Marawi (in the East) and Dongola (in the Center); insignificant at Al Burgaig (in the North) and Al-Dabbah (in the South West) or nil at Al Goled (in the West). In general terms, observed sero-prevalence's seemed to decrease from East to West and North (Figure 8).

Table 9. Prevalence of FMDV serotype-specific antibodies in cattle sera in different districts in the Northern State.

		O		A		SAT2	
District	No. tested	Positive (%)	Sero-prevalence estimate	Positive (%)	Sero-prevalence estimate	Positive (%)	Sero-prevalence estimate
Marawi	15	6/15 (40%)	10.3%	9/15 (60%)	15.45%	4/15 (26.66%)	6.86%
Dongola	16	7/16 (43.75%)	14.13%	7/16 (43.75%)	14.13%	3/16 (18.75%)	6.05%
Al-Dabbah	6	3/6 (50%)	5.71%	2/6 (33.33%)	3.81%	Nil	Nil
Al Burgaig	4	1/4 (25%)	1.98%	2/4 (50%)	3.97%	2/4 (50%)	3.97%

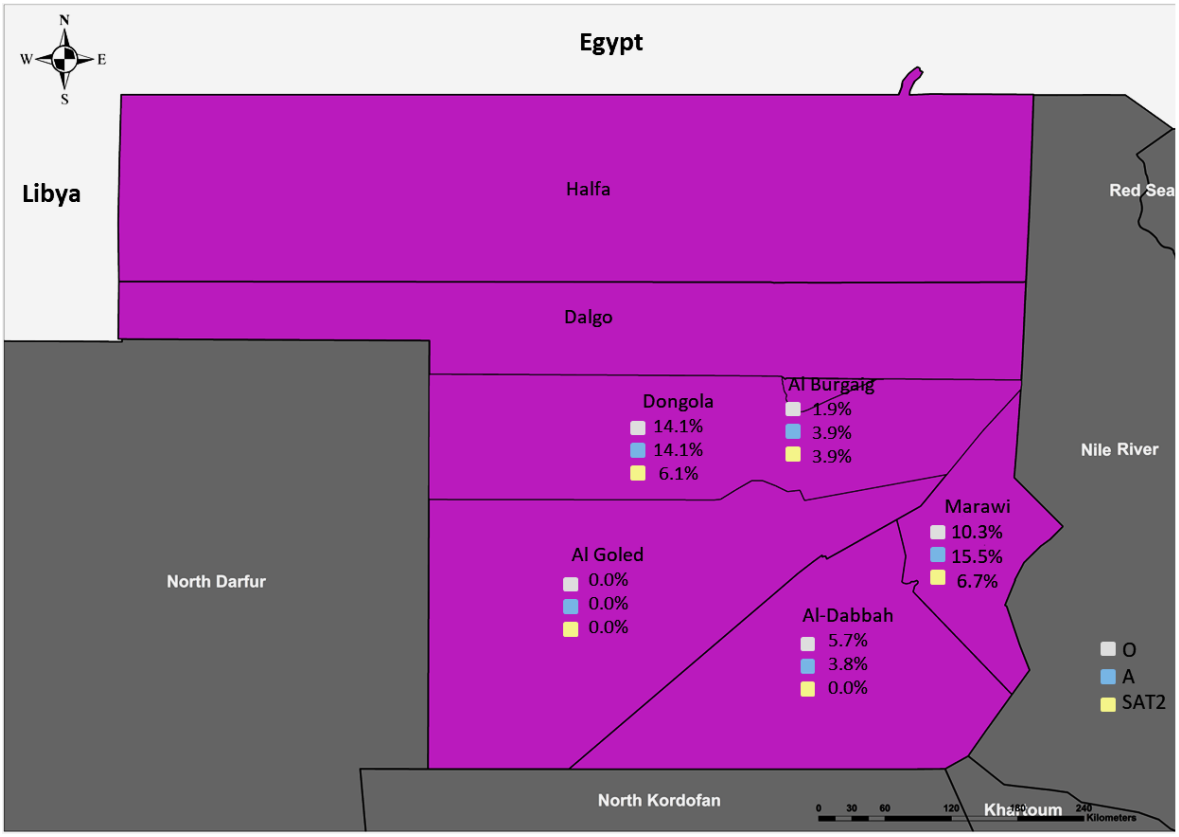

Figure 8. Prevalence of FMDV serotype-specific SPs antibodies in different localities in the Northern State.

3.4. Clinical disease investigation:

Between 2016 and 2018, clinical signs of FMD were reported only once. Suspicion has arisen in Dongola district in the Northern State. Cattle affected were dairy cattle of local breeds 'Kenana and Butana' and also cross breeds, resident in the area, and fattening calves smuggled to Egypt. Clinical signs included drolling of saliva, ulcerative lesions in the mouth and udder, lameness and drop in milk production. Morbidity reached 100% in affected farms where 27 animals out of 27 showed clinical signs. However, no similar clinical signs were seen in other ruminant species in the area. Active surveillance in four districts in the River Nile State in 2018, also, detected no clinical signs of FMD.

3.4.1. Serotyping and genotyping of FMD outbreak

Serotype O was detected in epithelium samples collected from clinically affected cattle originated at the international border from cattle smuggled to Egypt (5 samples) or from cattle at other locations in the Northern State (1 samples) using IZSLER ELISA (Table 10). The outbreak serotype was confirmed in one sample from the international border with Egypt (Table 11), the VP1 gene sequence of FMD virus - type O isolate O/SUD/1/2016 (GenBank accession number MK422563.1) was determined and the Sudanese FMDV was genotyped as an unnamed lineage within the topotype O-EA3 (Figure 9). In the generated phylogenetic tree, FMDV O/SUD/1/2016 was clustered in one subcluster under topotype EA-3 cluster and closer to the cluster contains other Sudanese strains from 2017 (O/SUD/4/2017, O/SUD/5/2017, O/SUD/15/2017), other Egyptian strains from 2016-2017, and Ethiopian strains from 2017-2018 (Figure 9).

The nucleotide sequence of VP1 gene (1D) region (639 nt) of FMDV O/SUD/1/2016 "serotype O, topotype EA-3" is closely related to FMDV Sudanese strains from 2017 (Raouf *et al.,* 2022) and shared the highest nucleotide sequence identity of 99.8-99.7% with many Egyptian strains (O/EGY/33/2017, O/Giza 1/Egy/2017, O/EGY/7/2017, O/EGY/9/2017, O/EGY/11/2017, O/EGY/22/2017, O/EGY/26/2017, O/Alexandria 1/Egy/2016, O/Behira 2/Egy/2017). Alternatively, the topotype EA3 sequence of FMDV O/SUD/1/2016 is closely related to FMDV Sudanese strain (O/SUD/2/86), Ethiopian strains (O/ETH/1/2007, O/ETH/3/2004), other African strains from Uganda (O/UKG/35/2001), Tanzania (O/TAN/2/2004) and Mali (O/MAL/1/98) (Figure 9).

Genotyping data is available at https://www.wrlfmd.org/sites/world/files/WRLFMD-2018-00020-SUD-GTR-O-O_001.pdf (WRLFMD, 2018).

Table 10. Detection and serotyping of FMD in Northern Sudan between 2016 and 2018.

Serial No.	Sample identity (CVRL Reference*)	Sample Origin	Description of Sample	Serotyping Result by IZSLER ELISA
1	Ep-/2017 (1)	Northern State (Dongola), resident cattle	Cattle, epithelium, collected on 25/12/2016	O
2	Ep-/2017 (2)			-ve
3	Ep-/2017 (3)			-ve
4	Ep-/2017 (4)			-ve
5	Ep-/2017 (border control-1)	Northern State (Dongola), Department of Border Control	Cattle, epithelium, collected on 25/12/2016	O
6	Ep-/2017 (border control-2)			O
7	Ep-/2017 (border control-3)			O
8	Ep-/2017 (border control-4)			O

CVRL = Central Veterinary Research Laboratory.

Ep = Epithelium.

*Samples were collected late in 2016 and are included in the disease season of the following year.

Table 11. Confirmation of serotype O outbreak in Northern Sudan in 2016-2017 at the WRL for FMD.

Sample identity (WRL Reference)	Sample identity (CVRL Reference)	Description of sample	Serotyping at CVRL	Serotyping at the WRL	
				PCR result	Serotyping result by cell culture/ELISA
SUD 1/2016	Ep-/2017 (border control-1)	Cattle, epithelium, collected on 25/12/2016	O	FMDV GD	O
SUD 2/2016	Ep-/2017 (2)	Cattle, epithelium, collected on 25/12/2016	-ve	FMDV GD	NVD
SUD 3/2016	Ep-/2017 (3)	Cattle, epithelium, collected on 25/12/2016	-ve	FMDV GD	NVD
SUD 4/2016	Ep-/2017 (7)	Cattle, epithelium, collected on 25/12/2016	N.D.	FMDV GD	NVD
SUD 5/2016	Ep-/2017 (5)	Cattle, epithelium, collected on 25/12/2016	N.D.	NGD	NVD

N.D. = Not detected.

FMDV GD = FMDV genome detected.

NGD = No genome detected.

NVD = No virus detected.

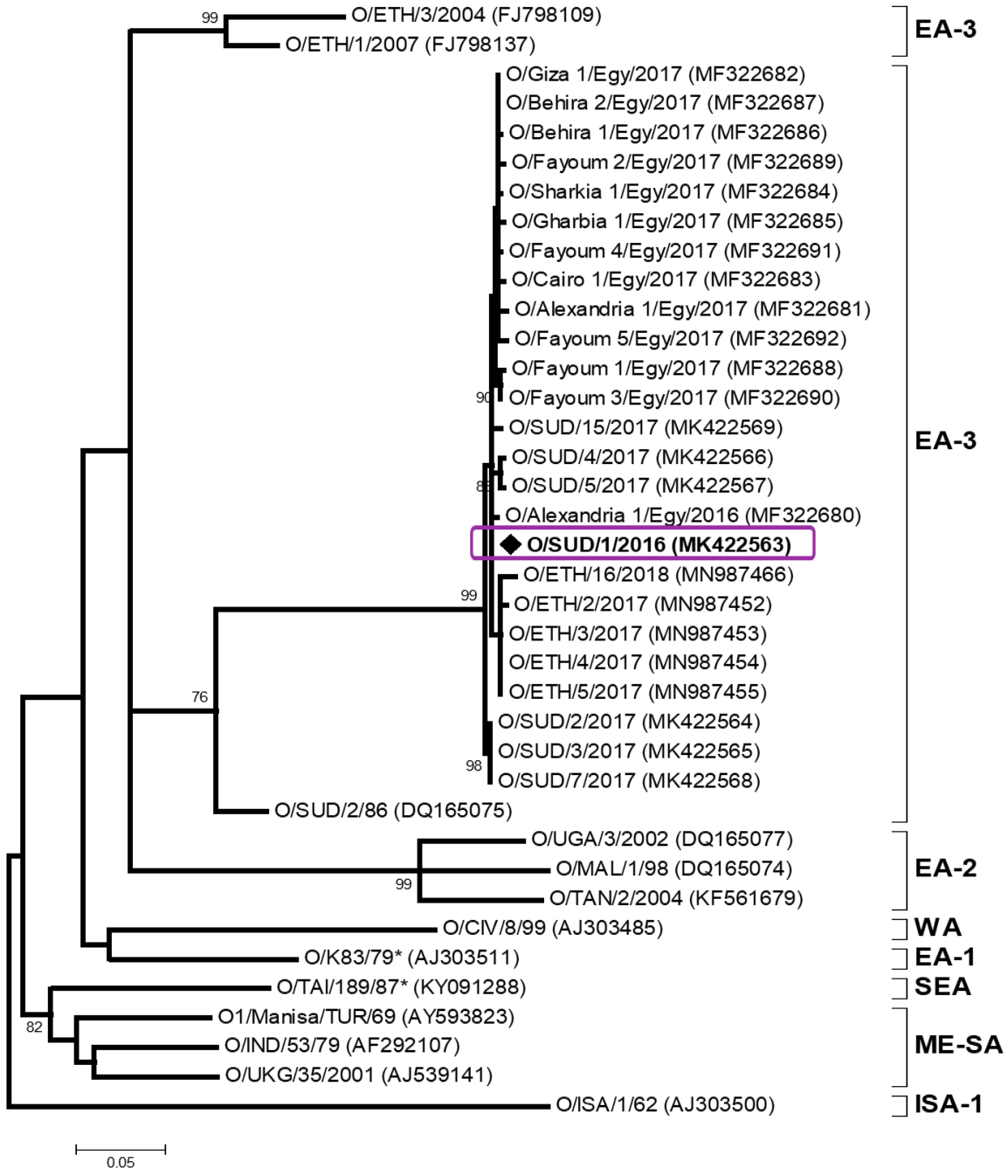

Figure 9. Neighbor-joining phylogenetic tree generated using nucleotide sequences (633 nt) of the VP1-coding region of serotype-O FMD viruses. The tree was constructed using the MEGA7.0.26 (Molecular Evolutionary Genetics Analysis) program (http://www.megasoftware.net/mega.html) by employing the Kimura 2-parameter nucleotide substitution model for FMDV serotype-O and using the Bootstrap method for test of phylogeny by analyzing 1,000 bootstrap replicates.

Chapter IV
Discussion

Foot-and-mouth disease is an important transboundary animal disease. It has been known in Sudan since 1903 (Eisa and Rweyemamu, 1977; Abu Elzein, 1983). Evidences for its endemicity, at least in some parts of the country, are currently accumulating (Habiela *et al.*, 2010b). On the other hand, data on circulation of FMD viruses in Northern Sudan, only recently, since 2014 have begun to mount up (Department of FMD Report, 2014; 2016; Saeed, 2019; Saeed and Raouf, 2020). Serotype O was typed in Northern Sudan in February 2012 in the Northern State (Saeed, 2019; Saeed and Raouf, 2020) and December 2012 in the River Nile State (Department of FMD Report, 2014). Similarly, estimated sero-prevalence rate of antibodies against SPs and NSPs of FMD virus in the study area were much lower than the latest estimates reported (Raouf *et al.*, 2016) in other parts of Sudan (Department of FMD Report, 2014; 2016; Saeed, 2019; Saeed and Raouf, 2020). Serotype O was identified in many instances as the predominant serotype in Sudan (Abu Elzein, 1983; Raouf *et al.*, 2016; http://www.wrlfmd.org/). In Sudan, FMD was usually diagnosed annually and is expected to be endemic, at least, in some parts (Abu Elzein, 1983; Habiela *et al.*, 2010b; Raouf *et al.*, 2016). The presented study monitored the field in Northern Sudan in 2016, 2017 and 2018 and analyzed the collected data for further evidence of FMD virus circulation in the area. It meant to compare current prevalence data with previous ones. It also aimed at evaluation of the multiple testing approaches for determination of sero-prevalence of FMDV antibodies under the low level of circulation in Northern Sudan.

In general terms, the passive and active surveillances (clinical and serological) carried out during the study period confirmed the lower circulation of FMD viruses in Northern Sudan compared to other parts of the country. Similar to earlier findings (Saeed, 2019; Saeed and Raouf, 2020), only serotype O was typed in the study area, estimated prevalence rates were low, ranged between 15.4% (serotype A) to 3.4% (serotype SAT2) compared to a range from 75% (serotype O) to around 5% (serotype SAT2) in other Sudanese States (Raouf *et al.*, 2016), and that of SAT2 in the Northern State was largely insignificant.

Previously, serotype O was typed in February 2012 in the Northern State (Saeed, 2019; Saeed and Raouf, 2020) and in December in the River Nile State (Department of FMD Report, 2014). In the three years of this study, serotype O was typed in the Northern

State only; in December 2016 in cattle smuggled to Egypt as well as in resident cattle. When four districts in the River Nile State were surveyed in March 2018, no clinical signs of FMD were further detected. Accordingly, in seven years period between 2012 and 2018, clinical FMD was confirmed three times in the study area. The sensed activity of clinical FMD in Northern Sudan was in general agreement with the NSPs antibody reactivity detected in the area; 15.5% in the Northern State and 39.4% in the River Nile State (Department of FMD Report, 2016). In absence of vaccination, SPs serology like NSPs serology is indicative of previous virus exposure. Reactivity to NSPs at the herd level is largely accepted as an indication to the degree of FMD virus circulation (Bergmann *et al.*, 2003; Bronsvoort *et al.*, 2004; OIE Manual, 2021).

Serotyping and genotyping of epithelium samples from the study area at the WRL for FMD confirmed incidence of serotype O clinical disease and indicated that the isolate, like all other Sudanese isolates, was of an unnamed lineage within the topotype O-EA3 (http://www.wrlfmd.org/). Genotyping data (WRLFMD, 2018) revealed that the serotyped isolate was part of a large temporal cluster (nt. id. > 95%) that involved Sudanese, Egyptian, Ethiopian viruses from 2016, 2017 and 2018 in addition to Israeli and Palestinian O viruses from 2017 (WRLFMD, 2018) rather than the Sudanese viruses that had been detected in the Northern State in 2012. It showed phylogenetic identity of above 99% with the Sudanese and the Egyptian member of this cluster which strongly suggests that it was the same virus or the same outbreak. Therefore, it is conceivable that it was introduced to Northern Sudan rather than being circulating in the study area. Interestingly, it was observed that the serotype O virus which had been detected earlier in the study area in 2012 had similarly showed phylogenetic identity of above 99% with Ethiopian and Eritrean viruses but only an identity of 95% with earlier Sudanese viruses (Department of FMD Report, 2016; Saeed, 2019; Saeed and Raouf, 2020). These sequences seemed to be introduced to Central Sudan in 2016 when serotype O disease was detected in September in Khartoum in a nearby village (Al Gadeeda) as suggested by the rise in frequency of typing serotype O disease events (Table 12) thereafter (Department of FMD Report, 2018) and by their phylogenetic distance from earlier Sudanese isolates (http://www.wrlfmd.org/). Similarly, O viruses that have been detected in Northern Sudan in 2012, though it had been circulating in Sudan since 2008, it has been linked to a large temporal cluster that involved Ethiopian and Eritrean O viruses in 2011 and also some Egyptian O viruses in 2012 (Saeed, 2019; Saeed and Raouf, 2020; Raouf *et al.*, 2022).

The described pattern strongly suggested that in seven years period, between 2012 and 2018, the two episodes of serotype O disease in 2012 and 2016 in Northern Sudan were caused by viruses of transboundary nature probably originated from outside the country (phylogenetic identity above 99%) rather than from within the study area. Similarly, Al-Hosary *et al.* (2019) characterized 2 groups of serotype O viruses from an outbreak of FMD in Southern Egypt in 2015-2016 showing nucleotide identity of 85% and 86% with previously characterized isolates from the area suggesting incursion of new viruses into Egypt. However, the important fact is that the circulating virus was detected in a large geographical area involving two neighbouring countries i.e. its transboundary nature is indisputable and particularly evident. The suggestion suited well the inferred low level of circulation of FMD virus in Northern Sudan. It is justifiable to conclude that introduction mechanisms of FMD virus to the study area were far more significant than circulation of FMD virus within the area. Also, country wise, such findings clearly emphasize the importance of introduction mechanism of FMD virus to Sudan. Raouf *et al.* (2016) recognized the Blue Nile State and border points surrounding the Upper Nile State of Southern Sudan Republic in the White Nile, Sinnar and Southern Kordofan States as probable points of entry of serotype O into Sudan.

The low level of circulation of FMD viruses in the study area has also been confirmed by the serological surveillances. The serial testing approach which was applied in the serological study is known for increasing specificity but decreasing sensitivity (Fletcher and Fletcher, 2005). Nonetheless, sero-prevalence rates in the Northern State detected in the course of this work were similar (overlapping C.I.) to those reported in the Northern State by Saeed (2019), and Saeed and Raouf (2020). The latter workers reported sero-prevalence rates of 9.05% - 16.83% (serotype O), 5.03% - 12.97% (serotype A) and 2.35% - 2.43% (serotype SAT2) compared to 3.5% - 9.3% (serotype O), 4.3% - 10.7% (serotype A) and 1.2% - 5.6% (serotype SAT2) in the course of this work. Previous studies reported that low sero-prevalence estimates, like that known in Northern Sudan, were more associated with NSPs positive SPs negative reactors ($NSP^{+}SP^{-}$) than with NSPs negative SPs positive reactors ($NSP^{-}SP^{+}$) (Bronsvoort *et al.,* 2008; Raouf *et al.,* 2017). Indeed that was one of the reasons to use the serial testing approach in this work since no significant reduction in sensitivity is to be expected. The fraction of NSPs positive SPs negative reactors was found to represent 7/11 and 20% of NSPs positive reactors when low sero-prevalence estimates prevailed in non-buffalo wild ungulates (Bronsvoort *et al.,* 2008) and small ruminants (Raouf *et al.,* 2017), respectively,

compared to 26/327 in buffalo and 4.54% in cattle (Bronsvoort *et al.*, 2008; Raouf *et al.*, 2017). In this work, around 30% of anti-NSPs positive sera failed to react in SPs serology. Such findings were related to epidemiological factors (Raouf *et al.*, 2017) such as mild repeated exposure to multiple serotypes or single predominant serotype rather than mere sensitivity or specificity of either test. Low sero-prevalence estimates were constantly observed to be associated with higher prevalence rates of NSPs antibodies compared to SPs antibodies (Ranabijuli *et al.*, 2010). However, in this study, sero-prevalence estimates of anti-NSPs and anti-SPs antibodies were not significantly different at the lower level of circulation of FMD viruses in the Northern State ($P = 0.106567$) but rather at the relatively higher level in the River Nile State ($P = 0.000725$).

Beside the lower circulation of FMD viruses, patterns of FMD in Northern Sudan showed stark differences from those in other parts of Sudan. Molecular data from Sudan (Habiela *et al.*, 2010b) indicated that within-country circulation is an important mechanism by which serotype O was maintained in the country. Likewise, serological data (Raouf *et al.*, 2016) detected predominance of serotype O antibodies in all studied Sudanese States. Currently, molecular data (http://www.wrlfmd.org/) indicated that recent serotype O isolates were likely exotic to Northern Sudan and perhaps to Sudan. Concurrently, serological data in Northern Sudan detected no predominance of serotype O antibodies in the area. Another difference was that; constantly Northern and Western districts in both States of Northern Sudan showed the lowest sero-prevalences. In contrast, in many other parts of the country, Northern areas were showing higher sero-prevalences than Southern areas; North Kordofan and Darfur States compared to Southern Kordofan and Darfur States, Port-Sudan compared to Kassala and Kassala compared to El Gedarif States (Raouf *et al.*, 2016; Department of FMD Report, 2016). Even in the Nile Basin, Khartoum showed higher sero-prevalence rates than the White Nile, Gezira and Sinnar States (Raouf *et al.*, 2016; Department of FMD Report, 2016). The nonappearance of predominance of serotype O antibodies and the lowest sero-prevalences in Northern and Western areas rather than in Southern and Eastern areas in Northern Sudan have, both, been previously observed (Department of FMD Report, 2016; Saeed, 2019; Saeed and Raouf, 2020). Apparently, neighbouring States of Khartoum and Kassala are the main routes of entry of FMD infections into Northern Sudan. Evidently, the high level of antibody to serotype O (Raouf *et al.*, 2011; 2016; Department of FMD Report, 2016), compared to A and SAT2, in neighbouring areas to

Northern Sudan (unlike other areas), in Kassala and particularly in Khartoum States (around 83%), has proportionally reduced infiltration of serotype O to Northern Sudan. Nonetheless, serotype O antibodies, unlike those of A and SAT2, were significantly higher (P = 0.0126) in areas neighbouring Khartoum and Kassala; Shendi, El Matamma and Ad-Damar, than elsewhere in the River Nile State what suggest comparatively more regular circulation with these States. It is interesting to draw a line here, in other parts of Sudan where antibody of serotype O predominated, Northern areas showed higher sero-prevalences of serotype "O" and NSPs antibodies than Southern areas (Raouf *et al.,* 2016; Department of FMD Report, 2016).

It was suggested that introduction mechanisms of FMD virus to Northern Sudan were far more significant than circulation of FMD virus within the area. Accordingly, recognition of portal of entries of FMD virus to Northern Sudan is equally important. The River Nile State showed consistently higher sero-prevalence rates than the Northern State. Both States fall almost between 16-22 ºN but the River Nile traverse the River Nile State first then the Northern State while North Kordofan State, not Khartoum State, comprise almost the whole of the Southern border of the Northern State. In Northern Sudan, FMD infection is expected to move from South to North while in the other parts of Sudan the case was consistent with the described intense within-country circulation of serotype O. In effect, FMD virus enters the River Nile State first then spread from there to the Northern State along the Nile basin. Particularly in Northern Sudan, animal distribution and movement as well as urban centers are largely confined along the River Nile. In Northern Sudan, apparently, low animal density and relatively limited animal movements coupled with high levels of antibodies to serotype O in neighbouring States of Khartoum and Kassala effectively decrease infiltration of endogenous O strains. Compatible with the suggestion, beside Southern districts in the River Nile State that are neighbouring Khartoum State and Marawi district in the Northern State which is neighbouring the River Nile State, districts containing the State capitals in both States showed high sero-prevalence rates. Sero-prevalences were highest at Ad-Damer at the center of the State and relatively high at the Southern districts of Shendi and El Matamma neighbouring Khartoum State. Also, in the Northern State high prevalences were detected at Dongola, the center of the State and at Marawi district which neighbor the River Nile State and where the River Nile enter the State. Similarly, Saeed (2019) and Saeed and Raouf, (2020) detected higher sero-prevalences at Dongola at the center of the State and at Marawi. Higher prices of meat and livestock in urban centers, such as

States capitals, drive trade animal movements and increase the risk of FMD (Jemberu *et al.*, 2015). State capitals beside Southern district in Northern Sudan were likely the most important portal of entry of FMD viruses into the area. In essence the presented study while confirmed previous findings of low circulation of FMD viruses in Northern Sudan has clearly demonstrated the significance of the introduction mechanism compared to the circulation of the infection within Northern Sudan and within Sudan. A pattern of FMDV infection in Northern Sudan where no predominance of serotype O antibodies, unlike many regions in Sudan, was recognized and explained largely on the high level of serotype O antibodies in the proximity of Northern Sudan. Obviously, a buffer zone in the upper stream areas of Khartoum and Kassala would be effective in controlling the infection in Northern Sudan.

Table 12. Frequency of serotyping O disease events in Sudan 2014-2018 (Department of FMD Report, 2018).

Year	Frequency				
	GZ	KH	RN	NS	Total
2014	1*	1	1		3
2015		3			3
2016		4		1***	5
2017		6**		6***	12
2018	-	-	-	-	-

Gz = Geziera state, Kh = Khartoum state, RN = River Nile state and NS = Northern state.

* SUD/4/2013 collected 30/12/2013 (disease season of 2014).

** Samples were collected from 3 different localities in Khartoum state.

*** Samples were collected in December/2016.

Conclusion and Recommendations

Conclusion:

In Northern Sudan, during the 3 years of the study period, FMD outbreak was reported and diagnosed only once. Low level of FMD infections in Northern Sudan was largely suggested by disease and serological surveillances between 2016 and 2018. Concurrently, unlike other parts of the country, no predominance of serotype O antibodies in bovine sera was detected. Molecular data were also compatible with the inferred low circulation of FMD viruses since a serotype O isolate from Northern Sudan in 2016 was probably originated from outside Sudan rather than being an endogenous strain circulating unabated. It could be concluded that low animal density and limited animal movement in Northern Sudan together with the high antibody levels against serotype O in immediately neighbouring States (Khartoum and Kassala) effectively decreased infiltration of endogenous O viruses.

FMD virus enters the River Nile State first then spread from there to the Northern State along the Nile basin. Also, the presented study while confirmed previous findings of low circulation of FMD viruses in Northern Sudan has clearly demonstrated the significance of the introduction mechanism compared to the circulation of the infection within Northern Sudan and within Sudan.

A pattern of FMD infection in Northern Sudan where no predominance of serotype O antibodies, unlike many regions in Sudan, was recognized and explained largely on the high level of serotype O antibodies in the proximity of Northern Sudan.

Recommendations:

1. Obviously, a buffer zone in the upper stream areas of Khartoum and Kassala would be effective in controlling the infection in Northern Sudan.
2. Restriction of animal movement and vaccination program are recommended for the disease control and should be applied to all animals entering to Northern area from neighboring state.
3. Sanitation and veterinary management is necessary for an effective control of the disease.

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Appendix

1. Preparation of buffers and reagents:

1.1. Deionized Distilled Water (DDW):

Water was distilled by passing through the water distillation device. Then the distilled water was deionized by passing through the Ionic equation device. Deionized Distilled Water (DDW) was sterilized by autoclave at 120°C for 15 minutes.

1.2. Phosphate diluent (PD):

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
DDW completed to	1 L

The solution was sterilized by autoclave at 120°C for 15 minutes then stored at 4°C.

1.3. Normal Saline (NS) (0.8%):

NaCl	4 g
DDW completed to	500 ml

The solution was sterilized by autoclave at 120°C for 15 minutes then stored at 4°C.

2. Cell Culture Medium and Reagents

2.1. Glasgow minimum essential Medium (GMEM) 5X (2L):

125.19 gram of GMEM powder (flow laboratories) were dissolved in DDW to form 2 liters of 5X stock solution, sterilized by filtration through Millipore filter under positive pressure and stored at -20°C.

2.2. Glasgow minimum essential Medium 1X (1L):

GMEM 5X	200 ml
$NaHCO_3$ (7.5%)	3.5 ml
Tryptose Phosphate Broth	100 ml
Penicillin/Streptomycin	1 ml
Gentamycin	1 ml
Fungizon (or mycostatin)	1 ml

(or Amphotercin B	6 ml)
DDW completed to	1000 ml/1 L

The medium was prepared after warming of GMEM 5X in water bath at 37°C, other components, antibiotics and antimycotic were added, mixed well and then stored at 4°C.

2.3. Tryptose phosphate broth (TPB) (1L):

TPB powder	29.5 g
DDW completed to	1 L

The solution was sterilized by autoclave and stored at 4°C.

2.4. Sodium bicarbonate 7.5% solution (Na_2HCO_3):

Na_2HCO_3 powder	7.5 g
DDW completed to	100 ml

The solution was sterilized by autoclave and stored at 4°C.

2.5. Hank's Balanced Salt Solution (HBSS):

Solution (A):

NaCl	80 g
KCl	4.0 g
$CaCl_2 2H_2O$	1.853 g
$MgSO_4.7H_2$	2.0 g
DDW completed to	1300 ml

Solution (B):

Na_2HPO_4	0.6 g
KH_2PO_4	0.6 g
Dextrose	10.0 g
DDW completed to	500 ml
Phenol red (1% solution)	16 ml

Solution (B) was added to solution (A) under continuous stirring, the volume was completed to 2 litres with DDW, sterilized by filtration through Millipore filter and stored at -20°C.

2.6. Tris buffer (0.05 M solution)

Tris	2.42 g

HCL (0.2 M)	76.8 ml (adjust pH to 7.6)
DDW completed to	400 ml

The solution was distributed in 20 ml aliquots, sterilized by autoclave and stored at 4°C.

2.7. Preparation of 0.04 M phosphate buffer:

Na_2HPO4 (0.04 M)	142 g/mol
NaH_2PO	42.84 g
DDW completed to	500 ml

The solution was sterilized by autoclave and the pH was recorded, stored at 4°C.

3. Preparation of cell dispersing agents:

3.1. Preparation of Trypsin (stoke solution 2.5%):

Trypsin powder	12.5 g
PD completed to	500 ml (cold)

The solution was sterilized by filtration through a Millipore filter (0.22 µ) and stored at -20°C.

3.2. Preparation of Versene (5%) (EDTA) solution:

Versene powder	5 g
PD completed to	100 ml (devoid of antibiotics)

The solution was sterilized by autoclaving and stored at 4°C.

3.3. Preparation of Trypsin-Versene solution:

Trypsin 2.5%	6 ml
Versene 5%	4 ml
PD completed to (sterile)	100 ml

The pH was adjusted by addition of few drops of sterile 0.5% phenol red solution, then the acidic pH was shifted to alkaline one by addition of few drops of 1 M NaOH, the solution was stored at 4°C.

4. Preparation of antibiotics for cell culture:

4.1. Penicillin/Streptomycin (Final concentration 200,000 IU/ml; 100 µg/ml):

Benzyle Penicillin powder	2,000,000 I.U (2 vials)
Streptomycin powder	1 g (1 vial)

DDW completed to	10 mL

One gram of streptomycin and the content of 2 vials of Penicillin were dissolved in 10 ml of sterile DDW into universal bottle then mixed well by shaking. The final concentration of the antibiotics in the mixed solution is 200,000 IU/ml Penicillin and 100 μg/ml streptomycin. The solution was stored at -20°C.

4.2. Gentamycin (Final concentration 10,000 μg/ml)

Gentamycin	2 mL (80 mg) (1 ampule)
DDW completed to	6 mL

Gentamycin in one ampule was mixed with 6 ml of sterile DDW to have a solution with final concentration of 10,000 μg/ml, stored at -20°C.

5. Preparation of stains and indicator:

5.1. Formal saline 10%:

Normal Saline (NS)	90 ml
Formalin	10 ml

Mix well and stored at room temperature.

5.2. Crystal Violet stain (0.1%):

Formal saline (10%)	100 ml
Crystal violet powder	0.1 g

The stain was dissolved by shaking and stored at room temperature.

5.3. Phenol red solution (0.5%):

Phenol red	2.5 g
1 M NaOH	20 ml
DDW completed to	500 mL

The solution was sterilized by autoclave and stored at room temperature.

5.4. Phenol red solution (1%):

Phenol red	5 g
1 M NaOH	20 ml
DDW completed to	500 mL

The solution was sterilized by autoclave and stored at room temperature.

6. Viral transport media for preservation of epithelium samples:

Phosphate buffer 0.04 M	50 ml
Glycerol cell culture grade	50 ml
Penicillin/Streptomycin	1 ml
Gentamycin	2 ml
Mycostatin	2 ml
Phenol red 0.5%	2 ml

The viral transport medium (pH 7.2-7.6) was sterilized by autoclave and stored at 4°C. The pH and sterility of the media were checked.

7. Preparation of diluents for ELISA:

7.1. Washing solution 1X:

PBS/Tween 10X	25 ml
DDW	225 ml

7.2. Conjugate:

Prepare both conjugate A and B as fresh preparations:

Conjugate A:

Conjugate A	400 µl
Dilution buffer	3600 µl

Conjugate B:

Conjugate B	200 µl
Dilution buffer	1800 µl