بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Epidemiological Studies of Foot-and-Mouth Disease in Northern and Southern Darfur States, Western Sudan

دراسات وبائية عن مرض الحمي القلاعية في ولايتي شمال وجنوب دارفور ، غرب 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 Laboratory (CVRL), Soba and the Department of Pathology, Parasitology and Microbiology at the College of Veterinary Medicine, Sudan University of Science and Technology from March 2017 to March 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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Dedication

To My Family

My mother: I learnt from you that only hard work makes me special

My father: for your love and patience

My brothers and sister for their understanding

And to all my friends and colleagues

I dedicated this work

Acknowledgements

First and foremost, I would like to thank Allah, who endowing me with health, patience, and knowledge to complete this work.

No word can express my high appreciation and sincere thanks to my supervisor Dr. Nussieba Ahmed Osman Elhag, Assistant Professor of Virology, Department of Pathology, Parasitology and Microbiology, Faculty of Veterinary Medicine, Sudan University of Science and Technology, for her stimulating supervision, valuable advice, kind help and with her aid this work was accomplished.

I wish to express my deepest thankfulness to my co-supervisor Dr. Yazeed Abdal Raouf, Head of the FMD Department, Central Veterinary Research Laboratory, Soba, for his valuable supervision, ideal guidance, fruitful advice, continuous encouragement and constructive criticism throughout the work. He gave me the moral example of what a supervisor professor should be.

I am very thankful to Dr. Selma Kamal Ahmed, Chief Researcher at the Epidemiology Department, Central Veterinary Research Laboratory, Soba, for her kindness, guidance, valuable help in drawing maps and designing the work.

I want to express my appreciation to all my colleagues in the FMD department, Central Veterinary Research Laboratory, Soba, for their kind help during the study; I am greatly indebted to all of them.

Finally, my thanks go to all the people who have supported me to complete my research work either directly or indirectly.

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Abstract

Western Sudan includes Kordofan and Darfur areas. The former is flanking the Nile basin while the latter constitutes the whole of the Western and South Western border areas of Sudan. Darfur area shares border with Libya, Chad, Central African Republic (CAR) and the Republic of South Sudan (SSR). Administratively, it is divided into 5 States; North Darfur in the North and East, South, Central and West Darfur States in the South. Darfur is a chief pastoral area in Sudan where around 10 million cattle and 20 million sheep and goats are reared. During the programme "Surveillances of Trade Sensitive Diseases" (STSD), in 2016, two levels of foot-and-mouth disease virus (FMDV) circulation, as indicated by non-structural proteins (NSPs) serology, were recognized in cattle, the main target species for FMD infection, in Darfur. The higher level was recognized in North and East Darfur States and the lower one in South, Central and West Darfur States.

The presented study meant to investigate FMD infection in Darfur area. Passive and active disease surveillances were utilized and NSPs positive bovine sera from the programme STSD from Darfur area were serotyped against the three current infections of FMD in Sudan; O, A and SAT2. Passive disease surveillance was carried out in South Darfur State; the State with the largest international border area with SSR and almost the whole international border area with the CAR. For logistic reasons, active disease surveillance was carried out in the White Nile State; the State with largest State border area with Western Sudan, rather than in Darfur States. For the serological study, NSPs positive bovine sera from two States were used; Northern and Southern Darfur States, representing respectively the higher and lower levels of FMDV circulating in the study area.

Presumptive diagnosis of clinical FMD was reached in the two surveyed States (South Darfur and the White Nile States) late in 2017 and early in 2018. The clinical signs were mild, hardly detectable and seen in limited number of cattle in limited geographical area. Epithelial samples from these mild cases were barely available and laboratory diagnosis was not achieved; neither locally nor abroad at the World Reference Laboratory (WRL) for FMD where sensitive cell culture system and reverse-transcription polymerase chain reaction (RT-PCR) were used.

In the serological study, 389 bovine sera representing the whole positive lot from North (243) and South (146) Darfur in the STSD stood for the tested sera. Accordingly, the

procedure for determining sero-prevalence of FMD virus (FMDV) serotype-specific antibodies involved serial testing approach i.e. only sera positive in two test systems, ID ELISA and virus neutralization test (VNT), were considered positive. The approach is known to decrease sensitivity but increase specificity. The decrease in sensitivity is not expected to affect interpretation of results since NSPs negative structural protein (SPs) positive reactors are expected to be more associated with the predominant rather than the subordinate serotypes. These reactors might be encountered due to mild repeated infection with the same serotype which is likely to be the predominant one. Indices of FMD infection by sero-prevalence estimates of SPs (combined) antibodies compared to estimates of NSPs antibodies were found to be 55.4% - 66.3% (95% C.I.) compared to 69.1% - 78.6% (95% C.I.) in North Darfur, and 28.8% - 39.1% (95% C.I.) compared to 37.6% - 48.2% (95% C.I.) in South Darfur. They were overlapping where lower level of FMDV circulation prevailed in South Darfur State.

Consistently, higher sero-prevalence rates were detectable in North rather than South Darfur State. The serological study revealed that serotype O was the predominant serotype in the North 43.3%-54.5% (95% C.I.) and in the South 22.3%-32% (95% C.I.) followed by A [21.3%-31.1% and 12.6%-20.7% (95% C.I.)] then SAT2 [9.7%-17.4% and 3.7%-12.4% (95% C.I.)]. It was the same order that was known in most parts of Sudan. The predominant serotype O exhibited a pattern of distribution where it showed statistically significantly higher sero-prevalence estimates in Northern rather than Southern localities in both states (North and South Darfur States); unlike serotype A and SAT2. The described pattern, compared to serotype A and SAT2, was consistent with the more regular circulation of serotype O and with its predicted circulation from the Nile valley to other parts of the country. Northern districts and urban centers that drive animal movements related to trade are important points for entry and circulation of FMDV in Darfur area. Simultaneously, no evidences of effective across border circulation were obtained.

It was concluded that Darfur area was unlikely an important source of FMD viruses to other parts of the country. No particular risk seemed to be associated with animal movement across the Western and South Western border. On the other hand, animal movement to the North and back as part of the pastoral system or due to trade was likely associated with a considerable risk of introduction of the infection to Darfur area.

ملخص البحث

غرب السودان يشمل منطقتي كردفان ودارفور. الأولى تحيط بحوض النيل بينما تشكل الثانية كامل المناطق الحدودية الغربية والجنوبية الغربية للسودان. تشترك منطقة دارفور في الحدود مع ليبيا وتشاد وجمهورية أفريقيا الوسطى وجمهورية جنوب السودان. ومن الناحية الإدارية ، تنقسم إلى خمس ولايات ؛ شمال دارفور في الشمال وشرق وجنوب ووسط وغرب دارفور في الجنوب. ودارفور هي منطقة رعوية كبيرة في السودان حيث يتم فيها تربية حوالي 10 ملايين رأس من الماشية و20 مليون رأس من الأغنام والماعز. خلال برنامج ترصد الأمراض الحساسة للتجارة (STSD) ، في عام 2016م ، تم التعرف على مستويين من الإنتشار لفيروس الحمى القلاعية (FMDV) ، كما إتضح من الإنتشار المصلي للبروتينات غير الهيكلية (NSPs) ، والتي تم التعرف عليها في الماشية ، والتي تمثل فصائل الحيوانات المستهدفة الرئيسية لعدوي الحمي القلاعية في دارفور. وتم التعرف علي أعلى مستوى في ولايتي شمال وشرق دارفور وأدنى مستوى في ولايات جنوب ووسط وغرب دارفور.

هدفت الدراسة الحالية إلى التقصي عن الإصابة بمرض الحمي القلاعية في منطقة دارفور. واستُخدمت عمليات الترصد السلبية والنشطة للمرض ، وتم تحديد النمط المصلي للبروتينات غير الهيكلية (NSPs) للفيروس في مصل الابقار عبر برنامج ترصد الأمراض الحساسة للتجارة من مناطق دارفور في مواجهة الإصابات الثلاث الحالية الناجمة عن مرض الحمى القلاعية في السودان ؛ O ، A وSAT2. وأجريت مراقبة سلبية للمرض في ولاية جنوب دارفور ؛ وهي الولاية التي تضم أكبر منطقة حدودية دولية مع جمهورية جنوب السودان وتقريباً كامل منطقة الحدود الدولية مع جمهورية أفريقيا الوسطى. ولأسباب منطقية ، أجريت مراقبة نشطة للمرض في ولاية النيل الأبيض ؛ وهي الولاية التي تضم أكبر منطقة حدودية مع غرب السودان ، بدلاً عن إجرائها في ولايات دارفور. وبالنسبة للدراسة المصلية ، استُخدمت الامصال البقرية الإيجابية للبروتينات غير الهيكلية (NSPs) من ولايتين ؛ وهما شمال وجنوب دارفور ، واللتان تمثلان على التوالي المستويات الأعلى والادني لإنتشار فيروس مرض الحمي القلاعية في منطقة الدراسة.

تم التوصل إلى التشخيص الإفتراضي السريري لمرض الحمي القلاعية في الولايتين اللتين شملتهما الدراسة الإستقصائية (ولايتي جنوب دارفور والنيل الابيض) في أواخر عام 2017م وأوائل عام 2018م. وكانت العلامات السريرية خفيفة ، وبالكاد يمكن اكتشافها ، وشوهدت في عدد محدود من الماشية في منطقة جغرافية محدودة. وكانت عينات النسيج الظهاري المأخوذة من هذه الحالات الخفيفة متاحة بالكاد ولذلك لم يتحقق التشخيص المختبري ؛ لا محليا ولا خارج البلاد في المختبر المرجعي العالمي (WRL) لمرض الحمي القلاعية حيث تم استخدام نظام زراعة الخلايا الحساسة والنسخ العكسي لتفاعل البوليميراز المتسلسل (RT-PCR).

في الدراسة المصلية ، 389 مصل من الابقار من شمال (243) وجنوب (146) دارفور والتي تمثل كل العينات الموجبة المتحصل عليها من برنامج ترصد الأمراض الحساسة للتجارة كانت هي العينات اتي تم إختبارها. بناء علي ذلك فإن طريقة تحديد الإنتشار المصلي للاجسام المضادة النوعية لفيروس مرض الحمي القلاعية شملت نهج الاختبار التسلسلي وهو يعني فقط أن أي مصل إيجابي في اختباري ، الاليزا ELISA واختبار معادلة الفيروس (VNT) ، يعتبر إيجابي. ومن المعروف أن هذه الطريقة تقلل نسبة الحساسية للإختبار ولكنها تزيد الفعالية. ولكن من غير المتوقع أن يؤثر الإنخفاض في الحساسية على تفسير النتائج لأن المفاعلات الإيجابية للبروتين الهيكلي السلبي (SPs) ترتبط أكثر بالنمط المصلي الاعلي إنتشاراً لفيروس الحمي القلاعية بدلاً عن النمط الاقل إنتشاراً. من

المحتمل ان هذه المفاعلات نتجت من عدوي بسيطة متكررة بواسطة نفس النمط المصلي للفيروس والذي من المرجح انه هو النوع السائد. كانت تقديرات الإصابة بفيروس الحمي القلاعية عن طريق تقدير الانتشار المصلي للأجسام المضادة للبروتينات التركيبية (مجتمعة) مقارنة مع الاجسام المضادة للبروتينات غير التركيبية هي 55.4 - 66.3% (C.I. 95) بالمقارنة مع 69.1 - 78.6% (C.I. 95) في شمال دارفور ، و28.8 - 39.1% (C.I. 95) بالمقارنة مع 37.6 - 48.2% (C.I. 95) في جنوب دارفور. وكانت النتائج متداخلة حيث كان المستوى الأدنى من الإنتشار سائدا في ولاية جنوب دارفور.

تم الحصول علي معدلات أعلى من الإنتشار المصلي للمرض بصورة دائمة في شمال دارفور بدلاً عن جنوبها. ووضحت الدراسة المصلية أن النمط المصلي O هو النمط السائد في الشمال بمعدل 43.3 - 54.5% (C.I. 95) وفي الجنوب بمعدل 22.3 - 32% (C.I. 95) ، يليه النمط المصلي A بمعدل 31.1 - 21.3% و 12.6 - 20.7% (C.I. 95) ، ثم النمط المصلي SAT2 بمعدل 9.7 - 17.4 % و 3.7 - 12.4% (C.I. 95). وهو نفس الترتيب المعروف في اغلب اجزاء السودان. وأظهر النمط المصلي السائد O نمطاً معيناً من التوزيع حيث أظهر تقديرات أعلى بكثير من الناحية الإحصائية للانتشار المصلي في المناطق الشمالية بدلاً عن المناطق الجنوبية في الولايتين (شمال وجنوب دارفور) ؛ بالمقارنة مع النمطيين المصليين A وSAT2. كان النمط الموصوف ، مقارنة بالنمط المصلي A و SAT2 ، متسقاً مع الإنتشار الأكثر انتظاماً للنمط المصلي O ومع الإنتشار المتوقع من حوض النيل الي المناطق الاخري من البلاد. وتشكل المناطق الشمالية والمراكز الحضرية التي تقود تحركات الحيوانات المتعلقة بالتجارة والتي تشكل نقاطا هامة لدخول وحركة فيروس مرض الحمي القلاعية في منطقة دارفور. وفي نفس الوقت لم يتم الحصول على أي ادلة تثبت الحركة الفاعلة عبر الحدود.

وتم استنتاج أن منطقة دارفور من غير المرجح أن تشكل مصدراً هاماً لإنتشار فيروسات مرض الحمي القلاعية إلى أجزاء أخرى من البلاد. ويبدو أنه لا يوجد خطر معين مرتبط بحركة الحيوانات عبر الحدود الغربية والجنوبية الغربية. ومن ناحية أخرى ، من المرجح أن تكون حركة الحيوانات إلى الشمال والعودة منها كجزء من النظام الرعوي أو بسبب التجارة مرتبطة بخطر كبير يتمثل في دخول العدوى إلى منطقة دارفور.

Introduction

Foot and mouth disease (FMD) is one of the most important diseases of livestock since its occurrence can have devastating consequences both for individual farmers and for the economy of a country. This can especially be the case when export trade in animals and animal products form an important component of the general economy of a nation. Foot-and-mouth disease can severely handicap attempts by developing countries to improve the dietary level of protein. This can occur through the drastic losses which FMD can inflict on high grade animals imported to improve productivity (Ferris and Donaldson, 1992). Foot-and-mouth disease is caused by an *Aphthovirus* of the family *Picornaviridae* of seven immunologically distinct serotypes; O, A, C, Asia1, SAT1, SAT2 and SAT3 that, apart from serotype C, show sustained activity (OIE Manual, 2021; Paton *et al.,* 2021).

Sudan has experienced FMD since 1902-1903 (Eisa and Rweyemamu, 1977; http://www.wrlfmd.org). Four FMD virus serotypes namely: O, A, SAT1 and SAT2, had been typed from disease events in cattle (Abu Elzein, 1983). Recent efforts indicated the maintained activity of O, A, SAT2 serotypes (Habiela *et al.,* 2010a; 2010b; Raouf *et al.,* 2010), while the fourth serotype SAT1 was showing insignificant serology and was not typed since 1976 (Raouf *et al.,* 2009). Of the Sudanese domestic ruminant species, cattle is the main target species while sheep and goats undergo silent infection, mostly show limited serology and are likely to play a minor role in the epidemiology of the disease (Abu Elzein *et al.,* 1987; Raouf *et al.,* 2017; Raouf, 2020; http://www.wrlfmd.org). Camels proofed to be refractory to FMD infection (Abu Elzein *et al.,* 1984; Habiela *et al.,* 2010a). Recently, the geographical distribution of FMD in Sudan was described as penetrating along the Nile basin up to Khartoum State whereas more favorable condition was expected in Eastern, Western and Northern Sudan (Raouf *et al.,* 2016). Within-country circulation and long-distance animal movement across the international border are important mechanisms for maintenance of FMD infections in Sudan (Habiela *et al.,* 2010b; Raouf *et al.,* 2016).

Western Sudan includes Kordofan area, that is flanking the Nile basin, and Darfur area that comprises the whole Western and South Western border areas of the country with four different countries. Darfur area is divided administratively into 5 States; North Darfur in the North and East, South, Central and West Darfur States in the South. Darfur area represents a major animal breeding area and a significant part of the pastoral

production system in the country where more than 30 million ruminant susceptible species (cattle, sheep and goats) are reared (Ibrahim, 1999; Department of FMD Report, 2012). In spite of that, information about FMDV infection in Darfur area is limited. The geographical distribution of different FMDV infections was not studied. Merely once, serotype O has been typed from disease events in South and North Darfur in 2005 (http://www.wrlfmd.org/). However, recently non-structural proteins (NSPs) serology identified a high level of infection ranging from 40% in Southern Darfur up to 70% in Northern Darfur (Department of FMD Report, 2016). There is a need to investigate the presence and current situation of FMD in Darfur States. The presented work serotyped NSPs positive cattle sera collected from Darfur area in 2016 during the programme "Surveillance of Trade Sensitive Diseases" (STSD) and investigated suspected FMD events in Darfur and neighbouring areas.

Objectives:

This study was designed to achieve the following objectives:

1- To better understand the epidemiology of FMD in Darfur States.

2- To determine the geographical distribution of different FMD viruses in Darfur.

3- To through light on the circulation and introduction mechanisms of FMD viruses in Darfur States.

4- To determine the epidemiological link of FMD viruses circulating in Darfur or in the nearby areas (e.g. Kordofan and White Nile States).

Chapter I
Review of Literature

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

Foot-and-mouth disease (FMD) could be the most economically important veterinary pathogen. Its high infectivity and transboundary nature, ability to cause persistent infections and its long term effects on the condition and productivity of the different animal species it affects are among the hallmark of the infection. In addition, many trade restrictions were placed against countries endemic with the disease (Knowles and Samuel, 2003).

FMD affects extensive areas worldwide and is included within the list of diseases notifiable to the World Organization for Animal Health (OIE Manual, 2021). It is recognized as a significant epidemic disease threatening cattle industry since the sixteenth century. Currently, FMD is considered a major global animal health problem (Longjam *et al.,* 2011).

1.2. History of the disease:

The first description of FMD was probably in Northern Italy in 1514 by Hieronymi Fracastorii (Knowles, 1990). Loeffler and Frosch described a disease caused by a filterable agent and made the first observation that an animal disease could be caused by a virus (Loeffler and Frosch, 1997; 1898; Mahy, 2005). In 1910, the first FMD research institute was built on the island of Riems in Germany, followed by other research institutes in Europe, e.g. in Pirbright, United Kingdom in 1925 and in Lindholm island, Denmark in 1926 (Klein, 2009). In search for experimental laboratory animals for the infection, Waldmann and Pape demonstrated the susceptibility of the guinea pig in 1920 and Skinner demonstrated the susceptibility of the suckling mouse in 1951 (Brown, 2003).

In the 1920s, Valle´e and Carre´ in France and Waldmann in Germany made the discovery of three distinct serotypes: O, A and C of FMD virus (FMDV). Subsequently, in the 1940s and 1950s, followed the recognition of the three Southern African Territory Types SAT 1-3, and Asia1 by the Pirbright group (Brown, 2003).

The development of in-vitro techniques for the growth of the virus has been crucial for the large-scale production of vaccines and for the accurate assay of virus infectivity. Hecke and the Maitlands in the early 1930s were successful in growing the virus in-vitro. Frenkel in

1947 had showed that large amounts of the virus could be produced in surviving tongue epithelium what formed the basis for the vaccination programmes initiated in Europe in the 1950s (Brown, 2003). Cell lines, in name, baby hamster kidney cells-21 (BHK-21) grown in monolayer (Mowat and Chapman, 1962) or suspension (Capistic *et al.,* 1962) have then replaced tongue epithelium in production of FMD vaccines. Fermenters technology permits further larger-scale production of FMD vaccines (Telling and Elsworth, 1965).

The last milestone has been the advent of molecular technology which permits rapid diagnosis (Longjam *et al.,* 2011), tracing of strains isolated from outbreaks (Knowles and Samuel, 2003) and designing new vaccines (Brown, 2003).

1.3. Etiology:

Foot-and-mouth disease virus is the type species of the genus *Aphthovirus* of the family *Picornaviridae*. It has a single-stranded positive-sense RNA genome that possesses high potential for genetic and antigenic variation. Seven recognized serotypes of FMDV (O, A, C, SAT1, SAT2, SAT3 and Asia1) and a wide range of variants and subtypes have been defined (Murphy *et al.,* 1999; Longjam and Tayo, 2011).

All FMDV serotypes are immunogenically different and infection or vaccination with one serotype does not develop immunity against other serotypes (Kitching *et al.,* 1989; Kitching, 1998).

1.3.1. Taxonomy of *Picornaviridae* family:

The family *Picornaviridae* belongs to the order *Picornavirales.* Currently, *Picornaviridae* consists of >75 species grouped into >30 genera such as: Aphthovirus, Enterovirus, Teschovirus, Cardiovirus, Erbovirus, Kobuvirus, Hepatovirus, and Parechovirus (Knowles *et al*., 2012; Zell *et al.,* 2017). The genus *Aphthovirus* contains beside FMDV, equine rhinitis A virus, bovine rhinitis A virus, and bovine rhinitis B virus (Maclachlan and Dubovi, 2011).

1.3.2. Resistance to physical and chemical action:

The technical disease card of the OIE on FMD (OIE, 2021a) described, comprehensively, the resistance and survival of FMDV as follow:

Temperature: The virus is preserved by refrigeration and freezing. It is progressively inactivated by temperatures above 50°C. Heating animal products to a minimum core temperature of 70°C for at least 30 minutes inactivates the virus.

pH: The FMDV is quickly inactivated by pH < 6.0 or > 9.0.

Disinfectants: Preparations of FMDV are inactivated by 2% sodium hydroxide, 4% sodium carbonate, 0.2% citric acid, 2% acetic acid, 3% sodium hypochlorite, 1% potassium peroxymonosulfate/sodium chloride, and chlorine dioxide but resistant to iodophores, quaternary ammonium compounds, and phenol, especially in the presence of organic matter.

Survival: The FMDV survives in lymph nodes and bone marrow at neutral pH, but get destroyed in muscle at pH < 6.0 i.e. after *rigor mortis*. Residual virus survives in milk and milk products during regular pasteurization, but is inactivated by ultrahigh-temperature pasteurization. The virus survives drying and may persist for days to weeks in organic matter under moist and cool temperatures. It can also persist in contaminated fodder and in the environment for up to one month depending on the temperature and pH conditions.

1.3.3. Morphology and structure of the virus:

The FMDV particle is roughly spherical in shape and about 25-30 nm in diameter. It consists of the RNA genome surrounded by a protein shell or capsid. The capsid is composed of 60 copies of the capsomeres. Each capsomere consists of four structural polypeptides (VP1, VP2, VP3 and VP4). The VP1, VP2 and VP3 are exposed on the surface of the virus while VP4 is located internally (Figure 1) (Belsham, 2005; Jamal and Belsham, 2013; Gao *et al.,* 2016; Malik *et al.,* 2017).

The protein coat surrounds a single-stranded, positive-sense RNA genome of about 8400 nucleotides (nt) in length. The RNA includes three separate parts i.e. the 5′ untranslated region (5′ UTR), a long coding region [a single long open reading frame (ORF)] and the 3′ untranslated region (3′ UTR) (Figure 2). A small protein termed VPg (24 or 25 residues long) which is encoded by the 3B portion of the viral genome region, is covalently linked to

the 5′ end of the genome. The 5′ UTR is about 1300 nt in length (Belsham, 2005; Gao *et al.,* 2016) and consists of an S fragment at its 5′ end, a poly-C tract, a series of RNA pseudo knot structures, a cis-acting replication element (cre) (also known as the 3B-uridylylation site “bus”), and the internal ribosome entry site (IRES) (Figure 2) (Jamal and Belsham, 2013; Gao *et al.,* 2016). The coding region, follows the 5′ UTR, is about 7000 nt in length and considered the major portion of the viral genome. It encodes a large polyprotein which is then cleaved by the viral proteases to form four different structural and eleven different non-structural proteins plus a variety of precursors, some of which have distinct functions (Jamal and Belsham, 2013; Gao *et al.,* 2016).

The 3′ UTR, much shorter than the 5′ UTR, is about 90 nucleotides long and folds to form a specific stem-loop structure, followed by a poly-A tract of variable length (Dorsch-Häsler *et al.,* 1975). The 3′ UTR plays an important role in viral genome replication (Figure 2) (Jamal and Belsham, 2013; Gao *et al.,* 2016).

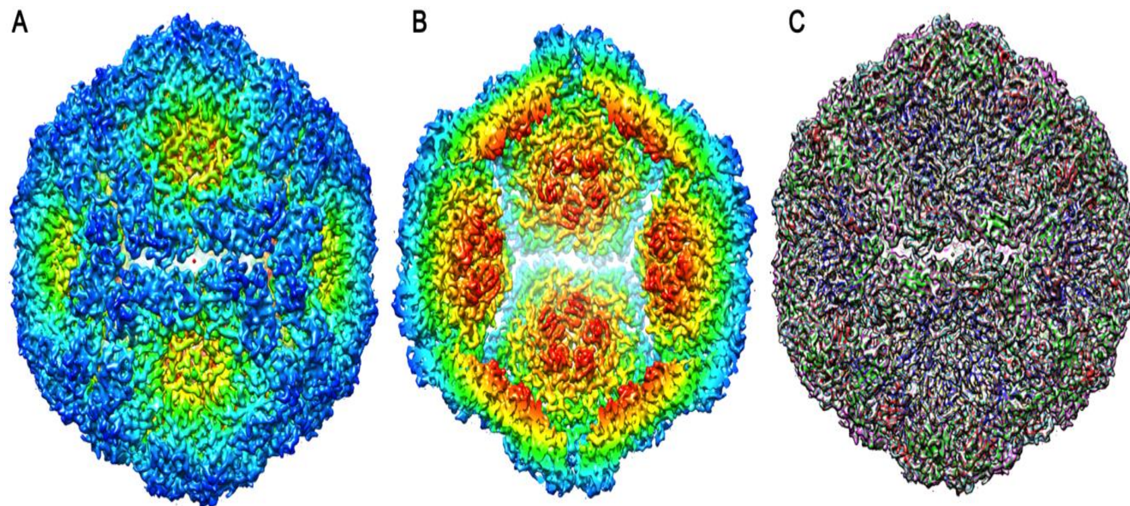

Figure 1. A structure of the FMDV inside-out particle. A and B: show the viral particle and a cross-section through the particle viewed down the two-fold icosahedral symmetry axis, coloured radially from the centre (<110 A red; 120–140 A: yellow; >150 A: blue). C: Atomic fitting of the structure into the electron density map (transparent grey render) to generate the whole virus structure of the inside-out particle. Individual protein chains VP1, VP2 and VP3 are coloured blue, green and VP3: red, respectively (Malik *et al.,* 2017).

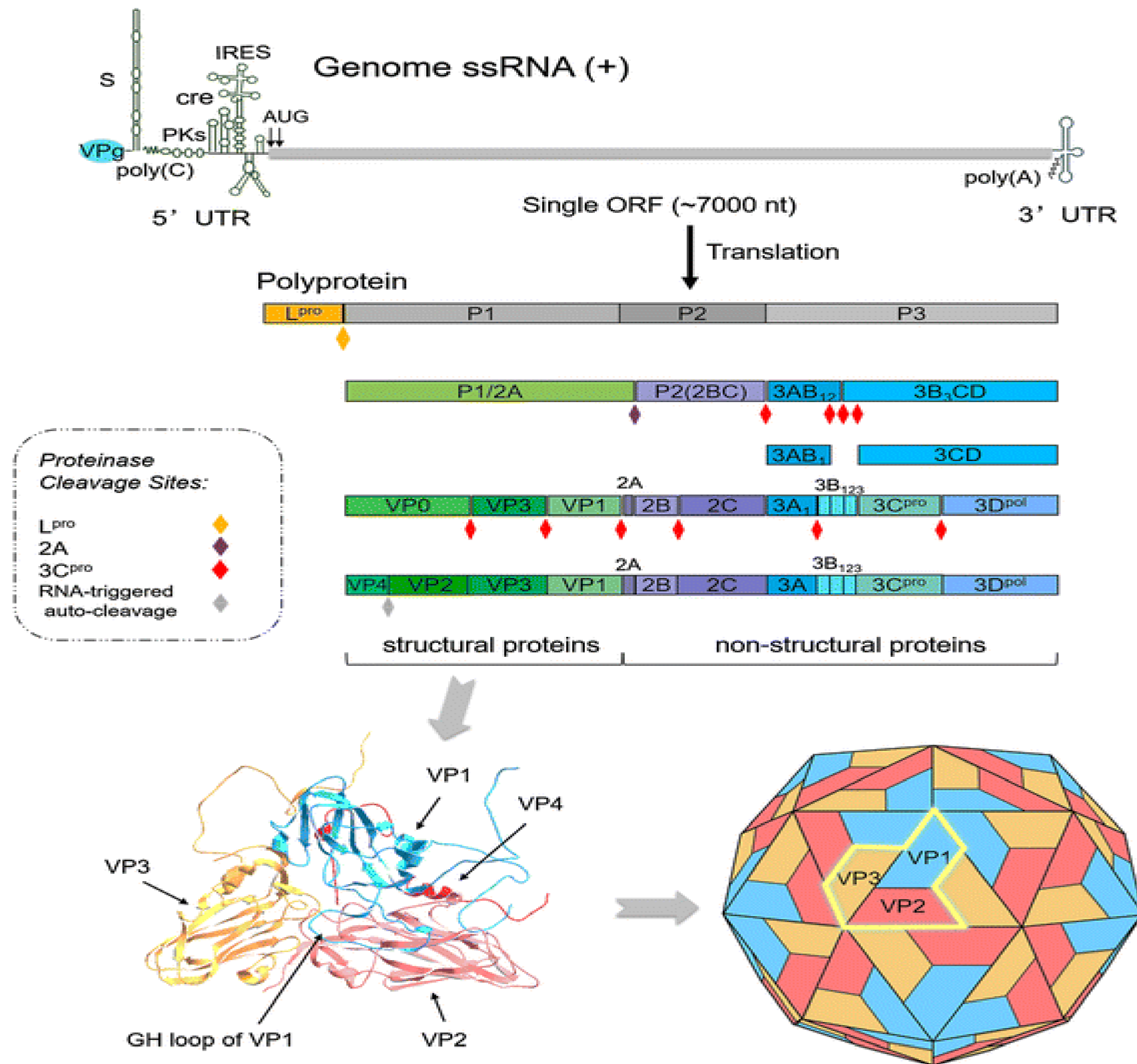

Figure 2. Schematic diagram of FMDV genome, processing of viral polypeptide and conformations of the structural proteins. FMDV RNA genome contains a single open reading frame (ORF) of about 7 Kb with two alternative initiation sites. The ORF is flanked by a long 5′-untranslated region (5′-UTR) and a short 3′-UTR. 3B (VPg) is covalently bound to its 5′ end. The ORF region is generally divided into four functional areas (L, P1, P2 and P3) due to the different functions of mature polypeptides. ORF-encoded polyprotein is processed into four products, L^{pro}, P1-2A, 2BC and P3 by L^{pro}, 2A and $3C^{pro}$. The precursors P1-2A, 2BC and P3 are further processed into mature viral proteins and some cleavage intermediates with relative stability, such as VP0 or 1AB, 3ABC, 3BCD, 3AB, and 3CD by $3C^{pro}$. Structural proteins form the biological promoter and viral capsid (Gao *et al.*, 2016).

1.4. Infection cycle:

Foot-and-mouth disease virus has a relatively short infectious cycle in cultured cells. Depending on the multiplicity of infection, newly formed infectious virions begin to appear at between 4 and 6 h after infection. The virus is cytocidal, and infected cells exhibit cytopathic effects which include cell rounding and alteration and redistribution of internal cellular Membranes. The virus also causes biochemical alterations including inhibition of host translation and transcription (Grubman and Baxt, 2004; Maclachlan and Dubovi, 2011).

The first step in infection of cells is attachment and adsorption. Foot-and-mouth disease virus utilizes different receptors to attach to cells. Molecules that might act as receptors for FMDV include mainly integrins and heparin sulphate but other types of molecules are also suggested (Ruiz-Sáenz *et al.,* 2009). Integrins are proteins used by cells to bind and respond to the extracellular matrix. Heparan sulfate [a glycosaminoglycan (GAG)] is the most abundant heteropolysaccharide in the body (Ruiz-Sáenz *et al.,* 2009). After adsorption, the virus can enter the cells using different pathways; the main one is binding integrins via the clathrin-mediated endocytosis pathway where the capsid rapidly dissociates in the acidified endocytic vesicles resulting in the release of the RNA genome. The second one using heparan sulfate in which FMDV enters the cells using the caveola-mediated endocytosis pathway and that caveolae can associate and traffic with endosomes to allow the low PH of the endosomes to trigger uncoating (Ruiz-Sáenz *et al.,* 2009). Other mechanisms of uncoating that do not involve receptor-mediated endocytosis, as described above, were also reported (Grubman and Baxt, 2004).

However, the infection process begins once the virus has delivered the viral RNA to the cytoplasm of the host cell. The viral RNA begins a round of viral translation before any transcriptional step. It serves immediately as a mRNA to direct synthesis single polypeptide which undergoes a series of cleavages reactions leading to the production of both structural and non-structural proteins (Gao *et al.,* 2016). Most of the primary cleavage reactions are performed by three virus-encoded proteinase; Lpro, 2A and 3C and involves production of many precursors and cleavage intermediates. The first translation product is Lpro which cleaves itself from the growing polypeptide chain. The role of the Lpro is to impair what is called cap-dependent translation required for cellular mRNA translation. Cellular mRNA

possesses a cap structure at the 5´ end while the viral RNA lacks this cap structure and requires cap-independent translation. Accordingly, rapid translation of viral proteins and shutoff most host protein synthesis are achieved. Ten of the remaining thirteen cleavages are performed by the 3Cpro apart from maturation cleavages (Figure 3) (Mason *et al.,* 2003; Gao *et al.,* 2016).

Transcription and replication of FMD viral RNA has not been well studied but models known for other picronaviruses and poliovirus are quite similar (Mason *et al.,* 2003; Grubman and Baxt, 2004). In some of these models, it was suggested that synthesis of the complementary minus-strand begins after translation of the plus-strand RNA ceases. In FMDV infected cells there are evidences for the opposite (Grubman and Baxt, 2004).Well known is that replication of the RNA is a function of the RNA-dependent RNA polymerase; earlier known as FMD virus infection associated antigen (FMD-VIAA). The synthesized minus-strand is not detected in infected cells since it is incorporated in a replicative form (RF) of double-stranded molecule. New plus-strand synthesis begins from the RF (Mason *et al.,* 2003; Grubman and Baxt, 2004).

The final steps in the replication cycle are the encapsidation (packaging of RNA into mature virions) and maturation. Only the plus-strand viral RNA is encapsidated. The maturation cleavage of VP0 to VP2 and VP4 (Figure 3) to form the mature virion occurs after encapsidation of the RNA (Grubman and Baxt, 2004; Gao *et al.,* 2016).

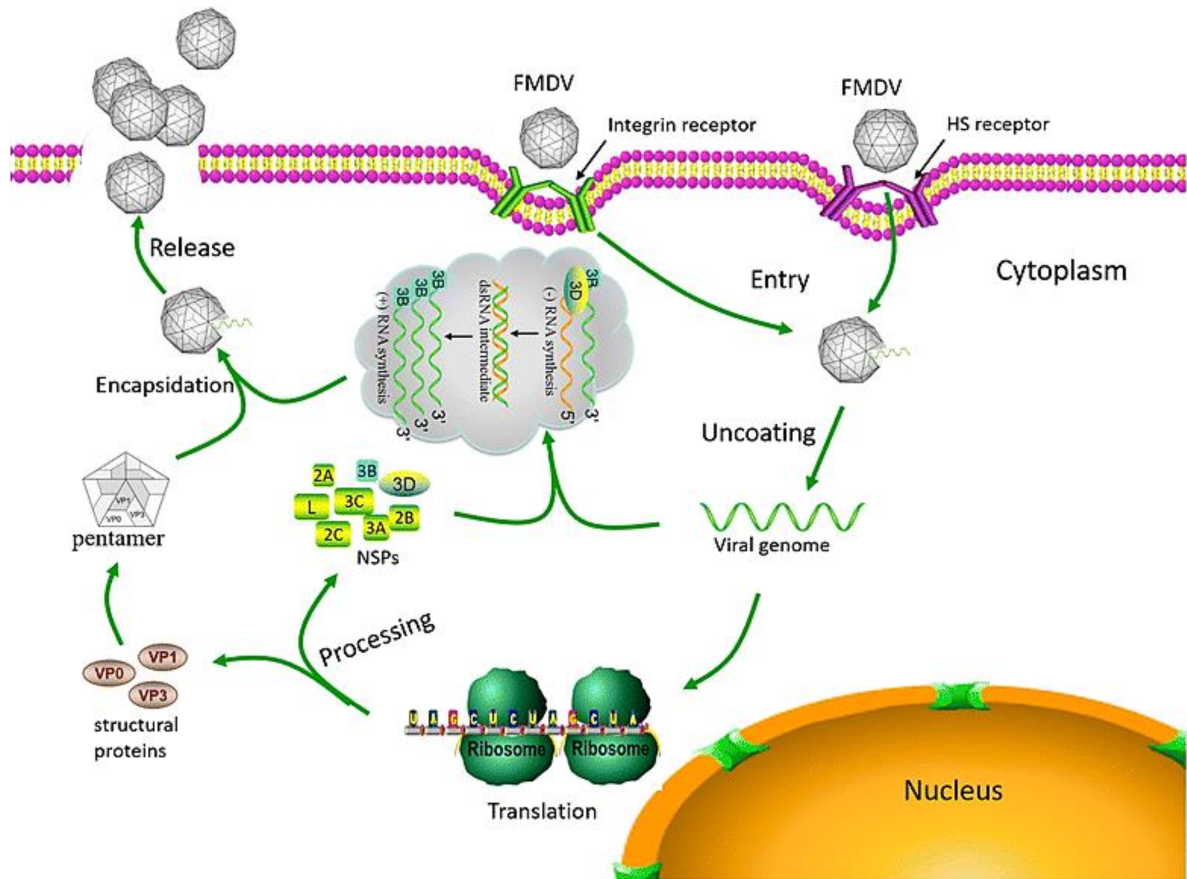

Figure 3. Life cycle of FMDV in the host cells. NSPs, non-structural proteins. HS, heparan sulfate. Green line, viral positive-strand (+) RNA. Orange line, viral negative-strand (-) RNA (Gao *et al.,* 2016).

1.5. Genetic and antigenic variation of FMDV:

Antigenic variation is one of the striking characters of FMD virus. There are seven immnologicaly/antgenically distinct serotypes of FMDV and multiple subtypes and variants within each serotype (Grubman and Baxt, 2004; Brito *et al.,* 2017). In general terms, antigenic variation is a process by which an infectious organism alters its surface proteins. Genetically, it is associated with mutations leading to amino acid replacement. These changes may happen either in the field due to continued circulation or in the laboratory during passage in cell culture or laboratory animals. It permits the pathogen to evade the host immune response, complicate diagnosis, challenge the vaccine and compromise control but facilitate traceability (Longjam and Tayo, 2011; Domingo *et al.,* 2002).

Changes in the VP1 region of the virus capsid proteins account for the low cross-reactivity observed among different serotypes of FMDV. This region is encoded within the P1 region of the open reading frame (ORF) of the FMDV genome. Sequence variability in the P1 region may occur through different mechanisms like high rates of mutation and genetic recombination (Longjam *et al.,* 2011). Mutations may also occur within other regions of the virus genome like the non-structural protein-coding regions. However, such mutations are probably less tolerated, since proteins encoded by these regions are necessary for viral replication and changes are more likely to be lethal (Grubman and Baxt, 2004).

RNA viruses in general have very high mutation rates, in the range of 10^3 to 10^5 per nucleotide site per genome replication, due to the lack of error correction mechanisms during RNA replication. The quasi-species concept was developed to explain the effects of errors in replication on the evolution of replicating RNA molecules (Domingo *et al.,* 2002). In the initial theoretical formulation (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 (Biebricher and Eigen, 2006; Domingo and Perales, 2019).

Usually, cross protection test (Periera, 1978) and virus neutralization test (VNT) (Rweyemamu, 1984), were used to identify intratypic antigenic differences of FMDV. Earlier efforts, based on cross protection, showed the ever increasing numbers of subtypes (over 60) (Periera, 1976). Later, a more pragmatic approach was adopted when field isolates were compared with reference vaccine strains using the VNT and r1 value to demonstrate protection and avoid the complexity of subtype classification (Rweyemamu *et al.,* 1977; Rweyemamu, 1984). In a similar manner, comparative sequence analysis of the P1 gene is used to measure genetic diversity of FMDV. It has revealed the circulation of groups of genotypes with, usually, less than 15% nucleotide difference within geographical boundaries and used the term topotypes to describe them (Samuel and Knowles, 2001). Ten topotypes were described for FMDV serotype O (Knowles and Samuel, 2003). Serotype A, the most genetically and antigenically diverse, was classified into three major geographical restricted genotypes; Europe, Asia and Africa (Knowles and Samuel, 2003) and Tosh *et al.* (2002) described 10 major serotype A genotypes (I-IX). Serotype C isolates from Europe and South America were classified into 8 topotypes (Knowles and Samuel,

2003). Asia1 is the least diverse and was included in one topotype (Ansell *et al.,* 1994; Knowles and Samuel, 2003). Southern African Territories (SAT) serotypes are normally restricted to sub-Saharan Africa with three distinct serotypes SAT 1-3. Serotype SAT-1 was classified into eight topotypes; serotype SAT-2 (the most diverse) into fourteen topotypes, and serotype SAT-3 into six topotypes (Vosloo *et al.,* 2004).

1.6. Geographical distribution of FMD:

Foot-and-mouth disease has historically occurred across most of the world. It is endemic in parts of Asia, Africa, the Middle East and South America but never known in a few isolated countries such as New Zealand and Iceland (Rweyemamu *et al.,* 2008). While serotypes O and A are widely distributed, SAT viruses occur mainly in Africa (with periodic incursions into the Middle East), Asia1 is currently found only in Asia (Kitching, 2005), and type C appears to be the first extinct serotype (Paton *et al.,* 2021). Currently, North and Central America, New Zealand, Australia, Greenland, Iceland and Western Europe are free of FMD. Western Europe was affected by some recent outbreaks (eradication was successful), but FMD has not been reported in North America for more than 90 years (Rweyemamu *et al.,* 2008). The last U.S. outbreak occurred in 1929, while Canada and Mexico have been FMD-free since 1952-1953 (Jamal and Belsham, 2013). The OIE identifies member countries and zones within thee status (Figure 4).

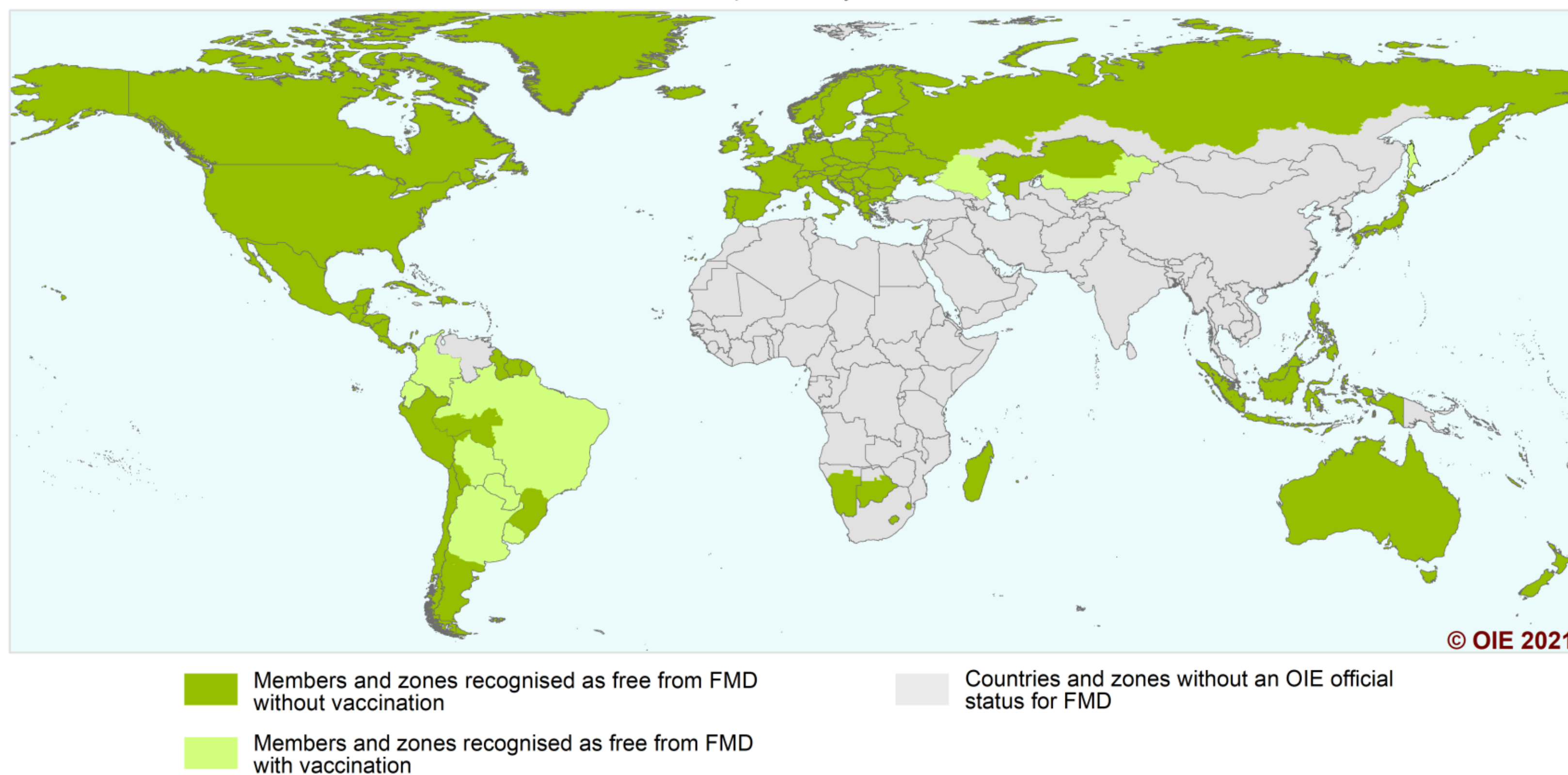

Figure 4. Official foot-and-mouth-disease status map. World Organization for Animal Health (OIE, 2021b).

(Available from: https://www.oie.int/app/uploads/2021/05/fmd-world-eng.png).

1.7. FMD in Sudan:

In Sudan, clinical FMD was initially reported in 1903 in cattle (Eisa and Rweyemamu, 1977). Four FMD virus serotypes namely: O, A, SAT1 and SAT2, have been typed from disease events in Sudanese cattle (Abu Elzein, 1983). Recent data in Sudan indicated the maintained activity of three serotypes, O, A and SAT2, while serotype SAT1 has not been serotyped since 1976 (Raouf *et al.,* 2009; 2010; 2016; 2017; Habiela *et al.,* 2010a; 2010b; http://www.wrlfmd.org). Clinical FMD has not been confirmed in Sudanese sheep and goats which undergo silent infection as evident by seroconversion (Abu Elzein *et al.,* 1987; Habiela *et al.,* 2009; 2010a; Raouf *et al.,* 2012; 2017; Raouf *et al.,* 2022). Sheep and goats are likely to play a diminished role in the epidemiology of the disease in Sudan (Raouf *et al.,* 2017; Raouf *et al.,* 2022) whereas camels are free of infection (Habiela *et al.,* 2010a).
Disease and serological surveillance in Sudan showed that serotype O FMDV was the most predominant followed by serotype A then SAT2 (Raouf *et al.,* 2010; 2016; Habiela *et al.,* 2010b; http://www.wrlfmd.org). Molecular data indicated that all Sudanese serotype O viruses belong to the O/EA-3 topotype (Habiela *et al.,* 2010b; http://www.wrlfmd.org).
Serotype A FMDV was recorded for the first time in Sudan in 1957 (Abu Elzein, 1983). All isolates of serotype A fell in the same genotype-strain linage G-IV within the topotype Africa (Habiela *et al.,* 2010b; http://www.wrlfmd.org).
FMDV serotype SAT2 was the last one to be detected in Sudan in 1977 (Abu Elzein and Crowther, 1979). Phylogenetically, serotype SAT2 viruses in Sudan were of two toptypes, VII and XIII (Habiela *et al.,* 2010b; http://www.wrlfmd.org).
The geographical distribution of FMD in Sudan was described as penetrating along the Nile Basin up to Khartoum State and more favorable in Northern, Eastern and Western Sudan (Raouf *et al.,* 2016; Saeed and Raouf, 2020; Ahmed *et al.,* 2021).

1.8. The disease:

1.8.1. Host range of FMD:

A wide range of cloven-footed animals contract FMD including cattle, pigs, sheep, goats, buffalo and various wildlife species of the order *Arteriodactyla*. Highly resistant animals include horses and carnivores (Alexandersen and Mowat, 2005). The disease is severe in

pigs, obvious in cattle, and in-apparent or mild in adult sheep and goats (Kitching, 2002). Foot-and-mouth-disease has been reported in at least 70 species of wild (or captive wild) artiodactyls, in addition to few animals that are not members of the Artiodactyla, such as hedgehogs (both *Erinaceus europaeus* and *Atelerix prurei*), armadillos, kangaroos, nutrias (*Myocastor coypus*), and capybaras (*Hydrochaerus hydrochaeris*) (Arzt *et al.,* 2011a). The Cape buffalo (*Syncerus caffer*) in Africa have been identified as natural hosts for the SAT serotypes of FMDV, although they may be infected by all serotypes (Hedger *et al.,* 1973; Hedger, 1976; Condy *et al.,* 1985; Dawe *et al.,* 1994a; Tekleghiorghis *et al.,* 2014) The Water Buffalo (*Bubalus bubalis*) can become infected and may also transmit infection to other species (Arzt *et al.,* 2011b).

Neither experimental data nor observations on natural infection of FMD have shown susceptibility of dromedary camels to FMDV while Bactrian camels and new world camelids [Llama (*Lama glama*)] have been shown to be susceptible (Wernery and Kaaden, 2004; Larska *et al*., 2009; OIE Manual, 2021). Foot-and-mouth-disease is not considered a zoonotic. Clinical cases in human were extremely rare in relation to human exposure during outbreaks (Davies, 2002; Depa *et al.,* 2012).

1.8.2. Pathogenesis:

Foot-and-mouth-disease virus spreads from infected to susceptible animals by direct contact, or indirectly through mechanical transfer. The virus gains entry into recipient animal through cuts and abrasions in the skin or mucosae, or by the deposition of droplets or droplet-nuclei (aerosols) in the respiratory tract (Alexandersen *et al.,* 2003a). 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; Weaver *et al.,* 2013).

During acute infection, transmission is facilitated by virus shedding from ruptured vesicles and by virus excretion in different bodily excretions and secretions, including breath, milk and semen (Alexandersen *et al.,* 2003a). Susceptible ruminants can be infected the inhaled virus through direct contact with the breath of other acutely infected animals, or indirectly by resuspension of aerosols from contaminated materials.

Pathogenesis of FMD has been most thoroughly investigated in cattle then pigs and minimally in other animals (Alexandersen *et al.,* 2003a; Grubman and Baxt, 2004; Arzt *et al.,* 2011a; 2011b). In these studies, different virus strains (McVicar and Sutmoller, 1976; Arzt *et al.,* 2017), different techniques of animal exposure, different methods of virus detection (McVicar and Sutmoller, 1976; Burrows *et al.,* 1981; Brown *et al.,* 1992; 1996; Pacheco *et al.,* 2010; Arzt *et al.,* 2010) and different tissues were used. It was suggested that lack of consensus in many aspects of FMD pathogenesis and apparently contradictory conclusions in these studies reflect intrinsic differences in the design of these experiments and should be accepted (Grubman and Baxt, 2004; Arzt *et al.,* 2011a). For convenience, Arzt *et al.* (2011a) used three terms to discuss pathogenesis; pre-viraemia, viraemia and post- viraemia. Pre-viraemia begins by infection, characterized by virus replication at the primary replication site and ends by detection of virus in blood. Viraemia is when the virus can be detected in blood and coincide with virus replication at the second replication site and with clinical disease. Post-viraemia starting with negative assay of virus in blood and including resolution of clinical disease, persistent infection (carrier state) and long term sequelae such as heat-intolerance and thyroid dysfunction.

When the virus gains entry through the respiratory tract, the primary site of virus replication is within the respiratory tract. However, reports conflict about the region of the respiratory tract of initial virus replication in cattle (Alexandersen *et al.,* 2003a, Grubman and Baxt, 2004; Artz *et al.,* 2011a). Some reports suggested that the pharynx (nasopharynx) and not the lungs is primary site of virus replication (Burrows *et al.,* 1981; Zhang and Kitching, 2001; Alexandersen *et al.,* 2003a; Stenfeldt *et al.,* 2016a). More recent studies (Pacheco *et al.,* 2010; Arzt *et al.,* 2010) suggested a certain dynamic of infection in cattle respiratory tract as the virus become more prominent within the lungs and less apparent in the pharyngeal tissues as viraemia approach. Brown *et al.* (1992; 1996) indicated the lungs as the primary site of virus replication. The primary site of virus replication is the known predilection sites in the tongue and the interdigital space when the virus gain entry through skin and mucosae or gain entry directly to the circulation (Alexandersen *et al.,* 2003a; Alexandersen and Mowat, 2005).

1.8.3. Incubation period:

The incubation period for FMD can vary with the species of animal, the dose of virus, the viral strain and the route of inoculation. It is reported to be one to 12 days in sheep, with most infections appearing in 2-8 days; 2 to 14 days in cattle; and usually 2 days or more in pigs (some experiments reporting clinical signs in as little as 18-24 hours). Alexandersen *et al.* (2003b) reported it would typically be 2-6 days. Other reported incubation periods are 4 days in wild boar, 2 days in feral pigs, 2-3 days in elk, 2-14 days in Bactrian camels, and possibly up to 21 days in water buffalo infected by direct contact (Arzt *et al.,* 2011a; 2011b).

1.8.4. Clinical signs:

FMD is typically an acute febrile illness with vesicles (blisters) localized on the feet, in and around the mouth, and on the mammary gland. Vesicles occur occasionally at other locations including the vulva, prepuce, or pressure points on the legs and other sites. The vesicles usually rupture rapidly, becoming erosions. Pain and discomfort from the lesions leads to clinical signs such as depression, anorexia, excessive salivation, lameness and reluctance to move or rise (Alexandersen *et al.,* 2003a; OIE Manual, 2021). Lesions on the coronary band may cause growth arrest lines on the hoof. In severe cases, the hooves or footpads may be sloughed. Reproductive losses are possible, particularly in sheep and goats. Deaths are uncommon except in young animals, which may die from multifocal myocarditis or starvation. Most adults recover within 2 to 3 weeks, although secondary infections may slow recovery. Possible complications include temporary or permanent decreases in milk production, hoof malformations, chronic lameness or mastitis, weight loss and loss of condition (Alexandersen *et al.,* 2003a; OIE Manual, 2021).

The clinical outcome of the disease may vary among the host species considered and the infecting virus strain. In cattle, especially the highly productive breeds often have severe clinical signs, they usually become gradual or sudden, severe decreased in milk production. However, in some cases milk may not be produced again until the next lactation, or milk yield may be lower indefinitely. Hoof lesions, with accompanying signs of pain, occur in the area of the coronary band and interdigital space. Young calves may die of heart failure without developing vesicles (Alexandersen *et al.,* 2003a).

Other complications include mastitis or hoof malformations. Some cattle that recover from FMD are reported to develop heat-intolerance syndrome (HIS; also called 'hairy panters') (Arzt *et al*., 2011b).

Severe cases of FMD in pigs may result in the sloughing of the hooves of one or more feet causing severe pain. Furthermore, secondary bacterial infection of these foot lesions may occur leading to severe and debilitating lameness, especially when animals are kept in unhygienic conditions. Generally, foot lesions heal more slowly than mouth lesions (Alexandersen *et al.,* 2003a).

Severe cases of FMD can occur in small ruminant, however, infection tends to be mild in sheep and goats. Most infected animals may be asymptomatic or have lesions only at one site (Kitching, 2002).

The symptoms of FMD in wildlife are similar to those described in domestic animals, although the pathogenesis of FMD virus in many susceptible wildlife species has not been extensively studied (Weaver *et al.,* 2013).

1.9. Carrier status (Persistence of infection):

Van Bekkum *et al.* (1959) showed the presence of infectious virus in the "saliva" [oesophageal-pharyngeal (OP) fluid] of asymptomatic cattle for many weeks after infection with FMDV. This discovery led to a number of studies noteworthy of Burrows (1966) demonstrating tissue-specific localization of persistence in dorsal soft palate and dorsal pharynx. Hence carrier state of FMD is known and defined as asymptomatic animals being virus positive for more than 28 days after infection (Sutmoller *et al.,* 1968). The subject received particular attention and in-depth reviews were made available (Salt, 1993; 1998; Alexandersen *et al.,* 2002a; 2003b; Grubman and Baxt, 2004; Arzt *et al.,* 2011a; Stenfeldt and Arzt, 2020). Carrier state was also detected in sheep and goats (Burrows, 1968; Alexandersen *et al.,* 2002a). Pigs were known to clear the infection in only 3 weeks and so do not become carriers. However, some recent studies detected FMDV RNA but not live virus beyond 28 days post-infection (Orsel *et al.,* 2008; Zhang and Bashiruddin, 2009; Mohamed *et al.,* 2011). A more recent work disputed the conclusion of these findings and concluded that pigs are unlikely to be competent long-term carriers of infectious FMDV; however, transient persistence of FMDV protein and RNA in lymphoid tissues is common

following clinical or subclinical infection (Stenfeldt *et al.,* 2016b). Regarding wildlife, it is well established that the African buffalo can carry the virus for several years (Hedger, 1972). Other cloven-hoofed wildlife species including deer and impala, which may get acutely infected, are unlikely to be involved in "the carrier problem" (Thomson *et al.,* 1984). Maximum duration of carrier state in cattle is 3.5 years, sheep 9 months, goat 4 months and buffalo 5 years (Alexandersen *et al.,* 2002a).

The target region involved in persistent infection in cattle is the pharynx, more specifically the dorsal soft palate and the dorsal part of the pharyngeal ceiling located above the soft palate (Alexandersen *et al.,* 2002a). Stenfeldt and Arzt (2020) observed that the term "pharynx" was not properly defined in earlier publications and distinguished between oropharynx and nasopharynx and pointed that beside the dorsal soft palate what relevant is the dorsal nasopharynx. In situ hybridization (Zhang and Kitching, 2001) and more recent studies (Stenfeldt *et al.,* 2016a; 2018) provided direct evidence of involvement of epithelial cells in these anatomical areas, rather than other components (lymphoid tissues), in the persistent infection.

The risk of disease transmission from carriers remains a controversial topic. Transmission from carrier animals has only been convincingly demonstrated from African buffalo for SAT2 virus (Dawe *et al.,* 1994a; 1994b; Parthiban *et al.,* 2015; Bertram *et al.,* 2018). Arzt *et al.* (2018) showed that FMDV in untreated OP fluid from carrier cattle is infectious and could cause FMD when deposited into the nasopharynx of naïve recipient cattle. However, that same work failed to initiate FMD in pigs by oropharyngeal deposition of the same material. Recently (Maree *et al.,* 2016), contradicting earlier work (Dawe *et al.,* 1994a; 1994b), direct contact exposure with carrier buffalo for 365 days failed to transmit FMD to cattle. Studying the evolutionary epidemiology of SAT1 and SAT2 in East Africa (Omondi *et al.,* 2019), it was concluded that there were limited evidence that buffalo serve as reservoirs for cattle though the effect of imperfect surveillance in East Africa could not be altogether ruled out. The role of carriers in the epidemiology of FMD remains unresolved and their impact on the legalization concerning international trade in animal products remains profound (Arzt *et al.,* 2018).

1.10. Epidemiology:

The epidemiology of FMD is affected by different viral, host and environmental factors. For instance, the variations in virus virulence, particle stability in different microenvironments and chances of long-term persistence in the environment are among such factors (Woods *et al.,* 2004). The movement of infected animals is the most important factor in the spread of FMD within the endemically infected regions of the world (Rweyemamu *et al.,* 2008).

The Epidemiology of FMD in sub-Saharan Africa is probably more complicated than in any other region of the world. Not only six of the seven serotypes had been known to be prevalent in Africa (only Asia1 has never been recorded), but also marked regional differences in the distribution and prevalence of serotypes and intra-typic variants occur (Wubshet *et al.,* 2019). Serotype C is probably the first extinct serotype (Paton *et al.,* 2021).

1.10.1. Transmission:

Foot-and-mouth disease virus (FMDV) can spread by a variety of mechanisms via direct or indirect contact (Alexandersen *et al.,* 2003b). FMDV can be found in all secretions and excretions from acutely infected animals, including expired air, saliva, milk, urine, feces and semen, the fluid from FMD-associated vesicles, and amniotic fluid and aborted fetuses in sheep. The amount of the virus shed by each route can be influenced by the host species and viral strain. Pigs produce large amounts of aerosolized virus, and the presence of large herds of infected swine may increase the risk of airborne spread (Alexandersen and Donaldson, 2002). Peak of the virus production usually occurs when vesicles are rupture and most clinical signs appear (Kitching, 2002). However, some animals can shed FMDV for up to four days before the onset of clinical signs. The virus can enter the body by inhalation, ingestion or through skin abrasions and mucous membranes. Susceptibility to each route of entry can differ between species. Sexual transmission could be a significant route of spread for the SAT type viruses in African buffalo populations. In sheep, FMDV has been shown to cross the placenta and infect the fetus (OIE Manual, 2021).

1.10.1.1. Airborne transmission:

The survival of airborne virus depends on its resistance to physical and biological decay factors. Viability of airborne FMD virus depends mainly on the atmospheric relative humidity (RH); at RH above 55% airborne virus is stable but at RH below 55% it is rapidly inactivated (Donaldson, 1972; 1973). The capability for the spread of FMDV through the airborne route can be decided by estimating the quantity of the virus released into the atmosphere and establishing the meteorological conditions in the location of infected animals (Sellers and Gloster, 2008).

Cattle commonly become infected through aerosol virus because of their extreme susceptibility to this route of infection, and their massive respiratory tidal volume as compared with smaller ruminants, which are also very susceptible to aerosol virus. Cattle may become infected at concentrations of FMD virus as low as 0.06 $TCID_{50}$ per cubic meter of air (Donaldson *et al.,* 2001; Kitching, 2005). Transmission from infected cattle or sheep could not be shown to occur over distances of more than approximately 3 Kilometer (Km).

A pig can release the equivalent quantity of airborne virus as 3000 cattle (Donaldson *et al.,* 1982). In many outbreaks, where airborne spread has been suspected, pigs were the source (Donaldson *et al.,* 2001), even as it significantly less susceptible to infection by aerosol virus, and might require up to 6,000 times higher concentrations (Donaldson and Alexandersen, 2001). The distance among which the outbreaks occurred in Brittany and at the Isle of Wight was approximately 250 Km (Sellers and Gloster, 2008) and there are many reports of aerosol spread over larger distances (Donaldson and Alexandersen, 2002).

Experimentally, the dose that will infect through nasal instillation is much larger than that given as an aerosol, i.e., around 104-105 $TCID_{50}$ for cattle and sheep (Alexandersen *et al.,* 2003b).

1.10.1.2. Oral route transmission:

Large amounts of infectious virus particles are required to cause infection by the oral route in cattle. In contrast, in pigs the oral route of infection is the most common. Infection resulting from the feeding of pigs with untreated swill has been a common source of FMD

outbreaks in Europe and Asia. There has been only one recorded case of this type of outbreak in southern Africa (Knowles *et al.,* 2001).

1.10.1.3. Transmission by people:

People can act as mechanical vectors for FMDV, by carrying the virus on their clothing or skin. The virus might also be carried for a time in the nasal passages, although several studies suggest prolonged carriage is unlikely. In one early study, nasal carriage was reported for up to 28 hours but less than 48 hours after contact with animals (Sellers *et al.,* 1970; OIE, 2021a).

1.10.1.4. Role of small ruminant in transmission:

FMD in small ruminants is generally silent, and is incriminated for the introduction of the disease in some countries in Europe, the Middle East, Asia, South East Asia and North Africa. Small ruminants from Iran and Iraq are considered one of the major risk factor for the transmission of the virus to the susceptible livestock population of European countries (Uppal, 2009). Small ruminants can play an important role in the spread of FMDV, but it is not clear whether the virus can be maintained in these species for long periods in the absence of infection of cattle (OIE Manual, 2021).

Sheep might have played a significant role in the 2001 UK FMD epidemic (Alexandersen *et al.,* 2002b). For reasons not fully understood, but probably to some extent related to the species predilection of SAT viruses, sheep and goats are only infrequently infected during FMD outbreaks in eastern and southern Africa but this is not invariably the case (Thomson and Bastos, 2004).

1.10.1.5. Role of wildlife in transmission:

Only African buffalo and impala (at least in southern Africa) have been implicated in the transmission of FMDV to cattle, particularly the SAT-type FMD viruses (Vosloo *et al.,* 2002; 2009). African buffaloes are a known reservoir for SAT-type FMDV (Condy and Hedger, 1974). Kudu may play a role in a similar transmission pathway in other parts of sub-Saharan Africa (Letshwenyo *et al.,* 2006; Vosloo *et al.,* 2007; Weaver *et al.,* 2013).

1.11. Diagnosis:

1.11.1. Clinical diagnosis:

In cattle, the disease is obvious and the acute phase lasts about 1 week then declines gradually coinciding with the emergence of a strong humoral antibody response. In sheep and goats, symptoms are frequently less severe and mild lameness may be the only sign of infection in these animals (Subramaniam *et al.,* 2012). Moreover there are other viral infections that cause vesicular diseases which cannot be clinically differentiated from FMD, swine vesicular disease (SVD), vesicular exanthema (VE) and vesicular stomatitis (VS) (Thomson and Bastos, 2004).

Preliminary diagnosis of FMD, in susceptible animals, is based on clinical signs and confirmation by laboratory methods (Rémond *et al.,* 2002).

1.11.2. Laboratory diagnosis:

The best source of material for diagnosis of FMD is vesicular fluid and fragments of epithelium from freshly-ruptured vesicles in the mouths or on the feet of affected animals (OIE Manual, 2021). In acute cases, unruptured vesicles are seldom seen and none putrefied epithelium from freshly-ruptured vesicles is available for 1-2 days following rupture of the vesicle. Ideally, at least 1 gram of epithelial tissue needs to be collected, kept cool in a buffered solution (pH 7.2-7.4), and transported to the laboratory as soon as possible (Thomson and Bastos, 2004). Other materials for laboratory diagnosis may include oro-pharyngeal fluid, throat swabs, blood samples and semen samples (Subramaniam *et al.,* 2012).

1.11.2.1. Identification of the agent:

1.11.2.1.1. Virus isolation:

Primary cultures of calf thyroid cells have been shown to be the most sensitive culture for virus detection; it is as sensitive as intradermal inoculation in cattle (Snowdon, 1966). Primary pig, calf or lamb kidney cells are generally more sensitive than the established cell lines such as baby Hamster kidney (BHK-21) (Longjam *et al.,* 2011). Cryo-preservation of the primary cells usually results in less susceptibility. The use of IB-RS-2 cells has the

advantage of differentiation between swine vesicular disease virus (SVDV) and FMDV (as SVDV will only grow in cells of porcine origin). It is also essential for the isolation of pig adapted strains, such as O Cathay (OIE Manual, 2021). Due to the high cost and intensive labor to get monolayer of primary thyroid cells always ready to be inoculated, attempts have been made to immortalize these cells by oncogen transfection. But so far, these immortalized cells display less sensitivity than the primary cells (Ferris *et al.,* 2002).
Suckling mice aged 2-7 days old are less susceptible and can also be used to isolate FMD virus (Rémond *et al.,* 2002; Poonsuk *et al.,* 2018).

1.11.2.1.2. Antigen detection ELISA:

Antigen detection ELISA is generally regarded as the primary test for FMD diagnosis especially at the regionally located FMD diagnostic laboratories (Sharma *et al.,* 2015). Widely used ELISAs include an indirect sandwich ELISA kit [World Reference Laboratory for FMD] (Roeder and Le Blanc Smith, 1987) and IZSLER ELISA kit [Biotech, Brescia, Italy] (Grazioli *et al.,* 2012; 2020). The former is based on the detection of FMDV structural proteins and utilizes the serotype-specific polyclonal antibodies generated in guinea pig and rabbits while IZSLER ELISA employs selected combinations of anti-FMDV monoclonal antibodies (MAbs) as coated and conjugated antibodies. The suspected clinical materials are processed into 10% suspension and sometime tissue culture supernatants showed cytopathic effect are also utilized (Clavijo and Kitching, 2003).

1.11.2.1.3. Polymerase chain reaction (PCR):

PCR based detection of viral RNA is the method of choice and several assays have been developed for FMD virus. It has the potential to be extremely sensitive due to the requirements for only small quantities of template and the possibility of making multiple cycles of PCR. Specificity can also be high as only targeted viral RNA regions are amplified. In the end, the test supplies genetic material suitable for sequencing thus providing the most detailed epidemiological information for tracing the origin of an outbreak (Longjam *et al.,* 2011).
Efficient extraction of viral RNA from field samples is a prerequisite for its isolation and subsequent successful amplification by PCR. In samples like oesophageal-pharyngeal fluid

or nasal discharge, the virus needs to be concentrated by polyethylene glycol precipitation before RNA is extracted (Marquardt *et al.,* 1995).

Development of real-time PCR has facilitated rapid quantitative analysis with reduced amount of post-PCR processing steps (Goller *et al.,* 2018).

1.11.2.1.4. Lateral flow device (LFD) pen-side test:

A pen-side test is essentially simple, cheap and disposable. It provides results within minutes of taking a clinical sample and could be used on suspected premises, typically, by a veterinarian. However, laboratory confirmation of FMDV and its serotype, further characterization and vaccine matching studies remain essential parts of FMD diagnosis. Many reports described the development of LFD for the pen-side diagnosis of FMD (Reid *et al.,* 2001; Ferris *et al.,* 2009; 2010). The test depends on identification of FMDV antigen by a monoclonal antibody (Mab) that reacted against all seven serotypes of FMDV (pan-reactive). Nonetheless, the OIE has not yet received a validation dossier for this test (OIE Manual, 2021). The pen-side test is advantageous when there is over-reporting of the disease e.g. in FMD-free countries where it would support veterinary clinical judgement and reduce time of confirmation of disease events in secondary outbreaks. In endemic countries, a simple and inexpensive field test like the pen-side test is hoped to increase FMD awareness and improve epidemiological information (Ferris *et al.,* 2009).

1.11.2.2. Serological methods:

1.11.2.2.1. Complement fixation test (CFT):

Complement fixation test (CFT) has been used extensively for distinguishing different serotypes of FMD virus. The test initially carried out in glass tubes and was later modified as micro-CFT that conducted in 96-well microtiter plates. This test was criticized largely for its lack of sensitivity and specificity (Longjam *et al.,* 2011; Jamal and Belsham, 2013).

1.11.2.2.2. Virus neutralization test (VNT):

Neutralization assay is the gold standard test for diagnosis of FMD (Golding *et al.,* 1976; OIE Manual, 2021). Briefly, the test was performed in microtitre plates using two fixed

doses of the virus and two-fold dilutions of sera and incubated at 37ºC for 1 h. The mixtures were then added to the monolayer of BHK-21 cells that were grown in flat bottom-96 well microtiter plates and incubated at 37ºC for three days in a humidified atmosphere containing 5% CO_2. It is found to provide a satisfactory means of differentiation between strains. Antibody titers were expressed as the logarithm of the reciprocal of the final dilution of serum in the virus/serum mixture that neutralized an estimated 100 $TCID_{50}$ at the 50% end-point (OIE Manual, 2021).

1.11.2.2.3. Enzyme-linked immune sorbent assay (ELISA):

ELISAs, simpler and quicker assays, have been developed as alternatives to VN tests (Hamblin *et al.,* 1986a; 1986b; 1987). A commonly used approach is to utilize tests such as ELISA for primary antibody detection, reserving VN tests for the confirmation of inconclusive and positive results**.** The test detects antibodies to FMDV structural proteins (SPs) or antibodies to FMDV non-structural proteins (NSPs). NSP tests are not serotype-specific, and can be used in both vaccinated and unvaccinated animals. However, they are less sensitive and may not detect cases with limited virus replication, including some vaccinated animals that become infected. Due to such limitations, serological tests that detect antibodies to NSPs are generally used as herd tests (Clavijo *et al.,* 2004).

1.12. Control:

Control of FMD depends on the slaughtering of affected and contact animals "the so called 'stamping out' procedure", mostly in FMD-free countries, or the regular vaccination of the major host species for FMDV, always cattle and when indicated also swine, mostly in endemic countries (Sobrino *et al.,* 2001).

Among control measures of FMD outbreaks are quarantines, movement restrictions and cleaning and disinfection of affected premises, equipment and vehicles. A good biosecurity measures should be practiced on uninfected farms to prevent entry of the virus (Sutmoller and Casas, 2002).

1.13. Protective immune response:

Protection against FMD is often associated with the induction of high levels of neutralizing antibodies in serum of animals. However, this immune response does not ensure clinical protection and animals with low serum neutralization (SN) titers may nevertheless be protected against infection with FMDV (McCullough *et al.,* 1992; Sobrino *et al.,* 2001). Ig M is the first neutralizing antibodies that appear at 3 to 4 days following infection or vaccination, in cattle this response peaks around 10 to 14 days post-infection then declines (Collen, 1994). The major antibody subclasses found in secretions are initially IgM then followed by IgA and IgG (Salt *et al.,* 1996). In both, infected and vaccinated cattle, IgG1 response is generally greater than that of IgG2 (Sobrino *et al.,* 2001). Early upon infection or vaccination, there is a detectable antibody response in the secretions of the upper respiratory and gastrointestinal tracts (Francis and Black, 1983).

1.14. Vaccination and vaccines against FMDV:

Vaccination against FMD is a key element in the control of the disease in addition to slaughter and movement restrictions. However, countries that vaccinate in the event of an outbreak will have to re-establish their FMD free status to the satisfaction of their trading partners. Vaccination inhibits local virus replication and excretion in the oropharynx and thus reduces or prevents virus transmission. It may also inhibit the development of the carrier state (Barnett *et al.,* 2004). Emergency vaccines contain higher antigen payloads than conventional vaccines thus induce rapid immunity (often within 4 days) and offer wider antigenic coverage. It is important to identify the optimum cross-protective vaccine strain for use in an outbreak (Barnett and Carabin, 2002).

Many FMD-free countries now have strategic reserves of concentrated, purified vaccine antigen at ultra-low temperatures for use in an emergency situation (Barnett and Carabin, 2002).

The first FMD vaccine was produced in 1938 using tongue epithelium harvested from cattle deliberately infected with FMD virus. Today, the large majority of FMD vaccines are produced in BHK-21 as cell culture suspension and the live virus is inactivated using binary ethyleneimine. The produced antigen is, then, usually blended with oil or aqueous adjuvant for vaccine formulation (Clavijo *et al.,* 2004; OIE Manual, 2021). Aqueous FMD

vaccines are formulated with aluminum hydroxide gel and saponin as adjuvants. Aqueous vaccines are commonly used in cattle, sheep, goats and buffalo but not pigs (Cloete *et al.,* 2008).

Oil adjuvant single and double emulsions are used to produce vaccine for immunization of all species of animals including pigs. Oil adjuvant vaccine should have potency of at least 3 PD50 and provide protective immunity within 7 days in cattle, swine and sheep. Revaccination must be carried out every 6 months. After multiple doses of vaccines in older animals vaccination frequency could be decreased to once a year, provided that no new strains are not covered by the vaccine formulation emerge or are introduced (Depa *et al*., 2012).

The purification of FMD viral antigens to remove non-structural proteins (NSP) allows differentiation between vaccinated and infected animals. Consequently, the combined use of purified vaccine and anti-NSP tests essentially provides a ‘marker’ system (Barteling, 2002).

1.15. Economic impact:

Although the disease is of low mortality, the global impact of FMD is huge due to the large numbers of animals affected. Economic impact of FMD includes direct losses due to reduced production and changes in herd structure and indirect losses caused by costs of FMD control, poor access to markets and limited use of improved production technologies (Knight-Jones and Rushton, 2013).

FMD impacts are not the same throughout the world; FMD production losses have a big impact on the world’s poorest where more people are directly dependent on livestock (Rushton, 2009). In countries with ongoing control programmes, FMD control and management creates large costs; these control programmes are often difficult to discontinue due to risks of new FMD incursion. The presence, or even threat, of FMD prevents access to international markets. In FMD free countries outbreaks occur periodically and the costs involved in regaining free status have been enormous (Knight-Jones and Rushton, 2013).

Chapter II
Materials and Methods

2.1. Materials:

2.1.1. Viruses:

Foot-and-mouth disease viruses used in this study were recent local Sudanese isolates, of cattle origin, adapted to grow in cultured cells, typed and retyped using reference antigen detection ELISAs (Pirbright and IZSLER Laboratories). They were of three serotypes; O, A and SAT2 and were designated according to that serotype, geographical origin within Sudan, year of isolation and order of isolation from that origin. Two SAT2 isolates were used, one was isolated from Khartoum in 2008 (SAT2-Kh 1/08), not genotyped at the WRL for FMD yet, however, topotype X111 of SAT2 serotype was circulating in Sudan in 2008 (http://www.wrlfmd.org; Raouf *et al.,* 2010). The other SAT2 isolate was isolated from North Kordfan State in 2010 (SAT2-NK 1/010) (Department of FMD Report, 2010) of genotype V11 and with the identity of SUD/4/2010 at the WRL for FMD (http://www.wrlfmd.org). Serotype A isolate was isolated from Khartoum in 2011 (A-Kh 2/011), of topotype Africa and with the identity of SUD/7/2011 at the WRL for FMD (http://www.wrlfmd.org; Raouf *et al.,* 2016). Serotype O isolate was isolated from Khartoum in 2015 (O-Kh 1/015) (Department of FMD Report, 2015) and was not genotyped at the WRL for FMD.

At the commencement of this work, all used viral materials were retyped using IZSLER antigen detection ELISA (Grazioli *et al.,* 2012).

2.1.2. Control sera:

Control sera for virus neutralization test (VNT) were known positive field bovine 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 sera.

2.1.3. Tested sera:

All sera analyzed in this study have been shown to contain anti-NSPs antibodies of FMD virus by the ID Screen® FMD NSP Competition ELISA i.e. mainly from FMD infected

cattle. The original serum samples had been collected, late in 2015 and early in 2016, during the programme "Surveillance of Trade Sensitive Diseases" (STSD) which was a joint programme between the government of Sudan and the African Union (AU). Sera had been collected from apparently healthy cattle, older than 1 year with no history of vaccination against FMD and discriminated as positive or negative to FMD anti-NSPs activity in the course of the programme.

In each State, sera were collected from an available sampling frame of 5 geographical districts (sampling units) (Table 1) and five sampling epi-units (herds or collection sites) per sampling unit. Accordingly, a minimal number of 25 epi-units per State was achieved which conforms 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. The approximate sample size required to estimate prevalence in an infinite population (large) in each sampling unit was calculated using the formulae (Thrusfield, 2007): $n = 1.96^2 P_{exp} (1- P_{exp})/d^2$. Where n is the required sample size, P_{exp} is the expected prevalence, d is the desired absolute precision and 1.654 is the approximate multiplier for the required level of confidence. The expected prevalence (P) was assumed to be 50%, the least favorable, and the desired absolute precision of 10% was applied under the level of confidence of 90%.

Selection of the sampling units (localities) depended to a large extent on security due to civil unrest in Darfur area. However, a total of 350 bovine sera were collected from each State (Table 1).

Table 1. Data of NSPs serology in North and South Darfur States: Numbers and origin of anti-NSPs positive sera.

States data				Districts data				
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
North Darfur	329	243	74.0%	El Kuma	61	47	77%	43
				Umm Keddada	65	45	69%	44
				El Taweish	66	36	55%	30
				El Lait	67	56	84%	53
				El Fasher	70	59	84%	58
Totals					329	243		228
South Darfur	340	146	43.0%	Niteaga	66	33	50%	29
				Nyala North	68	33	49%	30
				Nyala South	68	30	44%	28
				Marshang	68	26	38%	26
				Bielel	70	24	34%	26
Totals					340	146		139

* Out of 350 sera collected from each State; 21 (North Darfur) and 10 (South Darfur) sera were lost.

**15 (North Darfur) and 7 (South Darfur) sera positive to NSPs serology were lost.

2.1.4. Tissue samples:

Five Tongue epithelium tissue samples were collected from cattle showing suspected FMD lesions; from Nyala in South Darfur State and from Kosti in White Nile State. Tongue epithelium was collected in viral transport medium (appendix 6) and transferred under refrigeration to the Department of FMD, CVRL, Soba, Khartoum. Of these, two samples were submitted to the World Reference Laboratory of FMD, the Pirbright Institute, UK.

2.1.5. Cell and cell culture medium:

2.1.5.1. 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.

2.1.5.2. Cell culture medium:

Glasgow Minimum Essential medium (GMEM) (pH 7.4-7.6) (Sigma Aldrich, UK) supplemented with 10% Fetal calf serum (FCS) (Sigma Aldrich, USA) was used for cells growth and maintenance (appendix 2).

2.1.6. Antibiotics and Antimycotic:

Name	Company	Concentration/ml medium
Benzyl Penicillin	NCPC, China	200 I.U
Streptomycin Sulphate	NCPC, China	100 µg Streptomycin Base
Gentamycin injection Bp	Unique Pharmacoutical Laboratories, India	10 µg
Amphotericin B Solution	Sigma Aldrich, USA	12 ml/1000 mL

2.1.7. Equipment and apparatus:

Name	Company
Laminar Flow Cabinet class II	Labcaire, UK
Inverted microscope (Olympus CK×31)	Krüss, Germany
ELISA reader	BIO-TEK instruments ,USA

Water bath	Lauda, Germany
Mini orbital shaker	Stuart®, UK
Multichannel micropipette	Socorex, Swiss made
Single channel micropipette	Socorex, Swiss made
Vortex mixer	Appleton Woods, UK
Incubator	Scott Science, UK
Refrigerator (+1°C and +8°C)	COLDAIR, Sudan
Deep Freezer (-20°C)	COLDAIR, Sudan
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

2.1.8. Disposables, glassware and plastic ware:

Name	**Company**
Eppendorf tubes	-
Corning® 96-well clear flat bottom polystyrene TC treated microplates	Corning Incorporation, USA
Tips (Blue, 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. Chemicals:

Name	Company
Sodium Chloride	Sigma Aldrich, USA
Potassium Chloride	Applichem Biochem Synthesis Service
Sodium bicarbonate	Sigma Aldrich, USA
Potassium phosphate monobasic	Applichem Biochem Synthesis Service
Sodium dihydrogen phosphate monohydrate	Applichem Biochem Synthesis Service
Tryptose Phosphate broth	Sigma Aldrich, USA
Tris	Sigma Aldrich, USA
Hydrogen Chloride	Surechem Products LTD, England
Trypsin	Sigma Aldrich, USA
Versene (EDTA)	Sigma Aldrich, USA
Disinfectants:	
Dettol	-
Alcohol	-

2.1.10. ELISA for FMD Antigen Detection and Serotyping (FMDV O, A, C, Asia1, SAT1, SAT2) (Brescia, ltaly):

2.1.10.1. 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: a pan-FMDV MAb for detection of serotypes O, A, C and 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 (Tetramethylbenzidine (TMB)), ready to use.
7. Stop solution (H_2SO_4, 0.6N), ready to use.

2.2. Methods:

2.2.1. Study area:

Darfur is a large region in Western Sudan which covers an area of 493,180 square kilometers (190,420 sq mi) between 9-20 ºN and 24-25 ºE (Figure 5). Administratively, Darfur area is divided into 5 States: North Darfur State in the North and West Darfur, South Darfur, Central Darfur and East Darfur States in the South (Figure 5). The main capitals of Darfur States are El Fashir (North Darfur) and Nyala (South Darfur). It shares borders with four countries namely Libya and Chad in the West, Central African Republic in the South-East and Southern Sudan in the South (Morton, 2005). Darfur area falls for the most part in the savannah belt (low rainfall savannah) that traverses Sudan from the Western to the Eastern border. Small strips of the desert and semi-desert in the North and mountains ecological zones (Marrah Mountains) in the South are also known (Morton, 2005). Darfur area keeps around 9 million head of cattle, 11 million of sheep and 10 million of goats (Department of FMD Report, 2012). Animal distribution follows, to a large extent, the distribution of the ecological zones in the area. In the semi-desert zone, in the North, <5 cattle and <10 small ruminants per square Km are expected (FAO, 2005). To the South, cattle density is generally between 10, in the Northern areas of the savannah belt, and 30 head or more per square Km in the Southern areas of Darfur. Higher density of small ruminants between 25 and 100 head per square Km is expected in the savannah (FAO, 2005). Animals are mostly reared in the savannah free rangeland under nomadic or transhumant pastoralist systems of production. The latter pastoralists, unlike nomads, move their animals within limited diameters around tribal homeland "dar". Cattle owners in Darfur area are known for the large size of their cattle (200) and goat (200) herds compared to a smaller size of sheep (70) herds (Ibrahim, 1999). In large urban centers like El Fashir and Nyala, nuclei of the improved modernized systems of cattle rearing are represented by some individuals that own high producing milking cows. These cattle owners' rear mainly cross breeds of cattle while pastoralists own local cattle breeds; mostly Baggara.

North and South Darfur States were selected to represent the high (North Darfur) and the low (South Darfur) levels of FMD activity, as detected by NSPs serology, in Darfur area (Figure 5) by the programme STSD (Department of FMD Report, 2016). Sero-prevalence

rates of anti-NSPs antibodies were 74.0% and 69.0% in North and East Darfur States, respectively, but ca 40% in South, West and Central Darfur States.

Figure 5. Map of the Sudan showing Darfur area and studied States. Two levels of activity against NSPs of FMD virus; a higher level (streaked area) and a lower level (non-streaked area) were shown.

2.2.2. Serum testing:

2.2.2.1. Samples records and preparation:

At the commencement of the present work, all sera that had proved to be positive with anti-NSPs activity have been separated from serum lots of North and South Darfur States. Their numbers, the district of origin within each State (Figure 6) and associated anti-NSPs activity in States and districts (Department of FMD Report, 2016) are indicated in Table 1. Serum samples were inactivated at 56°C for 30 minutes, cooled, received 6 µl of Penicilline/Sterptomicine mixture, and kept at -20°C till use.

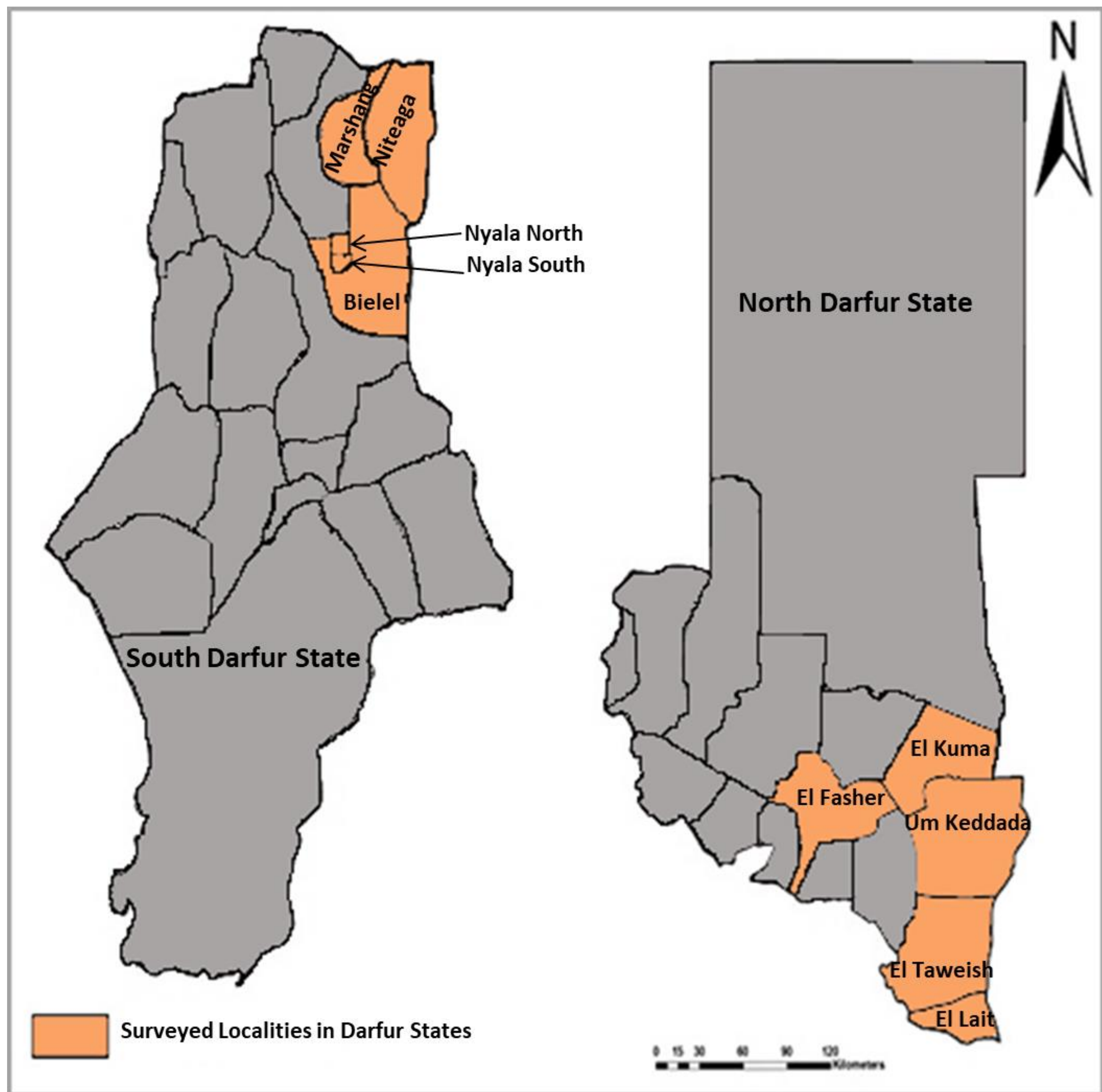

Figure 6. Districts (localities) of serum collection in South Darfur and North Darfur States.

2.2.2.2. Testing protocol:

All sera were tested for serotype-specific antibodies against FMDV serotypes O, A and SAT2 using a screening format of VNT (Raouf *et al.,* 2012). Sera from the two States and from different districts were tested simultaneously.

2.2.2.3. Virus neutralization test (VNT):

2.2.2.3.1. Description and principle:

All sera were tested for serotype-specific anti-structural proteins (SPs) antibodies against FMD virus serotype O, A and SAT2 using the standard procedure of VNT (OIE Manual, 2021) except that sera were tested at final two dilutions; of 1/32 ($10^{-1.5}$) and 1/64 ($10^{-1.8}$) each in two wells only, rather than several dilutions (Raouf *et al.,* 2012). In the VNT, sera are diluted in a two-fold dilution series, each serum was tested in 4 wells and each plate tested 20 sera in addition to control serum (positive and negative) and cell control (Figure 7). Adopting the procedure to include only two serum dilutions (1/32 and 1/64) while decreasing the test workload, span the standard cut-off of 1/45 ($10^{6.5}$) described for the purpose of sero-surveillance by the OIE. To further increase the sensitivity of the assay, the cut-off is lowered to 1/32 ($10^{1.5}$), which is usually considered inconclusive and needs to be retested, in case of individual serum screening (OIE Manual, 2021). Few sera were found positive at a titre of $10^{1.5}$ but negative at titres of $10^{1.65}$ and $10^{1.8}$ (Raouf *et al.,* 2012; 2016; 2017). Even fewer sera were found positive at titres of $10^{1.35}$ or $10^{1.2}$ (dilution 1/16) but negative at higher titres (Raouf *et al.,* 2012). Using the adopted VNT, previous serosurveillance determined seroprevalences as high as 75% (n = 531) (O) and 40% (n = 531) (A and SAT2) and detected subtle differences between States, regions and districts in Sudan (Raouf *et al.,* 2016).

The neutralization test was carried out in flat-bottomed microtitre plates in equal 50 µl volumes with suitable cells like BHK-21. The neutralizing virus was pre-titrated to contain 100 $TCID_{50}$, with an accepted range of 32-320 $TCID_{50}$, in a 50 µl volume. Results were readable microscopically after 48 hours and finally, after 72 hours, test plates were fixed and stained with vital stain. The virus would be neutralized in positive wells where no CPE is seen, cells remain intact and stained cell sheets appeared while in negative wells, where no neutralization took place, CPE would be seen, cells are damaged and wells appear

empty. Titre is calculated according to the method of Kärber (1931) and expressed as the final serum dilution where 50% of wells are protected.

2.2.2.3.2. Procedure:

First step, viruses were grown in BHK-21 cell culture, harvested 24 hours later, and clarified by centrifugation at 2000 rpm for 10 minutes in refrigerated centrifuge, distributed in 2 ml aliquots into cryogenic vials and stored in liquid nitrogen vapor. Virus stocks were titrated in the micro-titre system according to the method described by Raouf *et al.* (2010).

Second step, was serum diluents prepared in complete GMEM containing 10% Tris-buffer and distributed by using multichannel micropipette into the plate as 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. Tested sera were distributed in row A, C, E and G; 6 µl of each sera in each of 2 wells. Columns 11 and 12 of the mico-plate, were used for controls of normal sera, cell control serum, negative and positive controls, each in 4 wells. Serum dilution was performed using multichannel pipette by transferring 50 µl of the mixture from each well in one row into second row; mixed as above and discarded leaving only 50 µl of dilution. New tips were put onto a multichannel pipette before diluting different samples for each 2 rows (Figure 7).

Third step was subsequently, 50 µl of previously titrated viruses containing 100 $TCID_{50}$/50 µl was dispensed into all serum contained wells except that containing cell control were received only diluents. The plate was agitated lightly and shacked well for 10 minutes, virus-serum mixtures were allowed to react for one hour at room temperature (Figure 7).

Final step, 50 µl of BHK-21 cell suspension produced confluent or semi-confluent monolayer 24 hour later, in the above described growth medium, has been dispensed to all wells of the plate, suspension was agitated frequently to prevent sedimentation. The test plate was sealed with adhesive tape and incubated at 37°C for about 72 hours with humidity.

Results were read daily microscopically and thereafter stained with 0.1% crystal violet in 10% formal saline on the third day. Positive wells appeared as stained intacted cell monolayers in one or more well, while negative wells appeared as empty or with fragmented cell monolayers and patchy stain (Figure 13A, 13B and 13C).

Serum dilution starting		**Serum samples (Columns 1-10)** 1	2	3	4	5	6	7	8	9	10	**Controls (Columns 11-12)** 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 7. Layout of the VNT plate.

S1…..S20 = Serum samples

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

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

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

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

2.2.2.4. Statistical analysis:

In this study, sera were tested based on the results of a previous test, the ID Screen® FMD NSPs Competition ELISA. Only positive reactors to the NSPs Competition ELISA were tested by the VNT i.e. both tests are conducted serially "consecutively" (Fletcher and Fletcher, 2005). Calculations for serial testing were performed according to the standard procedure (Thrusfield, 2007). Prevalence was calculated as proportion positive to both tests, ID Screen® FMD NSP Competition ELISA (test A) and VNT (test B), according to the formula:

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 programme STSD (Table 1). Proportions positive by test B (VNT) in each sub-population were determined by dividing the number of positive reactors identified using the VNT by the number of sera tested in that sub-population. Sera eligible for the calculation of prevalence of combined serotype-specific antibodies (three serotypes) should be positive to one or more serotypes and/or negative to the three serotypes.

The priori (prospective power analysis) depended on the previous study and had been used to estimate sufficient sample sizes (Table 1) and *post-hoc* analysis (retrospective power analysis) was conducted to drive 95% confidence interval (C.I.) measures and *p-values*. In the *post-hoc* analysis, prevalence rates were compared by driving the 95% C.I. 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 test available at the Statistical Packages for Social Sciences (SPSS) at (www.sociostatistics.com); results were significantly different, if $p < 0.05$.

2.2.2.5. Clinical disease surveillance:

2.2.2.5.1. Passive surveillance:

Passive surveillance was organized in collaboration with Nyala regional laboratory in December 2017 and January 2018. The regional laboratory was contacted to participate in the effort and supplied with viral transport medium. Participation of regional laboratories in FMD surveillance has been one of the earlier recommendations for the study of the problem of FMD in the wide country area (Raouf *et al.*, 2009). Four samples of tongue epithelium were collected in viral transport medium and sent under refrigeration to the Department of FMD, CVRL.

2.2.2.5.2. Active surveillance:

A field trip was organized in March 2018 to the White Nile State which was the nearest point in the Nile Valley to the study area, Darfur. Earlier, virus spill from the Nile Valley was expected to spread to Western Sudan (Raouf *et al.*, 2017). The object was to gather information on the occurrence of clinical FMD in the area and to collect suspected virus material that is likely to spread to Darfur. One sample of tongue epithelium, from cattle showing salivation, was collected in viral transport medium and transferred under refrigeration to the Department of FMD, CVRL, Soba, Khartoum.

2.2.2.5.3. Tissue samples preparation:

Epithelium samples were removed from the transport medium, blotted dry, washed using GMEM containing five-fold concentration of antibiotics and dried on absorbent paper. Tissues were then weighted, minced into small pieces and grounded into a paste using a sterile pestle and mortar and sterile sand. A 10% suspension (W/V) was prepared and clarified by centrifugation at 2000 rpm at 4°C for 10 minutes. The supernatant was transferred to new sterile Eppendorf tubes and stored in liquid nitrogen or at -20°C until used.

2.2.2.5.4. ELISA for FMD antigen detection and serotyping (Brescia, ltaly):

ELISA plates were supplied pre-coated with MAbs (catching antibodies) directed against serotype O, A, Asia1, C, pan FMDV (Pan-O-A-C-Asia1), SAT1 and SAT2 and with positive (inactivated) and negative controls according to the plate layout shown in Figure 8. Samples were diluted 1:2 in ELISA diluents buffer (ready to use) and 50 µl/well of each sample was distributed in 8 wells of one column from column 1 to 10 (Sample 1-10: column 1 to 10). 50 µl/well of ELISA diluent buffer was distributed in column 11 to 12. The plate was covered and incubated for one hour at room temperature (25°C). The plate was then washed 3 times with washing buffer containing PBS-Tween 20. A washing cycle was effected by emptying the plate, tapping hard, filling with 200 µl/well of washing buffer for 3 minutes at room temperature then emptying and tapping hard onto an absorbent towel. 50 µl/well of the an appropriately diluted conjugate A (pan-FMDV type O, A, C, Asia1) was added to row A to F; and 50 µl/well of an appropriately diluted conjugate B (SAT1 and SAT2) was added to row G to H. The plate was covered and incubated for one hour at room temperature. At the end of the incubation time, the plate was washed for 4 times as before leaving the last wash for 5 minutes. Immediately after washing, 50 µl/well of the substrate/chromogen solution (TMB) was distributed to all wells. ELASA plate was covered and incubated for 20 minute in the dark then 50 µl/well of the stop solution (H_2SO_4 0.6N) was added to stop the reaction. Immediately, the optical density (OD) value of each well was read at 450 nm wave length.

For validation of the results, the positive inactivated control should give values ≥ 1.0. The negative control for serotypes O, A, Asia1, C and pan-FMDV should give OD values < 0.1., the negative control for serotypes SAT1 and SAT2 should give OD values ≤ 0.2. The calculated sample OD value (obtained by subtracting the OD value of the negative control of the corresponding catching MAb) of 0.1 or greater were considered positive while of < 0.1 were considered negative. Sample OD values ≥ 0.05 and < 0.1 should be considered suspected and should be retested.

Catching MAbs		*Sample 1* 1	*Sample 2* 2	*Sample 3* 3	*Sample 4* 4	*Sample 5* 5	*Sample 6* 6	*Sample 7* 7	*Sample 8* 8	*Sample 9* 9	*Sample10* 10	*+ Control* 11	*- Control* 12	Conjugate
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 8. Layout of ELISA plate for detection of FMDV antigen.

Chapter III
Results

3.1. Index of prevalence of FMD infection in North and South Darfur States (serial testing approach):

Index of prevalence of FMD infection as a function of prevalence of antibodies to NSPs and antibodies to SP (serial testing approach i.e. only sera positive in both tests were considered positive) decreased in North Darfur from 74.0% by NSPs serology to 61.0% by the serial testing approach. Similarly, in South Darfur State, it decreased from 43.0% to 34.0% (Table 2; Figure 9). Nevertheless, indices of FMD infection remained statistically significantly higher (non-overlapping C.I.) in North Darfur than in South Darfur (Table 3). In each State, ca 20% of NSPs positive sera failed typing by the VNT (Table 2).

Non-structural proteins positive sera that failed typing were negative to the three serotypes of FMDV; O, A and SAT2. Their proportions, among NSPs positive sera, were slightly higher in South Darfur [29/139 (21.0%)] than in North Darfur [40/228 (18.0%)]. Yet in both States, indices of prevalence of FMD infection by serial testing were statistically significantly lower than by NSPs serology (Table 3).

Table 2. Typing of anti-NSPs positive cattle sera.

State	Indices of prevalence of FMD infection by NSPs serology (Sero-prevalence of anti-NSPs antibodies %)	Typing of NSPs positive sera by the combined VNT (cVNT)				Indices of prevalence of FMD infection by serial testing (NSPs serology and cVNT)
		No. tested	No. tested positive to one or more serotype	% of typed sera	% of sera failed typing	
North Darfur	74.0% (243/329)	228	188	82.0% (188/228)	18.0% (40/228)	61.0%
South Darfur	43.0% (146/340)	139	110	79.0% (110/139)	21.0% (29/139)	34.0%

Table 3. Comparison between indices of prevalence of FMD infection by NSPs serology and by cVNT-O, A and SAT2 (serial testing approach)

State	Sero-prevalence of anti-NSPs antibodies		Sero-prevalence of anti-O, A and SAT2 antibodies-combined (serial testing approach)		P value (chi-squared test)
	Sero-prevalence%	95% C.I.	Sero-prevalence%	95% C.I.	
North Darfur	74.0%	69.0% - 79.0%	61.0%	56.0% - 66.0%	0.000448
South Darfur	43.0%	38.0% - 48.0%	34.0%	29.0% - 39.0%	0.01735

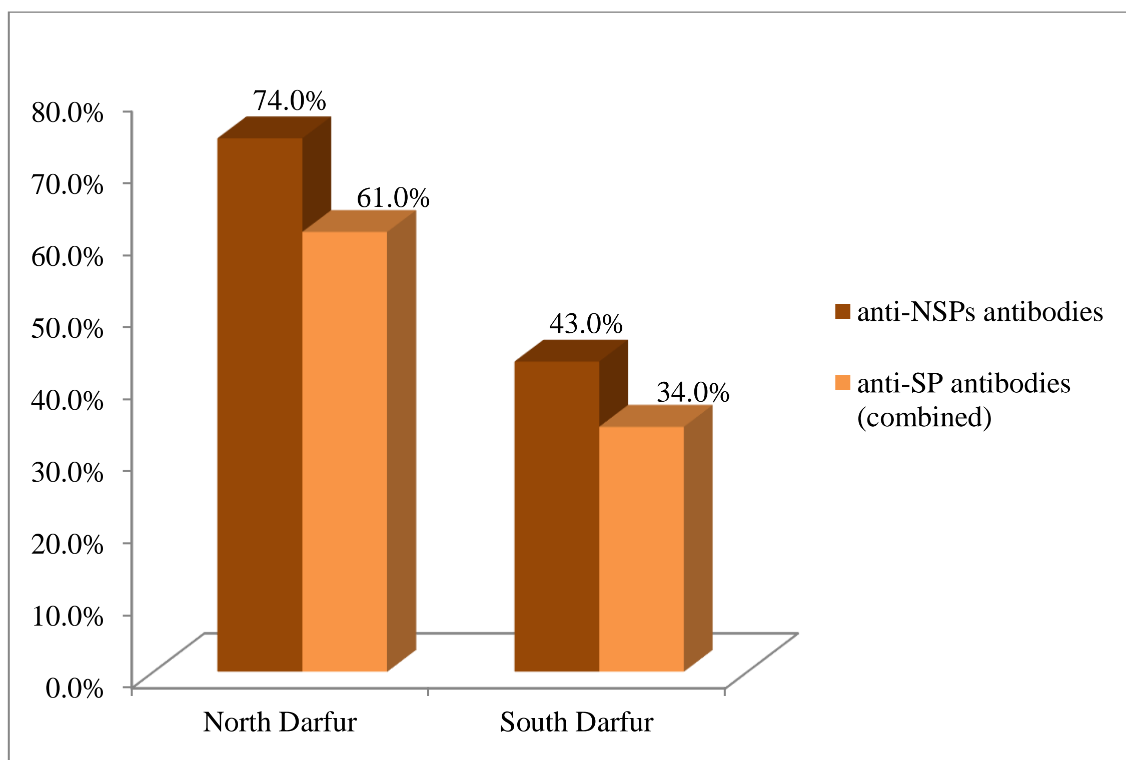

Figure 9. Indices of prevalence of FMD infection in North and South Darfur States by NSPs serology [anti-NSPs antibodies] and by serial testing [NSPs serology and combined VNT (O, A and SAT2)].

3.2. Sero-prevalence of FMDV serotype-specific antibodies in North and South Darfur States:

In both States, approximately two thirds of the typed sera were positive to serotype O, c.a. one third was positive to serotype A and c.a. one fifth was positive to serotype SAT2 (Table 4). Consistently, in both States, sero-prevalence of antibodies to serotype O was statistically significantly higher than sero-prevalence of antibodies to serotype A and that of serotype A was statistically significantly higher than that of serotype SAT2 (Table 4). Also, consistently, the three serotypes showed statistically significantly higher sero-prevalence of serotype-specific antibodies in North Darfur than in South Darfur (Table 4; Figure 5; 10).

Table 4. Sero-prevalence of FMD virus serotype-specific antibodies in North and South Darfur States.

FMDV Serotype	North Darfur			South Darfur			P value
	% positive in NSPs positive sera	Estimated prevalence	95% C. I.	% positive in NSPs positive sera	Estimated prevalences	95% C. I.	(chi-squared test)
O	66.0% (151/228)	49.0%	44.0%-54.0%	63.0% (88/139)	27.0%	22.0%-32.0%	0.00753
A	36.0% (81/228)	27.0%	22.0%-32.0%	39.0% (54/139)	17.0%	13.0%-21.0%	0.002788
SAT2	18.4% (42/228)	14.0%	10.0%-18.0%	19.0% (26/139)	8.0%	5.0%-11.0%	0.000003

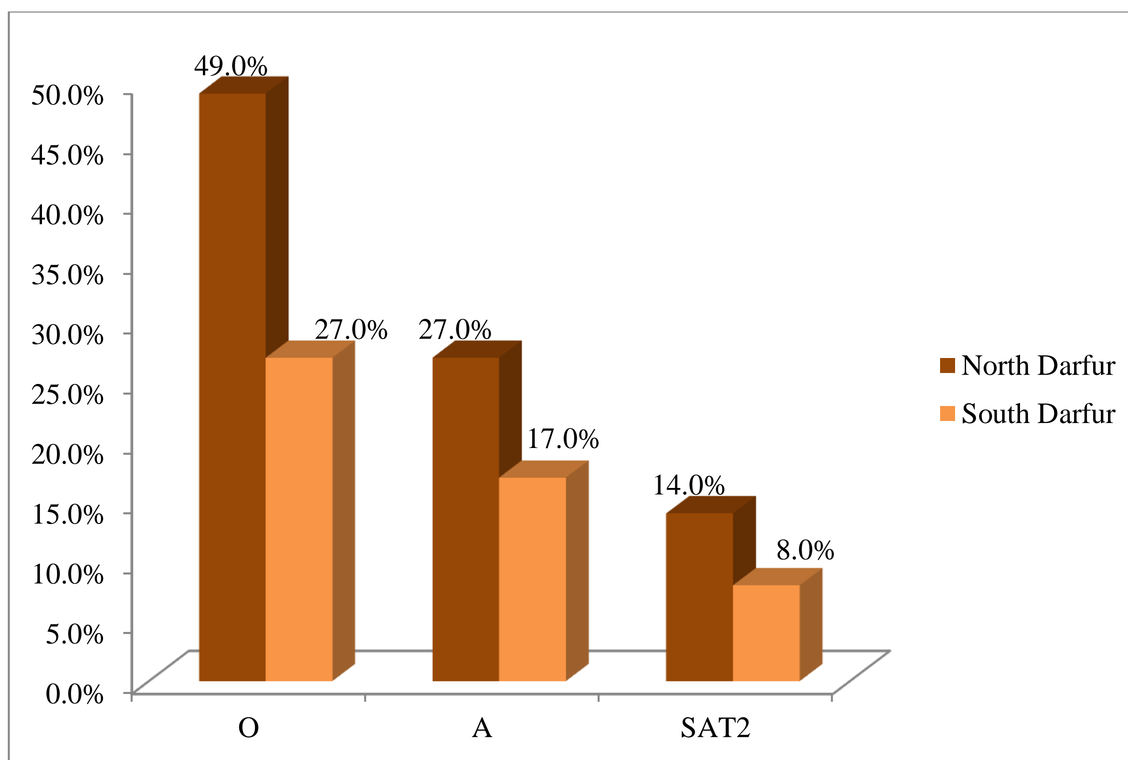

Figure 10. Consistently higher sero-prevalence of FMDV serotype-specific antibodies in North rather than in South Darfur States.

3.3. Sero-prevalence of FMDV serotype-specific antibodies in different localities of North Darfur State:

Serotypes O and A showed the highest sero-prevalence at El Fasher capital city but the lowest at Um Keddada district which neighbors, mainly, East Darfur State (Table 5; Figure 11). The Southern localities of Um Keddada, El Taweish and El Lait which share border with West Kordofan and East Darfur States presented in comparison lower sero-prevalence of serotype O antibody.

In contrast, serotype SAT2 showed the highest sero-prevalence at Um Keddada but the lowest at the Northern district of El Kuma where serotype O showed relatively high sero-prevalence. Serotype A showed relatively high sero-prevalence at the Southern locality of El Taweish and El Lait (Table 5; Figure 11).

It worth noting that the highest sero-prevalence of anti-NSPs antibodies, in North Darfur localities, at El Fasher (Table 1) was coinciding with the highest sero-prevalence of serotype-specific antibodies of serotype O and A but not SAT2 (Table 5).

Table 5. Sero-prevalence of FMD virus serotype-specific antibodies in different localities of North Darfur State.

Locality	No. of tested sera	O		A		SAT2	
		% +ve	Estimated Prevalence	% +ve	Estimated Prevalence	% +ve	Estimated Prevalence
El Fasher	58	83.0% (48/58)	70.0%	50.0% (29/58)	42.0%	19.0% (11/58)	16.0%
El Kuma	43	70.0% (30/43)	54.0%	30.0% (13/43)	23.0%	12.0% (5/43)	9.0%
El Taweish and El Lait	83	60.0% (50/83)	41.0%	53.0% (44/83)	37.0%	14.0% (12/83)	10.0%
Um Keddada	44	52.0% (23/44)	36.0%	11.0% (5/44)	8.0%	32.0% (14/44)	22.0%

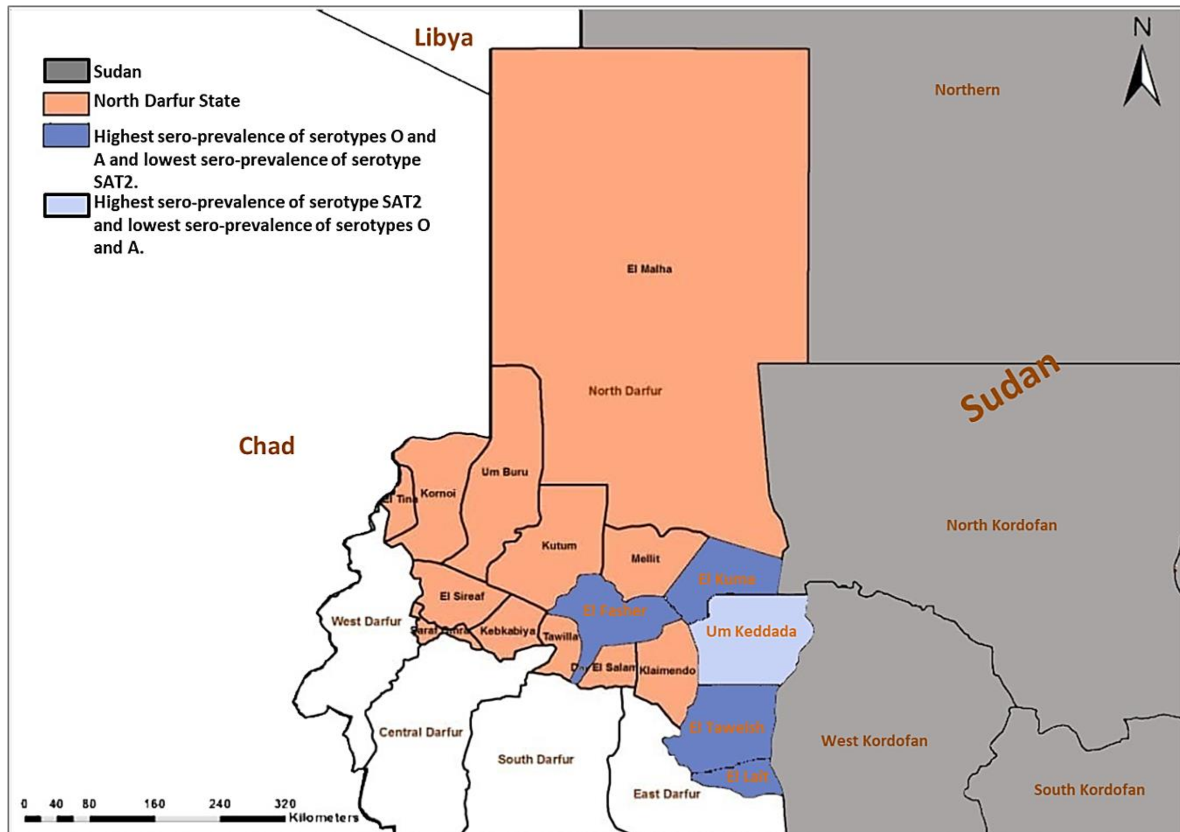

Figure 11. Prevalence of serotype-specific antibodies against FMD virus in cattle sera in different districts in North Darfur State.

3.4. Prevalence of FMDV serotype-specific SPs antibodies in different localities of South Darfur State:

Similar to North Darfur, serotype O showed relatively high sero-prevalence at the Northern district (Niteaga) and at the State capital city (Nyala) while it was lower at the Southern (Bielel) and Western (Marshang) districts (Table 6; Figure 12). Also, serotype A showed relatively high sero-prevalence at the capital city of Nyala but relatively low sero-prevalence at the North (Niteaga and Marshang) and at the South (Bielel). Sero-prevalence of serotype SAT2 was relatively low. However, remarkably, it showed the lowest sero-prevalence at the Northern district of Niteaga (Table 6; Figure 12).

Also similar to North Darfur, the higest sero-prevalence of anti-NSPs antibodies at Niteaga (Table 1) was coinciding with the highest sero-prevalence of serotype O specific antibody but the lowest sero-prevalence of serotype A and SAT2 specific antibodies in South Darfur (Table 6).

Table 6. Sero-prevalence of FMD virus serotype-specific antibodies in different localities of South Darfur State.

Locality	Total tested	No.	O		A		SAT2	
			% +ve	Estimated Prevalence	% +ve	Estimated Prevalence	% +ve	Estimated Prevalence
Niteaga	29		72.0% (21/29)	36.0%	28.0% (8/29)	14.0%	3.0% (1/29)	2.0%
Nyala North	30		70.0% (21/30)	34.0%	47.0% (14/30)	23.0%	20.0% (6/30)	10.0%
Nyala South	28		64.0% (18/28)	28.0%	39.0% (11/28)	17.0%	21.0% (6/28)	9.0%
Marshang	26		62.0% (16/26)	24.0%	42.0% (11/26)	16.0%	27.0% (7/26)	10.0%
Bielel	26		54.0% (14/26)	18.0%	31.0% (8/26)	11.0%	23.0% (6/26)	8.0%

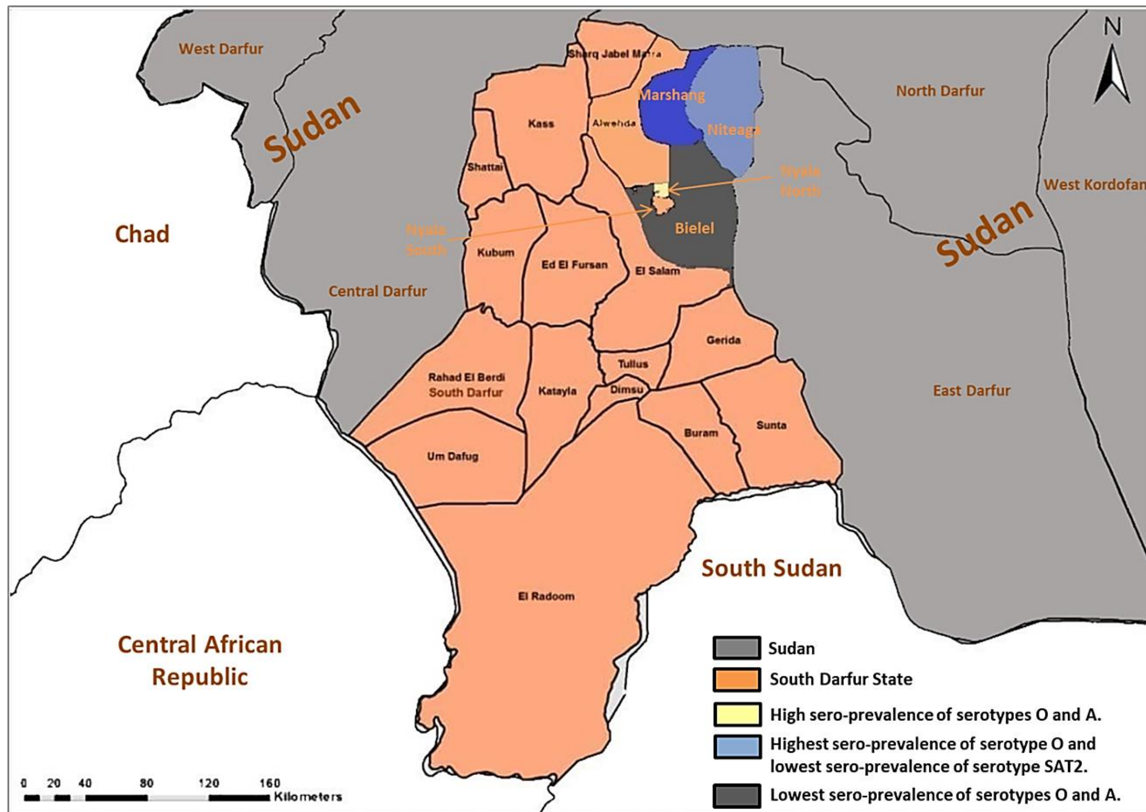

Figure 12. Prevalence of serotype-specific antibodies against FMD virus in cattle sera in different districts in South Darfur State.

3.5. Clinical disease surveillance:

3.5.1. Passive surveillance:

Clinical signs of FMD in cattle were observed between late 2017 and early 2018 in Nyala. Clinical signs were mild and involved salivation and small focal mouth lesions.

3.5.2. Active surveillance:

Seven location; Kaber Nile, Um Dbaker, Fangog, Aslaya, Lban, Gezira Aba, Shabsha were investigated late in March 2018. Clinical signs, which were mild involved salivation and lameness, were observed in cattle in Kaber Nile only.

Herd's men reported the occurrence of similar clinical signs earlier in winter months in Skerkila area bordering South Kordofan State and South Sudan Republic.

3.5.3. Detection and serotyping of FMDV:

Three samples were tested at the Department of FMD, CVRL, Soba, Sudan (Table 7) while two samples, because of their markedly poor quality, were dispatched to the World Reference Laboratory (WRL) for FMD at the Pirbright Institute, UK (Table 8). Detection and serotyping was effected locally by IZSLER ELISA and abroad by IZSLER ELISA, sensitive cell culture and PCR. Locally and abroad, samples failed typing (Table 7 and 8).

Table 7. Detection and serotyping of FMDV at the Department of FMD, CVRL.

Serial No.	Sample origin	Date of collection	Specimen	Species	Serotyping	
					Results	Test
250017Na	Nyala	12/2017	Epithelium tissue sample	cattle	Negative	IZSLER ELISA
250017Nb	Nyala	12/2017	Epithelium tissue sample	cattle	Negative	IZSLER ELISA
287018Na	Nyala	1/2018	Epithelium tissue sample	cattle	Negative	WRL
287018Nb	Nyala	1/2018	Epithelium tissue sample	cattle	Negative	WRL
298018	Kaber Nile	3/2018	Epithelium tissue sample	cattle	Negative	WRL

Table 8. Detection and serotyping of FMDV at the WRL for FMD.

WRL Reference Sample No.	Sample origin	Date of collection	Species	PCR results	Serotype results by cell culture and ELISA
SUD 1/2018	Nyala, South Darfur State	January, 2018	Cattle	FMDV NGD	NVD
SUD 2/2018	Nyala, South Darfur State	January, 2018	Cattle	FMDV NGD	NVD

Notes:

NVD = No Virus was detected.

NGD = No Genome was detected.

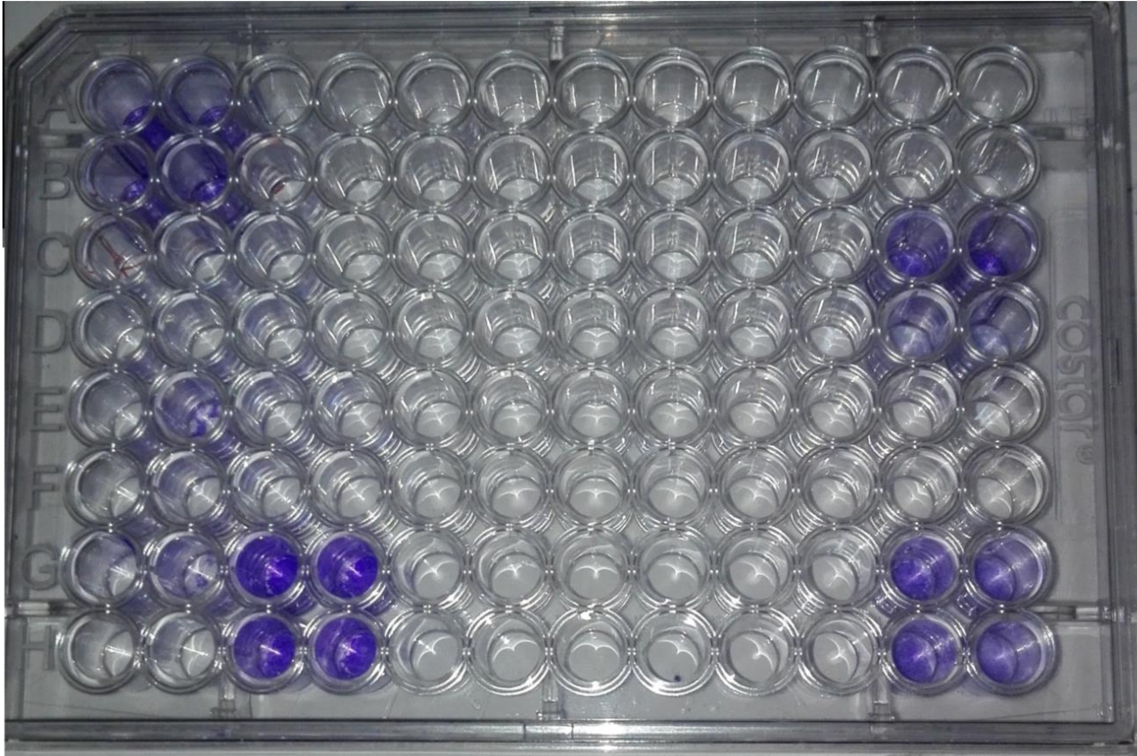

Figure 13A. Virus neutralization test (VNT) for typing of anti-SPs antibodies to type SAT2 FMDV. The VNT stained plate showing the stained VNT positive (+ve) and unstained VNT negative (-ve) cattle sera.

Figure 13B. Virus neutralization test (VNT) for typing of anti-SPs antibodies to type O FMDV. The VNT stained plate showing the stained VNT positive (+ve) and unstained VNT negative (-ve) cattle sera.

Figure 13C. Virus neutralization test (VNT) for typing of anti-SPs antibodies to type A FMDV. The VNT stained plate showing the stained VNT positive (+ve) and unstained VNT negative (-ve) cattle sera.

Chapter IV
Discussion

Control and eradication of FMD in likely endemic setting usually depends on vaccination in a step-by-step progression from one region in the country to another. For selection and prioritization of regions, use is made of geographical barrier then epidemiological and livestock production patterns that make an area distinct with regard to FMD spread. Merits also lie in selection of major livestock breeding areas. For efficient vaccination, it is more appropriate to vaccinate animals "upstream" where the virus is present in its ecological niche rather than in "downstream" where the virus enters a large susceptible population (Geering and Lubroth, 2002). Therefore, the present study of FMD in Darfur States, a major breeding area where pastoral system prevails, was preoccupied with two main concerns. The first was to define, with respect to the epidemiology of FMD in Sudan, whether Darfur area gets infected from other areas i.e. secondary endemic or it contains primary endemic areas. The second concern was to elucidate the influence of the prevailing pastoral system in Darfur on how and where FMD spreads. NSPs serology identified relatively low level of FMDV infection, of around 40%, in pastoral areas in Eastern (El Gedarif State) and Western (South Darfur area) Sudan (Department of FMD Report, 2016). Despite that introduction of serotype A with pastoralism through the international border areas of El Gedarif State was considered likely (Raouf *et al.,* 2016). Many other reports incriminated unrestricted animal movements and pastoralism for dispersal of FMD virus in sub-Saharan Africa (Sangare´ *et al.,* 2004; Ayelet *et al.,* 2009; Ahmed *et al.,* 2012 Kasanga *et al.,* 2014; Ularamu *et al.,* 2017). Findings of this study provided little or no data to support that Darfur area may be an important source of FMD viruses that could spread to other parts of the country. Also, no evidences were detected, that incriminated unrestricted animal movement and pastoralism across the Western border of Sudan for transmission of FMD virus. Active and passive surveillances in the disease season 2017-2018 spotted only mild form of clinical signs of FMD and in limited geographical areas; merely two locations. Samples from these disease events failed typing likely because of unavailability of sufficient amount of epithelial tissue. Concurrently, indices of prevalence of serotype-specific antibodies were higher in Northern rather than Southern localities (Figure 11 and 12) i.e. away from border areas and it followed the same order

(Table 5 and 6) that existed in other regions in Sudan where no or little pastoralism prevail; O, A then SAT2. Therefore, no particular effect for animal movement across the Western and South Western border could be realized.

The two studied States (North and South Darfur States) represented the two levels of FMDV infection detected in Darfur area by NSPs serology. South Darfur State was particularly selected because of two reasons. First, in the study area, it has the largest border area (Figure 5) with the Republic of South Sudan (SSR) and represented almost the whole border area with the Central African Republic (CAR). Secondly, it neighbours East Darfur State; the one Southern State in Darfur that showed high level of anti-NSPs antibodies activity (around 70%). For logistic reasons active disease surveillance was carried out in the White Nile State rather than in one of Darfur States. The White Nile State has long border area with Western Sudan and showed, in more than one occasion, similar geographical distribution of FMDV infection to Western Sudan (Raouf *et al.,* 2016; Department of FMD Report, 2016). It was hoped that active surveillance in the Eastern flank of Western Sudan and passive surveillance in South Darfur State with its large borders areas with two neighbouring countries (Figure 5) in South Western Sudan could prove useful to traceability efforts. It is evident that the two studied States in Darfur share border with the three neighbouring countries in the West and South West of Sudan; North Darfur State with Chad and South Darfur State with SSR and CAR.

Recently, to study the prevalence of serotype-specific antibodies against FMD virus in cattle in Sudan, VNT's have been employed extensively (Raouf *et al.,* 2016; Saeed, 2019; Saeed and Raouf, 2020). In this study, to decrease the load of the work, VNT's were used simultaneously with NSPs ELISA (ID ELISA); the latter being the primary testing method. This approach is known to increase specificity but decrease sensitivity (Fletcher and Fletcher, 2005). Particularly, NSPs-ELISAs are expected to be less sensitive than SPs serology in detecting mild FMD infection after vaccination; due to limited virus multiplication (Brocchi *et al.,* 2006; King *et al.,* 2015). In the field, where no vaccination is practiced, this is comparable to mild repeated infection with the same serotype which is more likely to happen with the predominant serotype than with the subordinate serotypes. Serotype O, the most predominant serotype in many parts of Sudan (Abu Elzein *et al.,* 1987; Raouf *et al.,* 2016) was also detected as the dominant serotype in this work whether the levels of FMD infection was high (North Darfur) or

low (South Darfur). Another concern was raised due to the known genetic heterogeneity of the 3ABC polypeptide of the SAT serotype (Van Rensburg *et al.,* 2002; Nsamba *et al.,* 2015). It was feared that NSPs-ELISA expressing 3ABC polyprotein derived from the classical "European/South American" types (O, A and C) may be less efficient in detection of NSPs-antibodies from FMD virus SAT infections. However, Chitray *et al.* (2018) have shown that NSPs-ELISAs irrespective of the origin of the 3ABC antigen, were reliable and accurate for the detection of FMD virus SAT 3ABC antibodies. As far as the specificity of the approach is concerned, some recent reports described cross reactions in the VNT (Tekleghiorghis *et al.,* 2014; 2015). This was observed in at least one of these two reports where sera were collected between 2 weeks to 2 months following confirmed FMD outbreaks (Tekleghiorghis *et al.,* 2014). In the second report, to increase the specificity of the neutralization assay, Tekleghiorghis *et al.* (2015) used a cut-off value different from the standard cut-off value of 1.65 $\log_{10}$ (OIE Manual, 2021). From our experience, in Sudan, although a cut-off of 1.5 $\log_{10}$, around the standard cut-off value or slightly lower, was used, significant differences in the prevalence and distribution of circulating FMD virus serotypes were observed previously (Raouf *et al.,* 2016; 2017) and also in this work.

For optimum sensitivity of the neutralization assay, the virus used in the assay should be closely matched to the strain circulating in the field (OIE Manual, 2021). Local FMD virus isolates used in the study were all recent isolates obtained in 2008, 2010, 2011 and 2015. Yet, approximately 20% of anti-NSPs positive sera in this work failed to show anti-SPs activity. Disease surveillance in Sudan in the last 15 years detected serotype O FMD viruses, mostly, followed by A then SAT2 (Raouf *et al.,* 2009; 2010; Habiela *et al.,* 2010a; 2010b; http://www.wrlfmd.org). Similarly, serosurveillance detected serum activity against these viruses, mostly, in that same order (Raouf *et al.,* 2016) which gave credibility to both types of surveillance. Accordingly, had there been any undetected activity of serotype SAT1 and SAT3 in Sudan, it is fair to expect it to be of minor importance and account for little or insignificant part of the un-typed sera. Alternatively, such reactors (NSPs +ve SP –ve) were also detected following vaccination and experimental challenge (Brocchi *et al.,* 2006). Brocchi *et al.* (2006) reported that these same experimental sera/reactors were detected repetitively by different NSPs-ELISAs and in different occasions. Therefore, they were unlikely to be non-specific reactors. In the field, on different occasions, studies that used different

NSPs-ELISAs and VNT also reported these reactors. Bronsvoort *et al.* (2008) reported 26/327 (8%) such reactors in buffalo and 7/11 (64%) in non-buffalo wild ungulates, Tekleghiorghis *et al.* (2015) reported 190/555 (34%) in cattle and Raouf *et al.* (2017) reported 49/215 (23%) in small ruminants and 3/66 (5%) in cattle. Bronsvoort *et al.* (2008) associated these reactors with low seroprevalence estimates whereas Raouf *et al.* (2017) expected that repeated mild exposure to different serotypes is likely to boost immune response to NSPs but not to SP what result in this type of reactors. In this study, it was remarkable that the proportion of such reactors remained similar at two significantly different levels of FMD virus activity in the North and in the South which suggested a likely minor role for the sensitivity of the testing methods.

One of the main objectives of the presented work was to define the extent of infection of different FMD virus serotypes in cattle in Darfur area. In absence of vaccination, prevalence of serotype-specific antibodies is indicative of previous infection. Prevalence of serotype-specific antibodies in Darfur was found to be highest for serotype O followed by A then SAT2 (Table 4) similar to the order detected previously in other part of the country (Raouf *et al.,* 2016), apart from Northern Sudan (Saeed, 2019; Saeed and Raouf, 2020). In every case, prevalence's detected were higher in North Darfur than in South Darfur State. In South Darfur, cattle graze most of the year in their Southern grazing fields away from trade routes and away from the Eastern areas of Western Sudan which are subjected to FMD virus spill from the Nile valley. On the other hand, in Northern Darfur the FMD-infected Eastern areas of Western Sudan are part of the cattle pastoral system. In general terms, prevalence's of serotype-specific antibodies in Darfur compared to prevalence's in other parts of the country in 2013 (Raouf *et al.,* 2016) were found to be lower for serotype O, similar for serotype A and higher for serotype SAT2. In neighbouring Kordofan area, prevalence's detected in 2013 were 67.5% (serotype O), 26.4% (serotype A) and 5.1% (serotype SAT2) in North Kordofan and 46.3% (O), 24.1% (A) and 4.5% (SAT2) in South Kordofan State. In comparison, corresponding figures detected in this work were 48.9%, 26.2% and 13.6% in North Darfur and 27.2%, 16.7% and 8% in South Darfur State. The lower frequency for serotype O antibodies in Darfur area compared to other parts of Sudan was consistent with its suggested pattern of circulation from the Nile valley to other parts in the country (Raouf *et al.,* 2016). The frequency of serotype SAT2 antibodies in Darfur in 2015/2016 is higher than in Kordofan in early 2013 which was consistent with the

detected wide dissemination of SAT2 infection in five Sudanese States (http://www.wrlfmd.org), after the surveillance performed late in 2013 and early in 2014.

Not only did the prevalence rates of the three FMD virus serotypes differ considerably but their distribution in different districts in the two States showed different patterns. Serotype O, unlike serotype A and SAT2, consistently showed high prevalence at the capital cities and at the Northern districts but low prevalence at the Southern districts. Serotype A clearly showed high prevalence at the capital cities while no particular pattern could be described for serotype SAT2. Because of the higher prices of meat and livestock in urban centers, capital cities drive trade animal movements and increase the risk of FMD (Jemberu *et al.,* 2015). The described pattern for serotype O was consistent with the indicated spread of serotype O from North to South (Raouf *et al.,* 2016) and significant within the country's circulation while the picture for SAT2 was more suggestive of occasional or sporadic spread. Therefore, though many border districts escaped examination in this work due to civil unrest, it could be concluded that the load of FMD infections crossing the international border of Darfur was negligible or too weak to impact prevalence data. Animal movement to the North during the wet season from June to October, as part of the pastoral system, and movement related to trade into urban centers seem to bear the risk of introducing and maintaining FMD infection in Darfur area. Otherwise, results presented little evidence to suggest presence of FMD primary endemic foci in Darfur area.

Conclusion and Recommendations

Conclusion:

The study presented little or no evidence to suggest that Darfur area is a primary endemic area of FMD infection in Sudan. Nevertheless, the low indices of FMD infection particularly in Southern Darfur areas, the active pattern of animal movement (pastoralism) and the known across border trade there highlight the significance of Darfur area as an important disease corridor cluster between East and West Africa. This information was supportive to the working hypothesis of how FMD is introduced and circulated in Sudan and vital to Sudan Risk Base Strategic Plan (RBSP).

Recommendations:

1. Laboratory confirmation of clinical FMD in Darfur area has not been achieved neither in this work nor, perhaps, since 2005. Enhanced disease and virological surveillances in Darfur area is highly recommended.
2. Further sero-surveillance studies should be conducted to increase our understanding of the epidemiological patterns of FMD in Darfur area and to cover areas that were not included in this work.
3. The serial testing approach employing VNT and NSPs ELISA (ID ELISA), the latter being the primary testing method that decreased the load of the work considerably while it compromised no result interpretation.

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Appendices

1. Preparation of buffers and reagents:

1.1. Deionized Distilled Water (DDW):

Deionized distilled water (DDW) was used for preparation of all reagents and buffers, DDW is sterilized by autoclaving at 120°C for 20 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	1000 ml

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

2. Cell Culture Medium and Reagents

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

GMEM powder	125.19 g
DDW completed to	2000 ml

GMEM powder was dissolved in DDW using magnetic stirrer, sterilized by filtration through Millipore filter under positive pressure and stored at -20°C.

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

GMEM 5X	200 ml
$NaHCO_3$	3.5-7.5 ml
Tryptose Phosphate Broth	100 ml
Penicillin/Streptomycin	1 ml
Gentamycin	1 ml

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

After thawing of GMEM 5X, by warming in water bath at 37°C, the medium was prepared, 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 bicarbonate7.5% 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):

2.5.1. Hank's Balanced Salt Solution (HBSS) 5X:

Solution (A):

NaCl	80 g
KCl	4.0 g
$MgSO_4.2H_2O$	2.0 g
$CaCl_2.HPO_4.2H_2O$	0.6 g
KH_2PO_4	0.6 g
DDW completed to	1000 ml
Add 10% TPB (v/v)	

Solution (B):

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

Add solution B to solution A under continuous stirring, complete to 2 litres with DDW, sterilized by filtration through Millipore filter and store at 20°C.

2.5.2. HBSS (1X) working solution:

HBSS (5x)	200 ml
Penicillin/Streptomycin	1 ml
Gentamycin	1 ml
Fungizon	1 ml
$NaHCO_3$ (7.5% solution)	10 ml
DDW completed to	1000 ml

2.6. Preparation of 0.04 M phosphate buffers:

2.6.1. Solution 1: 0.04 M Na_2HPO_4 (142 g/mol):

Na_2HPO_4	2.84 g
DDW completed to	500 ml

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

2.6.2. Solution 2: 0.04 M $NaHPO_4$ (120 g/mol):

$NaHPO_4$	2.4 g
DDW completed to	500 ml

The solution was sterilized by autoclave then the pH was recorded, stored at 4°C. Solutions 1 and 2 were mixed together in ratios that provide a pH of 7.3 as determined by a pH meter.

3. Preparation of cell dispersing agents:

3.1. Preparation of Trypsin solutions:

3.1.1. Trypsin (stoke solution 2.5%):

Trypsin powder	12.5 g
Phosphate diluent (PD)	500 ml

Trypsin powder was dissolved in cold PD, sterilized by filtration through Millipore filter and stored at -20°C.

3.1.2. Trypsin (working solution 0.45%):

2.5% Trypsin	180 ml
Phosphate diluents (PD)	1000 ml

3.1.3. Trypsin (working solution 0.25%):

2.5% Trypsin	1 ml
Hank's Solution (HBSS)	9 ml

3.2. Versene 5% (EDTA) solution:

Versene powder	5 g
P.D. completed to	100 ml

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

3.3. Trypsin-Versene solution:

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

A few drop of 0.5% phenol red solution (sterile) was added, some drops of 1 M NaOH were added to shifted to alkaline, 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

The antibiotics powder was dissolved in DDW, transferred into universal bottle then mixed well by shaking. The prepared solution contains 200,000 IU/ml Penicillin and 100 µg/ml Streptomycin per 1 ml, 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 solution, in one ampule, was transferred into universal bottle, then mixed well by shaking with DDW. The prepared solution contains 10,000 µg Gentamycin per 1 ml, stored at -20°C.

5. Preparation of stains and indicator:

5.1. Formal saline 10%:

Normal Saline (NS)	450 ml
Formalin	50 ml

Mixed well and stored at room temperature.

5.2. Crystal Violet stain (0.1%):

Crystal Violet powder	0.5 g
Formal saline 10%	500 ml

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 medium 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 autoclaving 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:

7.2.1. Conjugate A:

Conjugate A	400 µl
Dilution buffer	3600 µl

7.2.2. Conjugate B:

Conjugate B	200 µl
Dilution buffer	1800 µl