Foot-and-mouth disease sero-surveillance in Africa and vaccine matching

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2014

Cover design: Tesfaalem Tekleghiorghis

Printed by: proefschriftmaken.nl Uitgeverij BOXPress

ISBN: 978-90-8891-961-9

Foot-and-mouth disease sero-surveillance in Africa and vaccine matching

Mond-en-klauwzeer sero-surveillance in Afrika en vaccin matching.

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 9 oktober 2014 des ochtends te 10.30 uur

door

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geboren op 7 maart 1960 te Nielto, Eritrea

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The research described in this thesis was financially supported by the International Atomic Energy Agency (IAEA), Vienna, Austria, and by the ministry of economic affairs in the Netherlands (WOT-01-003-11).

Printing of this thesis was financially supported by the Central Veterinary Institute (CVI) part of Wageningen UR, Lelystad, The Netherlands.

To the memory of Michael Tekleghiorghis Sebhatu

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Chapter 1

General Introduction

The foot-and-mouth disease virus

Foot-and-mouth disease virus (FMDV) is the etiological agent of an important disease of domestic and wild ruminant animals. The first written description of foot-and-mouth disease (FMD) probably occurred in 1546, when Hieronymus Fracastorius described a similar disease of cattle in Venice, Italy [1]. In 1897, Loeffler and Frosch demonstrated that a filterable agent (i.e. virus) caused FMD [2, 3]. This was the first demonstration that a disease of animals was caused by a filterable agent and marked the start of the era of virology. The existence of immunologically distinct serotypes of FMDV has been shown by Vallée and Carrée in 1922, who demonstrated that there was no cross immunity between serotype O and A. These serotypes were named from their areas of origin; O for the department of Oise in France and A for Allemagne (the French word for Germany) [4]. Later serotype C was discovered by Waldmann and Trautwein in Germany in 1926. Subsequently, three new serotypes were identified in samples originating from southern Africa and they were named as Southern African Territories 1, 2 and 3 (SAT-1, SAT-2, SAT-3) [5]. The seventh serotype, Asia1, was initially detected in a sample collected from a water buffalo in Pakistan in 1954 [6].

Afterwards it was shown that the agent, FMDV, is a small (27 nm in diameter), non-enveloped, icosahedral, positive-sense, single stranded RNA virus, with an approximately 8.5 kb genome that encodes for structural proteins (SP) as well as non-structural proteins (NSP) [7-9]. It belongs to the *aphthovirus* genus and *Picornaviridae* family [10]. The four main structural proteins of FMDV are VP1, VP2, VP3 and VP4. VP1-3 have surface components and VP4 is internally buried within the capsid [11] (Fig. 1).

The FMD virus exhibits a high potential for genetic and antigenic variation [12], as shown by the presence of seven distinct serotypes (A, O, C, SAT-1, SAT-2, SAT-3 and Asia 1) [13, 14]. Within these serotypes, more than 65 subtypes have been recognized [15, 16]. The high degree of antigenic variation may be attributed to the high rate of mutation, genetic recombination and the quasispecies nature of the virus. This antigenic variation is the basis for maintenance of FMDV circulation resulting in severe economic loss in livestock productions [12, 17]. FMDV serotypes show genetically and geographically distinct evolutionary lineages (topotypes) based on nucleotide differences of up to 15% for serotype O, A, C, and Asia 1. In the case of the SAT

serotypes, the level for inclusion within a topotype was raised to 20% since the VP1 coding sequence of these viruses appear to be more inherently variable [18, 19].

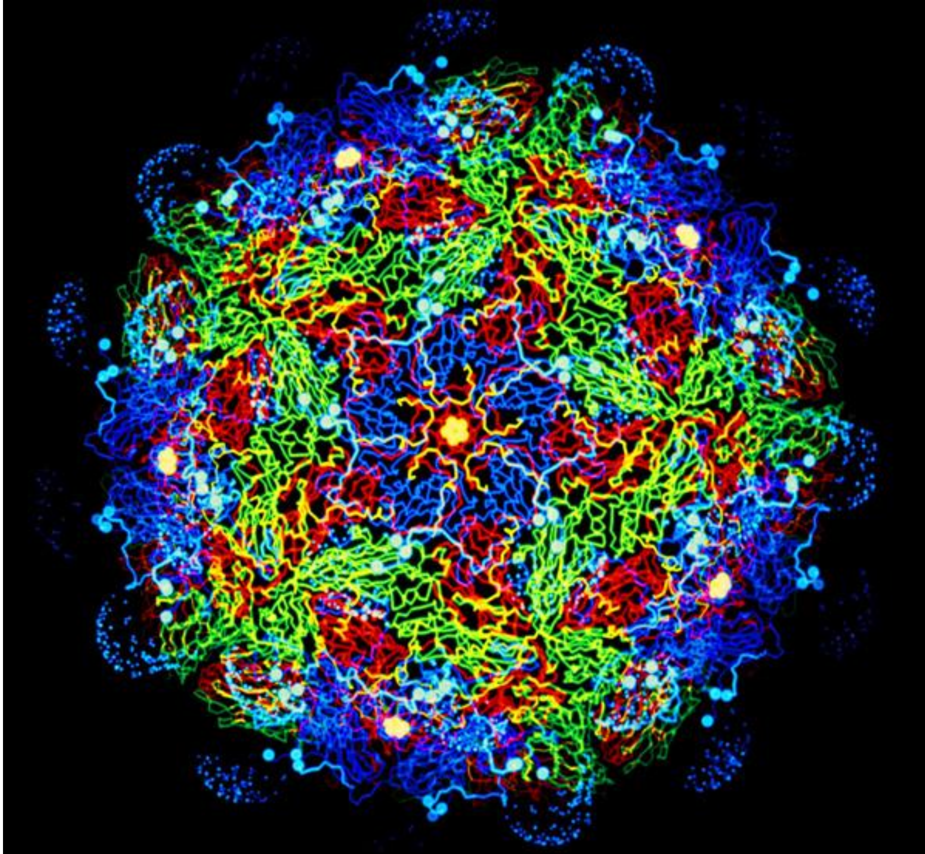

Figure 1. The three-dimensional X-ray crystallographic structure of foot-and-mouth disease virus at 2.9 Angstrom resolution [20].

Clinical disease

Foot-and-mouth disease is an important animal disease, severely affecting the production of livestock and disrupting regional and international trade in animals and animal products [21]. FMD is endemic in many countries of Africa (Fig. 2). In most developing countries the adverse effects of FMD are often underestimated. The disease affects domestic cloven-hoofed animals, including cattle, swine, sheep, goats and water buffalo as well as more than 70 species of wild animals, including African

buffalo [22], and is characterized by fever, lameness, and vesicular lesions on the tongue, feet, snout and teats of lactating animals. In sheep and goats the disease is generally mild and can be difficult to distinguish from other common conditions [23-25]. Although FMD does not result in high mortality in adult animals, the disease has debilitating effect, including weight loss, decrease in milk production, and loss of draught power, resulting in a loss in productivity for a considerable time [21, 26, 27]. Mortality, however, can be high in young animals due to myocarditis. In addition, cattle, sheep, and goats can become carriers, and cattle can harbor virus for up to 2 to 3 years [28]. Although a large number of FMD susceptible wildlife species is present in Africa, only the African buffalo is a true persistent carrier for the SAT (1-3) FMDV [29-33].

Epidemiology

Transmission of FMDV is generally effected by direct contact between acutely infected and susceptible animals [34, 35] or, more rarely, indirect exposure of susceptible animals to the excretions and secretions including expired air, saliva, nasal secretions, lachrymal fluid, milk, urine, faeces and semen [36, 37], products from infected animals (such as meat, milk, or uncooked meat) or inanimate objects contaminated with FMDV. Following recovery from the acute stage of infection, infectious virus disappears from the infected animal with the exception of low levels that may persist in the oropharynx of ruminants from which live virus or viral RNA may continue to be recovered from oropharyngeal fluids and cells collected from the oropharyngeal area [38]. Animals in which the virus persists in the oropharynx for more than 28 days after infection are referred to as carriers. There is some experimental evidence, particularly in the African buffalo, that carriers are able, in rare occasions, to transmit the infection to susceptible domestic animals with which they come in close contact [39]. Pigs do not become carriers, but can excrete large amounts of virus in their expiration, and are therefore by some researchers considered as key amplifiers of the virus [40-42].

In 2001, the FMD outbreak in the United Kingdom was associated with pigs fed on swill obtained from restaurants. The most likely source believed to be waste food contaminated with FMD virus [43]. Based on the sequence data information the Pan Asia O strain of the virus is assumed to originate from Asia [43-45]. Live animal movement was the main route of further transmission of FMD to France and the

Netherlands [46]. In South-East Asia the management of animal movement is quite variable across the region and animal movement is mainly market-driven often irrespective of borders [47]. Outbreaks of FMD is linked to animal trade movements in the region [47]. In Africa, FMDV transmission is considered to happen through long distance animal movement for grazing and water and trade often irrespective of borders, but also wildlife involvement most likely plays an important role. The differences in different parts of Africa on the role of animal husbandry, trade and livestock involvement is not completely known.

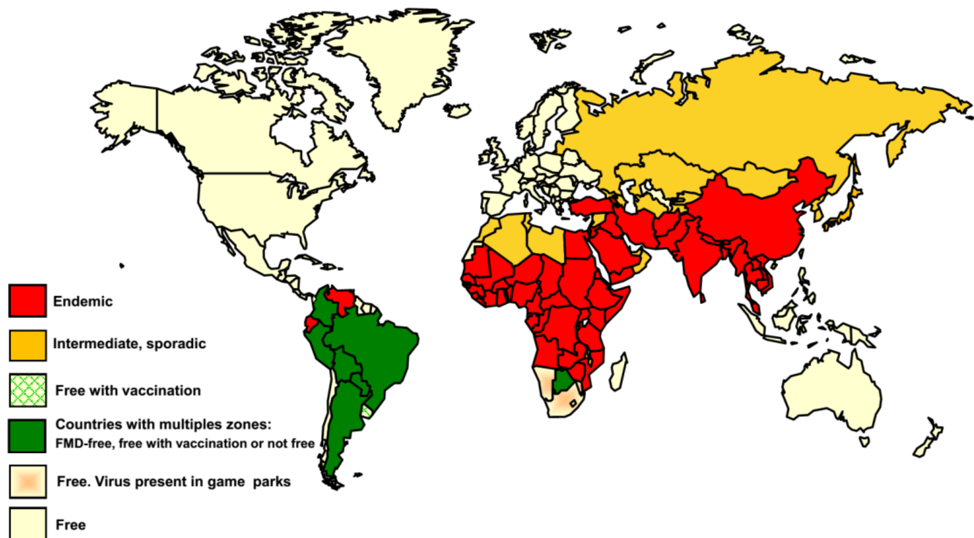

Figure 2: Conjectured status of FMD in 2012 (www.pirbright.ac.uk).

It has been possible to genetically group many FMDV's based on their geographic origin and this has led to their being referred to as topotypes [48]. The implications of this are that inter-regional spread of FMD viruses can often be easily recognized and any evolutionary changes which subsequently occur can be monitored. Topotype/genotype identification has been used in FMD epidemiology to trace or detect recent or previous outbreaks in Europe, Asia and South America but in Africa there was not comprehensive genetic analysis of the links between outbreaks. There is lack of information on FMD in some African countries because of not submitting outbreak

samples to diagnostic and reference laboratories. As described in this thesis a sero-prevalence study has been carried out in cattle to bridge the gaps on the inadequate data on FMD in Eritrea.

FMDV vaccine

The oldest known strategy used by cattle owners in the distant past to confer active protection on their herd was to practice 'aphtisation' as soon as the first case of FMD was observed in the herd or in the neighborhood [49, 50]. Later, Waldmann produced a vaccine using epithelium and vesicles from tongues of infected cattle, inactivated by formaldehyde in the presence of aluminium hydroxide gel [51]. In 1947, Frenkel developed the large-scale production of vaccine by growth of virus in surviving tongue epithelium [52, 53]. FMD virus growth in monolayers of BHK-21 cell cultures [54] and later also in suspension [55], allowed large scale production of vaccine [56]. The large majority of FMD vaccine today is produced in BHK-21 suspension cell culture, and the live virus is inactivated using binary ethylenimine (BEI) [57].

Adjuvants were originally described by Ramon as 'substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone [50]. For control of FMD, inactivated whole virus vaccines against FMD are formulated as mono or polyvalent products with suitable stabilizers, buffers and adjuvant to enhance their potency. Both aluminium hydroxide [$Al(OH)_3$]-saponin and oil adjuvanted FMD vaccines are used. In aqueous formulations, the inactivated viral antigen is adsorbed to $Al(OH)_3$-saponin adjuvant. $Al(OH)_3$-saponin adjuvanted FMD vaccines are effective in cattle, sheep and goats, but function poorly in pigs, while oil-adjuvanted vaccines can be used in all species [58, 59]. Some researchers consider oil adjuvanted FMD vaccines superior, but this opinion is probably based on the fact that in South America the development of oil adjuvants coincided with improved quality control of the vaccine. Others claim that oil adjuvanted FMD vaccines are at least as effective as $Al(OH)_3$-saponin adjuvanted vaccines, with some studies suggesting that the duration of immunity is longer in oil-based adjuvants [37, 59, 60]. In a comparative study both the $Al(OH)_3$-saponin and Montanide ISA 206B containing saponin vaccines protected 4 out of 6 cattle almost 1 year after primo vaccination and booster at 4 and 8 weeks after vaccination, respectively against virulent virus challenge [61]. There was no

information on the difference in the breadth of the immune response using different adjuvants.

FMDV shows a high degree of antigenic variability [17, 62-64], as evidenced by the seven serotypes and numerous subtypes distinguished to date [16]. Therefore, the extensive antigenic diversity of the virus is considered one of the major obstacles for the control of FMD by vaccination in the endemic areas [28, 65-68]. The strength and duration of antibody immune response of a vaccine depends upon vaccine potency and antigenic match between the vaccine and the field outbreak strain [69, 70]. There is a need for FMD vaccine matching due to the antigenic changes caused by spontaneous mutations and recombination that occur during replication of the single-stranded RNA genome of positive polarity [67] and some serotypes are more antigenically diverse than others. The antigenic relationships within a serotype may be also quite distant. The relationship between field and vaccine strains is often calculated based on the quotient between heterologous titre and homologous titre (r-value). A high correlation between r_1-values and in vivo cross-protection, however, has not always been observed [70]. There are documented cases where cross-protection was found in spite of low r_1-values [71] and vice versa. The r_1-value was found to be an inaccurate indicator of cross-protection in contrast to the VNT titers against the field isolates [72]. The latter takes also the vaccine quality into account, as a low titre induced by a good matching strain might not protect, whereas a very high titre against a poor matching strain could protect [73]. Therefore, quality control of FMD vaccines is also essential.

Aims and outline of the thesis

The research work described in this thesis has been performed for three important aspects. The first was to get an overview of the role that animal husbandry, trade and wildlife have on the transmission of FMD virus and to provide a scientific basis for different FMD control measures in Africa (chapter 2). On the same track, two surveillance studies on FMD were carried out to know the level of sero-prevalence and identify the serotypes in cattle in Eritrea as described in chapter 3 and 4. The second was to determine which serological test method is best to be used to test cross-reactions in serology and address the inherent variability of the r_1-value. To this end we analysed the cross-reactions in serology using serum samples from cattle vaccinated

with 10 different FMDV serotype A strain vaccines using three serological test methods (virus neutralization test, neutralization index test and the liquid phase blocking ELISA) (chapter 5). The third was to investigate whether the formulation of using different adjuvants, antigen composition, antigen payload and administration route of an FMD vaccine had any influence on the breadth of the antibody response in cattle (chapter 6).

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Chapter 2

Foot-and-mouth disease transmission in Africa: implications for control, a review

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Transboundary and Emerging Diseases (2014),

DOI:10.1111/tbed.12248

Summary

In Africa, for the control of foot-and-mouth disease (FMD), more information is needed on the spread of the disease at local, regional and inter-regional level. The aim of this review is to identify the role that animal husbandry, trade and wildlife have on the transmission of FMD and to provide a scientific basis for different FMD control measures in Africa. Review of literature, published reports and databases show that there is more long distance spread of FMD virus serotypes within North, West, Central and East Africa than in southern Africa. In North, West, Central and East Africa migratory animal husbandry systems often related with search for grazing and water as well as trade are practiced to a greater extent than in southern Africa. In southern Africa, the role of African buffalo (*Syncerus caffer*) is more extensively studied than in the other parts of Africa, but based on the densities of African buffalo in Central and East Africa, one would assume that buffalo should also play a role in the epidemiology of FMD in this part of Africa. More sampling of buffalo is necessary in West, Central and East Africa. The genetic analysis of virus strains has proven to be valuable to increase our understanding in the spread of FMD in Africa. This review shows that there is a difference in FMD occurrence between southern Africa and the rest of the continent; this distinction is most likely based on differences in animal husbandry and trade systems. Insufficient data on FMD in wildlife outside southern Africa is limiting our understanding on the role wildlife plays in the transmission of FMD in the other buffalo inhabited areas of Africa.

Keywords: foot-and-mouth disease; serotype; topotype; Africa; transmission; animal husbandry; wildlife

Introduction

Foot-and-mouth disease (FMD) is an economically important contagious disease of domestic (cattle, pigs, sheep and goats) and wild cloven-hoofed animals [1]. After rinderpest, FMD will be the next disease the World Organization for Animal Health (OIE), and the Food and Agricultural Organization (FAO) of the United Nations target for worldwide eradication. To be able to plan such eradication, more information is needed on the epidemiology of the disease and its spread in the world.

The causative agent of FMD is a small positive sense single-stranded RNA virus (approx. 8.4 kb) which belongs to the *Aphthovirus* genus of the family *Picornaviridae* [2]. The FMD virus (FMDV) is an antigenically variable virus, reflected in seven serotypes (A, O, C, Asia 1, Southern African Territories (SAT)-1, SAT-2 and SAT-3), that do not confer cross immunity to each other in addition to having many subtypes and variants within each serotype [3, 4].

The genetic diversity of FMDV is a consequence of the high mutation rate due to error-prone RNA polymerase-lacking proofreading activity. Four structural proteins VP1, VP2, VP3 and VP4 form the virus capsids. These structural proteins are coded respectively by the 1D, 1B, 1C and 1A coding region of the genome. Of these viral structural proteins, VP1 is immunogenically the most important as it contains an essential cell receptor site on the GH loop that forms an important neutralising epitope together with the C-terminus of VP1 [5, 6]. The 1D coding region that encodes for VP1 has the highest variability and is therefore used to study the molecular epidemiology of FMD in various parts of the world, including Africa [7-10]. These molecular epidemiological studies help us to understand the source of the spread of the disease. Within each FMDV serotype, genetic analysis can group viruses together in distinct clusters, which often correspond to geographically defined regions. These region-specific clusters are called topotypes and reflect the presence of genetically and geographically distinct evolutionary genotypes [11, 12].

The aim of this review is to identify the role that the current animal husbandry, trade and wildlife situations have had on the transmission of FMDV in Africa. This information will provide a scientific basis for different FMDV control measures in Africa. Using data available in literature, conference papers, reports of international organisation and databases (e.g. the website of the World Reference Laboratory (WRL)

for FMD in Pirbright, www.wrlfmd.org, OIE, http://www.oie.int/), we will first present an overview of the occurrence and distribution of different serotypes and topotypes of FMDV in Africa in the period 1990 to 2013. We focus on serotyped and genotyped isolates from the various regions, that is, North, West, Central and East and southern Africa. In the second part, we review the livestock husbandry systems, trade and involvement of wildlife in Africa. Finally, we discuss the roles that animal husbandry, trade and wildlife might play in the transmission of FMDV.

Review of the epidemiology of FMD in Africa

Occurrence of FMDV serotypes and topotypes

The information on FMDV serotypes and topotypes recorded in Africa obtained from the various data sources is summarised in Table 1, and in Figs 1 and 2. In sub-Saharan Africa, two cycles of FMD that impact on livelihoods occur, one in which the virus circulates between wildlife hosts and domestic animals and another in which the virus spreads among domestic animals, without the involvement of wildlife [10]. In many papers, it is made clear that the epidemiology of FMD in sub-Saharan Africa is very complex and each serotype behaves differently. Firstly, six of the seven serotypes (i.e. all but Asia 1) have been recorded in the African continent [13]. Secondly, the African buffalo plays a role in virus maintenance and disease transmission [14, 15]. This long-term relationship with buffalo has only been shown with convincing evidence for the three SAT serotype viruses (SAT-1, SAT-2 and SAT-3) [10].

In Africa, six topotypes have been identified for serotype O, two for serotype A, three for C and 9, 14 and 5 topotypes for SAT-1, SAT-2 and SAT-3 respectively [16, 17]. Africa has been divided into 3 FMDV pools: East Africa (pool 4) with serotypes O, A, SAT-1, SAT-2 and SAT-3; West Africa (pool 5) with serotypes O, A, SAT-1 and SAT-2; and southern Africa (pool 6) with serotypes SAT-1, SAT-2 and SAT-3 [18]. These pools only relate to countries currently infected with FMDV. Because we want to describe the FMD situation in all of Africa, also the non-endemic countries, here we have divided the African continent into three regions: North Africa, a combination of East, West and Central Africa, and finally southern Africa.

Although FMDV SAT serotypes are mainly found in Africa, serotype SAT-1 and SAT-2 incursions have been recorded outside Africa, mainly in the Middle East - very

likely through live animal importation from Africa. SAT-1 had spread to Bahrain, Israel, Jordan and Syria in 1962, to Iran in 1964, Turkey in 1965 and Kuwait in 1970 [19]. SAT-2 had been reported in Yemen in 1990 [20], in Kuwait and Saudi Arabia in 2000 [8] and in the Palestinian Autonomous Territories (PAT) and Bahrain in 2012 [20].

Occurrence of FMDV in North Africa

Countries in the North African region are Morocco, Western Sahara, Algeria, Tunisia, Libya and Egypt (Fig. 1). In North Africa, incursions of FMDV from sub-Saharan Africa and the Middle East are mainly by livestock trade [16, 21] and the virus maintenance is very likely within the domestic animals [22]. Sheep populations have previously been implicated in maintaining FMDV in this region [23, 24]. In the Western part of this region, that is, Morocco, Algeria and Tunisia, FMD occurrence was sporadic and usually originated from exogenous sources [25]. These countries have limited trade with the rest of Africa, but movement of herds of small ruminants within the region may have played a role in the spread of FMD from Tunisia to Morocco between 1989 and 1991 [19]. The FMDV strains found in the western part of North Africa were related to those from countries adjacent to the southern borders of this region. For example, the Sudan-Sahel type O strain (WA topotype) from West Africa (Cote d'Ivoire, O/CIV/8/99 and Ghana O/GHA/5/93 and O/GHA/9/93) was most likely responsible for the outbreak in the Maghreb region (Morocco, Algeria and Tunisia) in 1999 [12, 24]. At present, serotypes O and A have been controlled in Morocco, Algeria and Tunisia by vaccination and animal movement restrictions. These three countries have submitted control programme dossiers according to the provisions of chapter 8.6 of the OIE Terrestrial code [26] and were officially endorsed by the OIE in 2012, [27].

In recent years (2003-2012), FMD has become endemic in Libya and Egypt [16]. Before 2003, FMD outbreaks in Egypt and Libya were considered to be new introductions, although no evidence of absence during the inter-epidemic period was provided. In 2009, FMDV serotype O was introduced into Egypt (ME-SA Sharquia-72) and in 2011 into Libya from the Middle East (ME-SA PanAsia-2^{ANT-10}) [21, 28]. In Libya, in 2011, an outbreak in sheep and goats and in Egypt, in 2012, in cattle and sheep caused by serotype O topotype EA-3 was closely related (99.6% nucleotide identity) to viruses from Eritrea (2011) and northern Ethiopia (2011). This was the first record of

Table 1. Summary topotype distribution of FMDV serotypes O, A, C, and SAT-1-3 in Africa; in the period 1990 to 2013 (earlier isolates are included when there is no representative of the genotype in reports after 1990).

Serotype	Topotype	Genotype/ strain	Representative country / Countries	Reference
O	EA-1		Kenya (2010), Uganda (1996)	[16]
	EA-2		Kenya (2011), Burundi (2003), DRC (2011), Malawi (1998), Rwanda (2004), Tanzania (2009), Uganda (2007), Zambia (2010), Sudan (1999)	[16]
	EA-3		Ethiopia (2011), Eritrea (2011), Niger (2007), Nigeria (2009), Somalia (2007), Sudan (2011), Kenya (1987), Libya (2011), Egypt (2012)	[16]
	EA-4		Ethiopia (2013), Kenya (2010), Uganda (1999)	[16, 29]
	ME-SA	Sharquia-72	Egypt (2009)	[16]
	ME-SA	PanAsia-2	Libya (2011), Egypt (2007)	[16]
	ME-SA	PanAsia-1	South Africa (2000)	[16]
	ME-SA		Algeria (1990), Egypt (1993), Ethiopia (1994),Eritrea (1996), Tunisia (1994), Tanzania (1998), Libya (1994)	[23] [12]
	WA		Algeria (1999), Cote d'Ivore (1999), Burkina Faso (2002), Cameroon (2005), Ghana (1994), Guinea (1999), Gambia (1999), Mali (2007), Mauritania (2001), Niger (2005), Senegal (2006), Togo (2005), Tunisia (1999), Morocco (1999)	[23] [12], [16]
A	AFRICA	G-I	Kenya (2009), Tanzania (2012, 2013), Uganda (2002), Zambia (1990), Burundi (1990), DR. Congo (2011)	[16]
	AFRICA	G-II	Ethiopia (2005)	[16]
	AFRICA	G-III	Kenya (2005), Ethiopia (2005), Sudan (2007), Uganda (2002), Cameroon (2005), Egypt (2006)	[30]
	AFRICA	G-IV	Egypt (2012), Eritrea (2009), Mali (2006), Nigeria (2009), Togo (2005), Cameroon (2005), Sudan (2006)	[16]
	AFRICA	G-V	Ghana (1973)	[16]

	AFRICA	G-VI	Mali (1997, 2006), Mauritania (2006), Gambia (1998), Senegal (1996), Burkina Faso (1994), Cote d'Ivore (1996)	[16]
	AFRICA	G-VII	Egypt (2009), Ethiopia (2009), Kenya (2006)	[16]
	AFRICA	G-VIII	Kenya (1964)	[16]
	ASIA	Iran-05^{BAR-08}	Egypt (2011), Libya (2009)	[16]
C	AFRICA (I)	Ken-67	Kenya (2004)	[30]
	AFRICA (II)	Eth-71	Ethiopia (1983)	[28]
	AFRICA (III)		Angola (1973)	[8]
SAT-1	I (NWZ)		Kenya (2011), Tanzania (2010*, 2012), South Africa (2010), Zimbabwe (2003), Mozambique (2009), Zambia (2009), Malawi (2001)	[16]
	II (SEZ)		Botswana (1998*), Namibia (2010), Zambia (2010), Zimbabwe (2004), Swaziland (2000), Mozambique (2010*)	[16]
	III (WZ)		Tanzania (1999), northern Zimbabwe (1997*), Botswana (2006*), Zambia (2012), Namibia (2011)	[11, 16]
	IV (EA-1)		Uganda (2007*)	[31]
	V		Nigeria (1976), Niger (1976)	[32]
	VI		Nigeria (1981), Sudan (1976)	[16]
	VII (EA-2)		Uganda (1974)	[16]
	VIII (EA-3)		Uganda (1997*)	[16, 33]
	IX		Ethiopia (2007)	[34]
SAT-2	I		Botswana (2011), Malawi (2008), Mozambique (2010), Zimbabwe (2010), South Africa (2012), Burundi (1991), Kenya (1999), Zambia (1996*), Namibia (1998*)	[16, 35]
	II		Botswana (2008), Zimbabwe (2010), Namibia (1998*), Malawi (2008), Ghana (1991)	[16]
	III		Botswana (2006*, 2012), Namibia (2008), Zambia (2009), Zimbabwe (2002), South Africa (2011)	[16]
	IV		Kenya (2009), Tanzania (2012), Ethiopia (1991), Burundi (1991), Zambia (2012)	[16]

	V	Ghana (1991), Rwanda (2000), Senegal (1975)	[34]
	VI	Gambia (1979), Senegal (1983)	[32]
	VII	Egypt (2012), Libya (2003, 2012), Cameroon (2005), Eritrea (1998), Niger (2005), Nigeria (2008), Senegal (2009), Sudan (2007, 2010)	[16]
	VIII	Rwanda (2001)	[7, 35]
	IX	Kenya (1996), Uganda (1995)	[34]
	X	Uganda (2007*)	[31]
	XI	Angola (1974)	[35]
	XII	Uganda (1976)	[36]
	XIII	Sudan (2008), Ethiopia (2010)	[16]
	XIV	Ethiopia (1991)	[16, 34]
SAT-3	I (SEZ)	Zimbabwe (1999), Kruger National Park (1997*), Mozambique (2010*), South Africa (2011)	[7, 16, 37]
	II (WZ)	Zimbabwe (1994*), Namibia (1998*), Botswana (1998*), South Africa (2011)	[7, 16, 35]
	III (NWZ)	Zimbabwe (1991*)	[7]
	IV	Zambia (1996*)	[7]
	V	Uganda (1970*), Uganda (1997*), South Africa (2011)	[7, 16]

*: Foot-and-mouth disease (FMD) virus isolated from African buffalo.

EA-3 topotype in North Africa [16]. In Egypt, the large outbreak of serotype A virus seen in 2006 (African topotype G-VII$^{KEN\text{-}05}$) was genetically related to serotype A isolates from East Africa [16, 38]. Egypt experienced another exotic serotype A outbreak in February 2012 (topotype: AFRICA, genotype/strain: G-IV) genetically linked with strains occurring in sub-Saharan Africa [16]. Although Egypt is part of Africa, it borders on the Middle East, and has therefore also experienced introductions from Asia. Serotype A (A/IRN/05) was confirmed between 2006 and 2012 in Egypt and in 2006 in Libya [16].

Figure 1. Occurrence of FMD serotypes in Africa based on outbreaks reported in 1990 to 2013. Countries coloured with light grey: North Africa, white: West, Central and East Africa, dark grey: Southern Africa.

In Libya in 2003, an outbreak with SAT-2 (topotype VII) occurred that was genetically related to isolates from viruses from Cameroon in 2002, Saudi Arabia in 2000 and Eritrea in 1998 [39]. The SAT-2 virus was probably introduced from Western or Eastern Africa with imported livestock. In 2012 again, the serotype SAT-2 introduced to Libya (topotype VII) was genetically related to isolates in Sudan (SAT2/SUD/1/2007) and Nigeria (SAT2/NIG/5/2008). Later in 2012, Egypt reported FMDV SAT-2 topotype VII virus outbreaks, the first known occurrence of this serotype since 1950.

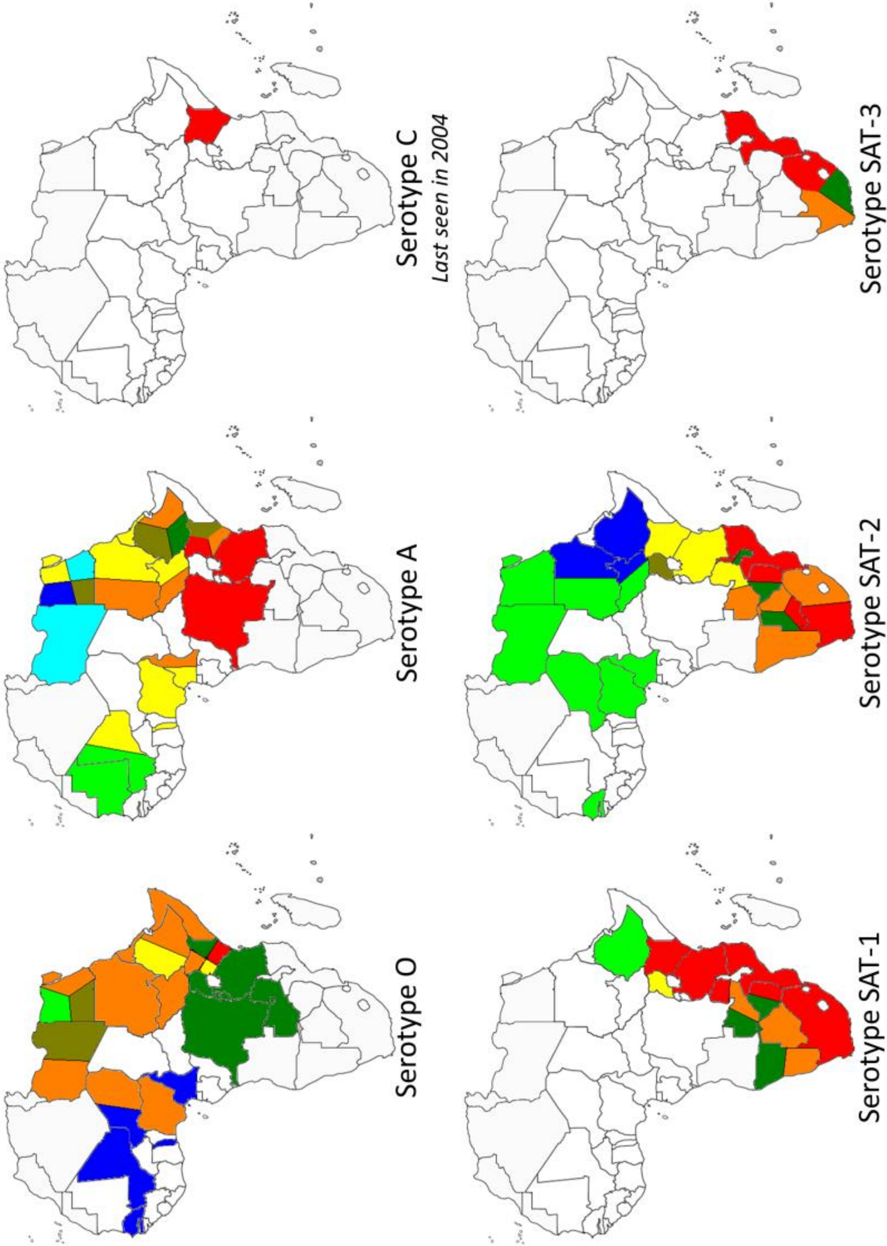
Serotype O
Serotype A
Serotype C
Last seen in 2004
Serotype SAT-1
Serotype SAT-2
Serotype SAT-3

Figure 2. Map with genotyped isolates where the different topotypes and genotypes reported from 2003 to 2013 are shown in different colours. If more than one topotype and/or genotypes was present in a country, it was divided into more than one section, without taking the location of the isolate (which is often unknown) into account.
For type O the topotypes and genotypes were:

● EA-1 ● EA-2 ● EA-3 ● EA-4 ● ME-SA Sharquia-72 ● ME-SA PanAsia-2 ● WA

For type A the topotypes and genotypes were:

● AFRICA G-I ● AFRICA G-II ● AFRICA G-III ● AFRICA G-IV ● AFRICA G-VI ● AFRICA G-VII ● AFRICA G-VII BAR-08 ● ASIA Iran-05

For type C the topotypes and genotypes were:

● AFRICA (I) Ken-67

For type SAT-1 the topotypes and genotypes were:

● I (NWZ) ● II (SEZ) ● III (WZ) ● IV (EA-I) ● IX

For type SAT-2 the topotypes and genotypes were:

● I ● II ● III ● IV ● VII ● X ● XIII

For type SAT-3 the topotypes and genotypes were:
● I (SEZ) ● II (WZ) ● V

Although these SAT-2 viruses belonged to the same topotype (VII) as the Libyan SAT-2 serotype, in this outbreak in Egypt, three distinct sub-lineages were identified (one Libyan and two Egyptian) [40]. Libya and Egypt in particular import a considerable number of livestock from FMD endemic countries. These imports could explain why all recent outbreaks of FMD are from sub-Saharan Africa and the Middle East.

Occurrence of FMDV in West, Central and East Africa

The countries in the West African region are Mauritania, Mali, Niger, Cape Verde, Senegal, Gambia, Guinea-Bissau, Guinea, Sierra Leone, Liberia, Cote d'Ivoire, Burkina Faso, Ghana, Togo, Benin and Nigeria. Central African countries are Chad, Cameroon, Central African Republic (CAR), Equatorial Guinea, Gabon, Republic of Congo and the Democratic Republic of Congo (DRC). The East African region comprises the countries Sudan, Eritrea, South Sudan, Ethiopia, Djibouti, Somalia, Kenya, Uganda, Tanzania, Rwanda and Burundi (Fig. 1). The reason for combining this large part of Africa is the

overlap in FMDV serotypes and topotypes isolated in West, Central and East Africa (Fig. 2). In this region six serotypes (O, A, C, SAT-1, SAT-2 and SAT-3) have been found (Fig. 1). Although in 2005, SAT-3 was reported in Central Africa (in the DRC) [28], no virus genotype or species affected was documented and therefore this outbreak is not mentioned in Table 1 and Fig. 2.

The West, Central and East Africa region is divided into two virus pools (4 and 5) [18]. There is a considerable co-occurrence in virus serotypes and topotypes within the two pools, for example, isolates of serotype O (topotype EA-3, orange in Fig. 2) from Niger (2007) and from Nigeria (2007 and 2009) in West Africa (pool 5) were genetically related to the serotype and topotype virus found in Eritrea (2004 and 2011), in Ethiopia (2005, 2006, 2008 and 2010-2012) and in Sudan (2005, and 2008-2011) in East Africa [16] (Fig. 2). The FMDV serotype A topotype AFRICA (G-IV genotype, yellow in Fig. 2) was found in cattle samples from Togo (in 2005), in Nigeria (2009) in West Africa, and in Cameroon (2005) in Central Africa. Sequence analysis on the 1D coding region indicates that these isolates have a close relationship with the serotype A viruses from Eritrea (1998) and from Sudan (2006 and 2011) in East Africa, [16, 41]. In addition, for serotype SAT-2 topotype VII, similar isolates were found in the two pools in the East and West African countries [16, 41] (green in Fig. 2). The last reported isolation of the FMDV serotype C was in Kenya in 2004 from cattle [42], the isolate being suggested as re-introduction of the vaccine strain into the field [43]. Serological studies to prove absence of circulation of type C have not been performed, in fact recent serological tests indicate presence of antibodies against serotype C [34, 44-46]. More serological studies need to be carried out in this region of Africa using specific test methods to prove type C is most likely no longer circulating.

While serotypes O, A, and SAT-2 were found throughout the region, SAT-1 has limited presence in East Africa. SAT-1 has been found in Uganda, Ethiopia, Kenya, Tanzania and the DRC (Fig. 1). The first recorded occurrence of FMDV serotype SAT-1 in Ethiopia was identified from cattle, sheep and goat samples collected in 2007 from an area bordering Kenya [34]. Moreover, in 2007, FMDV isolates of serotype SAT-1 (topotype IV) and SAT-2 (topotype X) were isolated and genotyped from African buffalo oropharyngeal probang samples collected in Queen Elizabeth National Park in Uganda [31]. We could not find reports on isolation of SAT-1 virus from domestic animals or wildlife in Central and West Africa for the last 3 decades, although the WRLFMD

considers it one of the strains circulating in pool 5 (West and Central Africa). There is a serological evidence in sheep and cattle for antibodies against SAT-1 and SAT-3 in Nigeria [47], but this has to be confirmed using virological methods. East Africa has recorded serotype SAT-3 only twice: in 1970 [48], and in 1997 [7, 35, 49]. Serotype SAT-3 was isolated from African buffaloes in Uganda, but it has never been isolated from livestock in this region [24]. In West Africa, SAT-1 has not been isolated from domestic and wild animals for the last 3 decades, it is possible that either the virus has stopped circulating or most likely remains undetected due to inadequate surveillance both in domestic animals and wildlife.

Occurrence of FMDV in southern Africa

Countries in the southern Africa region are Angola, Zambia, Malawi, Namibia, Botswana, Zimbabwe, Comoros, Mozambique, Madagascar, Swaziland, Lesotho, South Africa, Mauritius and the Seychelles (Fig. 1). In southern Africa, there is strong evidence that livestock-wildlife interface influences the FMD dynamics in livestock populations [15, 35, 50-52], specifically for the SAT (1-3) virus transmission and maintenance. In this region, four serotypes (O, SAT-1, SAT-2 and SAT-3) have been isolated since 1991, with the SAT-1-3 serotypes of FMDV most frequently detected [18, 24]. Before 1991, serotype A and C were reported, but seem to have disappeared since.

Of the four serotypes, serotype O is the least prevalent in southern Africa. Serotype O has mainly been isolated in the region bordering Central and East Africa and spread by illegal movement of infected animals, for example, in 2010 and 2012; O has also been isolated at Mbala in the Northern Province of Zambia [53]. The VP1 sequence of this virus belongs to the serotype O EA-2 topotype and was most closely related to viruses from the DRC (Central Africa) isolated in 2006, from Uganda (East Africa) isolated between 2004 and 2007, and from Tanzania (East Africa) isolated in 2009 [54] (Table 1). However, not all FMDV serotype O strains are introduced from East and Central Africa. In 2000, Kwa Zulu Natal in South Africa experienced a one-time outbreak in pigs and cattle caused by serotype O topotype ME-SA PanAsia-1 virus, most likely introduced from Asia traced in uncooked swill from a ship in Durban [55, 56]. Since 2000, the Pan-Asian serotype O virus has not been reported in South Africa again.

The SAT serotypes are maintained by large numbers of African buffalo in the region, which provides a potential source of infection for both domestic livestock and other wild, cloven-hoofed animals [51, 57], such as impala, which can be transiently infected and transmit the disease to susceptible livestock [58]. In southern Africa, where FMDV transmission has been studied extensively by genotyping the SAT serotypes, outbreaks in domestic animals have been shown to come regularly as spillsover from buffalo reservoir to cattle [14, 50, 51, 58-60]. The most recent SAT-1 outbreaks in southern Africa were recorded in Botswana (2013), Zambia (2012), Namibia (2011 and 2013) and South Africa (2010 and 2013), but SAT-1 had been detected in cattle, buffalo and impala earlier in most countries in this region [33, 61, 62]. Foot-and-mouth disease virus serotype SAT-2 caused widespread outbreaks in domestic cattle in 2011 and 2012 in Botswana, Zambia and Namibia. In Botswana in 2013, SAT-2 was isolated from goats, impala and greater kudu [54]. In the same year, SAT-2 was also isolated from cattle in South Africa and Mozambique [54]. Of the three SAT serotype FMDVs, SAT-3 has the most restricted distribution and essentially occurs only in southern Africa [51], in the south-western region of Uganda [13, 63]. SAT-3 has been reported in South Africa in 2006, 2008, 2010 and 2011, in Mozambique in 2010 [16].

Animal husbandry, trade and involvement of wildlife in FMD transmission

Livestock management systems

The African livestock husbandry system varies from region to region mainly due to differences in availability of grazing and watering grounds. The five livestock production systems identified in Africa by Robinson et al., [64] can be described in terms of the amount of movement of animals: (i) total nomadism: no permanent place of residence, no regular cultivation, (ii) semi-nomadism: a permanent place of residence exists and supplementary cultivation is practiced, but for long periods of time animal owner's travel to distant grazing areas, (iii) transhumance: a permanent place of residence exists, and herds are sent to distant grazing areas, usually on seasonal cycles, (iv) partial nomadism: farmers live continuously in permanent settlements and have herds at their disposal that graze in the vicinity, and (v) stationary or sedentary animal husbandry: animals remain on the holding or in the village throughout the year.

The animal husbandry system along the Mediterranean coast of North Africa is a transhumance system with sedentary small holders practising mixed farming. In the arid and semi-arid part of the southern Mediterranean climate zone, a nomadic, semi-nomadic and transhumance husbandry system is practiced, mainly with small ruminants. In West, Central and East Africa, all five farming systems can be found [64], with total nomadism, semi-nomadism and transhumance the main rearing systems for domestic ruminants (cattle, sheep, goats and camelids). Just south of the Sahara in the Sahel region (which includes most of the countries Senegal, Mauritania, Mali, Burkina Faso, Niger, Chad, the Sudan and the northern fringes of Nigeria) a significant share of the livestock (estimated 70 to 90 %) is reared under this system. There are nomadic, semi-nomadic and transhumance systems in which farming societies travel with their livestock from the drier parts of East Africa, across the Sahel and back to the Atlantic south of the tropical forests [65]. In the nomadic, semi-nomadic and transhumance systems, livestock move freely (crossing international borders) for long treks and frequently intermingle with more sedentary domestic cloven-hoofed animals. The moving animals also have the possibility of exposure to wildlife on route, due to the concentration of stock at rivers, watering points and lake shores, where grazing is possible during the driest part of the year.

In southern Africa, livestock husbandry (cattle, sheep and goats) is mainly sedentary. However, risks of FMD transmission have increased and will in future inevitably increase further, given the expansion of game farming conservancies in livestock ranching areas and the establishment of Transfrontier Conservation Areas (TFCAs) [66]. Therefore, the contact between domestic livestock and wildlife might increase, even when livestock is not moving.

Livestock Trade in Africa

Although livestock rearing is one of the main economic activities on which the poorest populations depend for food and income, official figures on livestock trading are limited. According to official FAO data [67] in 2010 more than 780 million cattle, camels, buffaloes and small ruminants were kept in Africa. North African countries in the West (Algeria, Morocco and Tunisia) mainly import cattle and small ruminants from Europe and South America. According to FAO data, additional meat and dairy products are

imported from the EU, Australia, New Zealand and Argentina. For some of the imports, this database gives information on the origin of the animals, but not for all.

While such trade is expected to be considerable in Africa, the FAO data did not record informal trade or the transboundary movements of cattle of nomadic and transhumance herds. For example, because the north-west of Africa is separated from sub-Saharan Africa by the Sahara desert, it is likely that informal trade into north-west Africa from countries to the south is limited, but in north-east Africa no data on formal trade from Sudan to Egypt and Libya is found, but informal trade is very likely. Furthermore, the FAO figures do not include animal products that can represent a risk of transmission. North Africa is the continent's largest beef (76%) and milk (56%) importer (http://www.africalivestockdata.org/afrlivestock/content/about-us).

In West, Central and East Africa, there are well-established livestock trade routes across the arid and semi-arid sub-Saharan region. The 2010 FAO data on West, Central and East Africa show that 2.9 million livestock were imported, two thirds small ruminants and one third cattle. In this region, Nigeria is the largest importer (over 1.4 million animals in 2010, including over 1 million live cattle, sheep and goats from Niger) [67]. Limited information is found in the FAO database on trade in the Greater Horn of Africa, which includes the countries Sudan, Eritrea, Ethiopia, Djibouti and Somalia. This region has the main livestock trading routes for sheep and goats, largely towards the Arabian Peninsula and the Gulf States. Livestock export in East Africa is important and supplies the Arabian Peninsula and the Gulf States with mostly small ruminants, cattle and the Arabian one hump camel. Somalia, Sudan and Ethiopia are the continent's largest sheep meat (61%) exporters (http://www.africalivestockdata.org/afrlivestock/content/about-us). Although export out of Africa is not a risk for FMD transmission within Africa, these exports cause considerable trade within Africa, as not all animals come from the countries that export to the Arabian Peninsula and the Gulf States. Many are brought in from neighbouring African countries, that is, assembled along the trade routes and finally trucked or trekked to the sea ports in the Gulf of Aden and the Red Sea for shipment to the Arabian Peninsula and the Gulf States. There are also significant livestock markets in large East African cities (such as Nairobi and Mombasa in Kenya) due to the high local demand, and these also cause trade within the region. Kenya, theoretically, is a meat deficient country, with its shortfall covered through cross-border imports from Tanzania, Ethiopia and Somalia and to some extent,

from South Sudan [68]. These animal movements are of major importance for the dissemination of new strains of FMD [69]. For example, serotype SAT-2 topotype VII isolates recorded in Saudi Arabia (SAU/6/00) in 2000 were most closely related to published sequences of isolates from Eritrea (ERI/1/98) in 1998 [41], indicating that the northeast was the most likely source of the virus [35]. This trade linkage between West, Central and East Africa suggests that a control strategy should be implemented on a wider, region-based scale.

In southern Africa, based on the FAO data, the highest number of livestock is imported into South Africa (650,000 head). Again actual livestock trade is difficult to document; statistics are poor and unreliable, and informal trade most likely overshadows formal trade in many regions [70]. Southern Africa is the largest beef (94%) exporter in Africa (http://www.africalivestockdata.org/afrlivestock/content/about-us); this beef is mainly from Namibia, South Africa and Botswana. The FAO data show that most (57%) of the meat exports from Botswana go to South Africa, and within the formal FAO data also a considerable amount (40%) goes to the European Union (EU). The risk of FMDV transmission to disease free countries could be minimised by importing live animal and animal products from OIE recognised FMD free zones.

Involvement of African wildlife in FMD transmission

Some wild animals play a potential role in the transmission of FMD. Serotype SAT (1-3) viruses have been shown to be constantly evolving in the African buffalo (*Syncerus caffer*) and are a true maintenance host for the SAT serotypes [10, 14, 33, 35, 60, 63, 71, 72]. Most healthy buffalo populations maintain SAT viruses and usually become sub-clinically infected [62]. Furthermore, virus has been shown to persist in an individual buffalo for at least 5 years and, in small free-living populations has been maintained for at least 24 years [14]. For the SAT serotypes, it is assumed that most buffalo calves are infected during "childhood" epidemics, that is, horizontal transmission between calves <1 year old when the maternal antibodies wane [10, 73] during which time they can infect susceptible cattle [74].

The role of persistently infected animals in initiating new outbreaks remains highly controversial [73, 75]. While transmission of FMDV from buffalo to cattle is considered a rare event [76, 77], transmission of SAT serotypes from carrier buffalo to cattle has been unequivocally demonstrated under both experimental and natural

conditions [59, 60, 71, 77]. Natural transmission of FMDV between African buffalo and impala in the Kruger National Park in South Africa has also been described [59]. In one experiment, transmission of FMDV was observed from carrier male buffalo to female cattle, suggesting sexual transmission from the buffalo bull to cattle cows [78].

Other wild species can also be transiently infected by the SAT serotypes [51] and transmit the disease to susceptible livestock. It can be assumed that other wild species can also play a role in transmission of type O and A, although this has not been studied in Africa. From the wild cloven-hoofed animals, the FMD susceptible species other than buffalo are impala (*Aepyceros melampus*) [51, 59, 61, 79-83], antelope, warthog, kudu, bush pig [80, 84, 85], deer [86], and giraffe [84, 85, 87]. Warthogs are clinically and experimentally susceptible to FMD infection but unlike domestic pigs, they are not major virus amplifiers [88]. While no other wild species other than buffalo has been shown to become persistently infected with FMDV SAT serotypes [85, 88], transiently infected wild cloven-hoofed animals might play a role in its spread. Still, the main contact believed to be important for the transmission of SAT serotypes of FMDV is between buffalo and cattle and/or impala [59, 61, 77, 89, 90] in southern Africa. It has been supported by phylogenetic studies, that impala isolates in the Kruger National Park cluster with buffalo lineages present in the same park, indicating interspecies relationships of impala and buffalo SAT viruses [59].

There is no evidence of an important role of African buffalo in the epidemiology of the FMDV serotypes O, A, and C [51, 91]. Although serological findings from African buffalo in Uganda (sera collected in 2001-2008) [31, 45, 49, 92] and Kenya [93] indicate that the African buffalo can be infected with serotype O and perhaps also with C. Further virological evidence is needed to substantiate the importance of buffalo in the epidemiology of serotype O. One should, however, realise that four subspecies of African buffalo are usually distinguished. The southern savannah buffalo (*Syncerus caffer caffer*) described above lives in the savannah of eastern and southern Africa from the southern part of Ethiopia and the upper White Nile as far as the Cape reaching the southwest coast. The forest buffalo (*S. c. nanus*) is found in the region spanning from the southern part of West Africa up to Central Africa. The West African savannah buffalo (*S. c. brachyceros*) occurs in the northern part of the region spanning from Guinea to Cameroon. Lastly, the Central African savannah buffalo (*S. c. aequinoctialis*) is found in the region spanning from the northern part of Central Africa (Chad, Sudan

and South Sudan) up to the western part of Ethiopia (see Fig. 3 or for detailed information on distribution of the subspecies zoom in on the map supplied on the following website: http://maps.iucnredlist.org/map.html?id=21251). It is unknown if the West African savannah buffalo and the forest buffalo can be persistently infected with FMDV SAT serotypes. The role that subspecies other than the southern savannah buffalo play in the maintenance and transmission of FMDV in Africa is not clear.

In East Africa, there are no sufficient data to substantiate the occurrence of FMDV SAT (1-3) serotypes in wildlife, although recently, positive titres for SAT-3 were found in domestic cattle in Uganda that had direct contact with African buffalo [94]. In a study by Molla et al., [95] in Ethiopia, the highest sero-prevalence to FMDV was observed in a cattle herd that had contact with wild animals, suggesting that also in East Africa, wildlife is important in the maintenance of FMDV. Foot-and-mouth disease virus serotype SAT-1 and SAT-2 have been isolated from African buffalo in Botswana in 2006 [16] and Uganda in 2007 [31]. The SAT-3 serotype is the virus type least frequently recovered from the African buffalo [51]. Nevertheless, there are no reports of SAT serotype virus isolation from buffalo inhabiting regions of East, Central and West Africa except in Uganda (East Africa). As is shown in Fig. 3, the African buffalo can mostly be found only south of latitude 10° N, where it appears in a variety of habitats, including open savannahs, woodlands, swamps and rainforest. Serological studies on buffalo samples collected between 1994 and 2004 from East and Central Africa tested by virus neutralisation for SAT serotypes indicated that 37.3% had SAT-1, 67.1% SAT-2, and 17.0% SAT-3 [96]. Antibodies against O, SAT-1 and SAT-2 serotypes were found in both West African and Nile buffaloes by Di Nardo et al., [97] in West and Central Africa in sera collected in 1986-2007, although validation of the possible cross-reactions in the tests used has not been given.

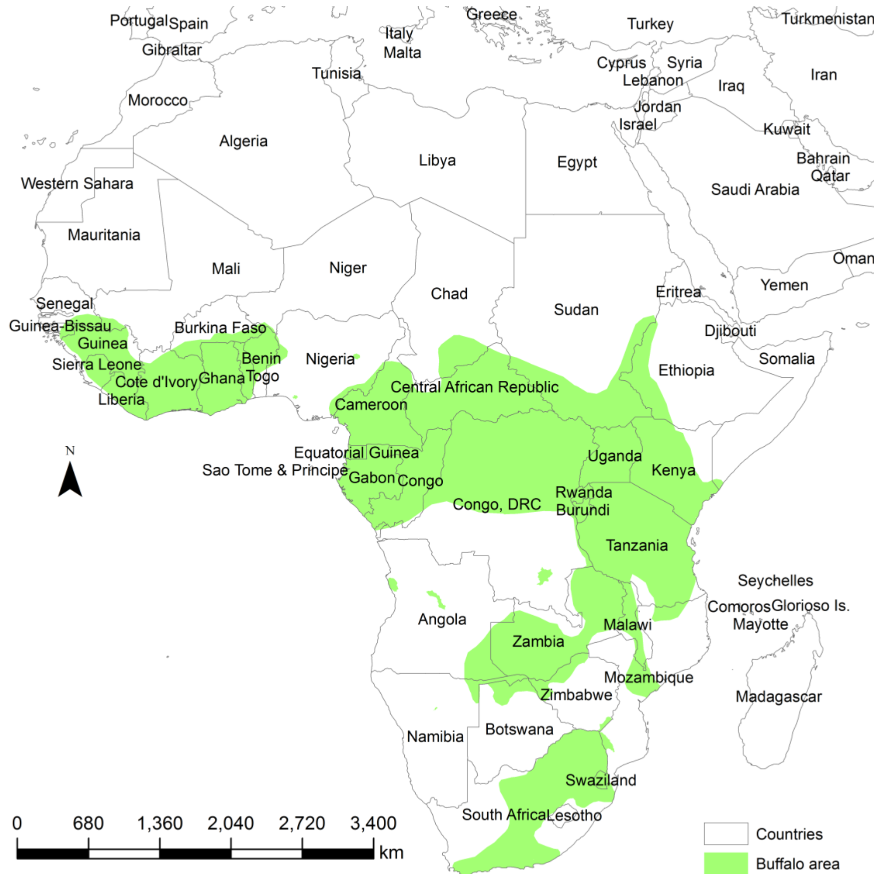

Figure 3. Distribution of African buffalo (*Syncerus caffer*) in sub-Saharan Africa (green area) (IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1. http://www.iucnredlist.org. Downloaded on 23 September 2013).

Transmission of FMD in Africa

The aim of this review is to identify the role animal husbandry, trade and wildlife have on the transmission of FMD in Africa, providing a scientific basis for different FMD control measures. The most remarkable result is the limited spread of FMDV, which lead to the designation of topotypes. For serotype O and A, the distribution is limited to North Africa (Libya and Egypt), West, Central and East Africa. For serotype C there has been no virus isolation since 2004, with serological data suggesting that it has not disappeared although the serology is difficult to interpret. Serotype SAT-1 is limited to

southern and eastern Africa and SAT-1 has not been isolated in West and Central Africa since 1981, although serological studies reported antibodies to SAT-1 in cattle and sheep against the virus [47]. Serotype SAT-2 is also limited to southern and eastern Africa, but SAT-2 topotype VII has spread to the North and West Africa. Serotype SAT-3 is restricted to South Africa, Namibia and Mozambique since 2006. The Namibian SAT-3 isolates have not been genotyped and not shown in Table 1 and Fig. 2.

The distribution of FMDV serotypes has not changed significantly in the last 10 years, except for the recent spread of SAT-2 into North Africa. The distribution can be explained by animal husbandry system, trade and close contacts to wildlife. For some topotypes (e.g. serotypes O topotype EA-3, orange in Fig. 2, and A topotype AFRICA (G-IV genotype), yellow in Fig. 2 and SAT-2 topotype VII, green in Fig. 2) the distribution has increased, and this is most likely linked to the husbandry system and trade in the area where livestock move from West to East and vice versa as well as northward to North Africa from sub-Saharan Africa. The fact that in southern Africa there is a more sedentary type of animal husbandry, explains most likely why virus topotypes occurring in northern, western, central and eastern Africa do not occur in the southern part. The SAT strains mostly reported in the southern part (with the exception of SAT-2 topotype VII and XIII), are more linked to infected buffalo. In fact some topotypes such as SAT-1 topotype IV and VII, SAT-2 topotype X and SAT-3 topotype III and IV have only been isolated from buffalo and have not been reported in livestock. This shows that the SAT strains (with the exception of SAT-2 topotype VII and XIII) are very probably associated with infected buffalo and therefore most likely do not spread to regions with low buffalo density. This hypothesis is supported by a study described by Hall et al., [22] and the fact that although SAT strains have escaped from Africa to the Middle East, they only caused outbreaks that lasted for a short period and did not spread much further. The fact that FMD serotype SAT-2 topotype VII was found in regions without buffalo led to the conclusion that some variants of SAT-2 can be maintained in domestic animals [9, 22, 52].

This review indicates that distribution of topotypes in Africa can be explained by animal husbandry system and trade. Therefore livestock movement is one of the main risk factors involved in the spread of FMDV, especially in Africa where such movements are poorly regulated [17]. There are a few studies on risk factors relating to

FMD infection and animal husbandry. In one of the studies, the odds ratio (OR) for mobile herds is 2.6 which means mobile herds are more likely to get FMD infection [98]. Also mixing at watering points added to the risk of transmission (OR = 2.4). Similarly in Ethiopia, a study made by Megersa et al., [99], in a pastoral (nomadic and transhumance) production system has shown that, cattle movement is a potential risk factor for FMD seropositivity (OR = 16.3). The substantial diversity of the circulating FMDV serotypes and topotypes is directly linked to the complexity of the livestock husbandry system, the marketing structure, and livestock-wildlife interface reflecting the significant influence on the risk of transmission of the virus.

Although FMD infection can be caused by contaminated animate and inanimate material (fomites), infected animal products, aerosols containing FMDV and carrier animals, as stated earlier our review indicates that most transmissions are probably caused by close contact between acutely infected and susceptible animals [17, 50, 52, 100-102].

Implications for control

This review shows that distribution of FMDV topotypes can be explained by animal husbandry systems, trade routes and exposure to wildlife. Nevertheless, the distribution of some topotypes of FMDV is very limited, as serotype A and O are not found in southern Africa, and SAT-3 is not found north of Tanzania except in Uganda. These naturally occurring limitations on the spread of the virus should be used when designing control measures for FMDV. We believe that the direction of trade often limits transmission of FMDV, e.g. the Arabian Peninsula and the Gulf States import live animals from both Africa and Asia, but African FMDV strains have not spread further East. This is most likely due to the fact no animals are moved eastwards from the Arabian Peninsula and the Gulf States. When trade is necessary due to demand on animal proteins, animals that are exported should not be a risk in transmitting the disease; this might be accomplished by vaccinating animals few weeks before shipment. When designing strategies for FMD control in Africa; information on animal husbandry, trade and wildlife is necessary. Control should be coordinated for larger regions that are likely to be part of the same ecosystem. For the control of FMD in Africa, mass vaccination with good quality conventional vaccine containing the antigens

that are relevant to the specific region is likely the preferred approach and could be used to limit primary outbreaks and spread of the virus. Monitoring the result of vaccinations in the field is an essential tool for success. This is in line with the assumptions of the FMD progressive control pathway (PCP) promoted by FAO and OIE that states control should be risk based.

When using vaccination to contain transmission the proportion of protected animals *p* should be > $1 - 1/R_0$ [103] . The reproduction ratio R_0 is the average number of new infections caused by a typically infectious individual during its whole infectious period in a completely susceptible population. This critical proportion is equal to $h \times f$, where *h* is the proportion of vaccinated animals that are protected after vaccination and *f* is the vaccination coverage. In situations where the R_0 is high, for example, in markets and during transport, the coverage and the fraction of protected animals should increase to prevent disease spread. Both coverage and fraction of protected animals are improved by repeated vaccination, and both should be monitored. Animals that have been previously vaccinated could be boosted (higher probability of protection), and animals not vaccinated in the past will, after vaccination, improve the coverage. This strategy can be applied to countries where nomadic and transhumance movement of animals are important in the transmission of FMDV. One could envisage a policy to vaccinate all animals before they are moved. Similarly animals traded for religious festivals should be vaccinated before being transported from endemic areas. The concept can also be applied in countries where infections come from wildlife, where one must assume a higher R_0 compared with the R_0 in the vaccinated population due to mixing of non-vaccinated infected wildlife (African buffalo) with vaccinated domestic animals. By improving the vaccination status of the domestic animals, for example, by repeated boosting, transmission can be reduced.

Equally, the risk of development of clinical disease in cattle exposed to buffalo infected with FMDV decreases when the cattle have been vaccinated (i.e. in the vaccination zone), as long as the level of herd immunity achieved is high [104, 105]. In fact, the concept is currently applied in southern Africa where livestock populations are at risk of spillover infection from wildlife populations are vaccinated [50]. Besides, in South Africa not only this immune barrier but also the physical barrier has been important. These strategies could be weakened by the establishment of the TFCAs and human encroachment into the wildlife domain resulting in increased wildlife migration

and the pressure to remove fences in southern Africa. Therefore, FMD remains a major challenge for the livestock trade in southern Africa, given its opportunities for market access, including trade with the European Union, direct exports to large retailers, export to emerging markets (particularly Asia) and regional trade in southern Africa and domestic urban and rural markets [106]. It is unlikely that the risks involved in trading with FMD infected countries will be acceptable without investing in disease control.

The future of FMD control in Africa is difficult due to a number of challenging issues. The most significant problems are: (i) presence of multiple FMDV serotypes having great genetic and antigenic diversity, which makes the application of vaccine challenging, (ii) involvement of wildlife (African buffalo) in maintenance of the virus and disease transmission with the three SAT (1-3) serotypes, (iii) poor quality vaccines having low stability and lacking matching with field strains, (iv) unregulated cross-border animal movement for grazing, water and trade practices, (v) poor veterinary services and inadequate infrastructure, and (vi) inadequate data on FMD epidemiology.

Nevertheless, there have been successes in parts of the continent. For example, Botswana, Namibia and South Africa have FMD free zones certified by the OIE as free of FMD without vaccination. Furthermore, Morocco, Algeria and Tunisia have OIE endorsed control plans. These successes indicate that FMD control is possible in Africa, and can be extended.

Acknowledgement

This work is supported by the International Atomic Energy Agency (IAEA), Vienna, Austria. We thank Gert Jan Boender for preparing the map on the distribution of African buffalo in sub-Saharan Africa. We would like to thank the FAO, Representative Office in Eritrea for providing internet access during preparing this manuscript.

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Chapter 3

Serological evidence indicates that foot-and-mouth disease virus serotype O, C and SAT1 are most dominant in Eritrea

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Transboundary and Emerging Diseases (2013),
DOI: 10.1111/tbed.12065

Summary

Foot-and-mouth disease (FMD) is endemic in Eritrea and in most parts of Africa. To be able to control FMD using vaccination, information on the occurrence of various foot-and-mouth disease serotypes in Eritrea is needed. In this cross-sectional study, 212 sera samples were collected from FMD infected and recovered animals in Eritrea. These samples were tested for the presence of antibodies against FMD non-structural proteins (NSP) and neutralising antibodies against six of the seven (all but SAT-3) serotypes of FMD virus (FMDV). Out of these, 67.0% tested positive to non-structural protein antibodies in the FMD NS ELISA. By virus neutralisation FMDV serotype O antibodies were shown to be the most dominant (approximately 50%). Virus neutralisation test results indicate that infection with serotype C and SAT-1 might have occurred, although there are no reports of isolation of these two serotypes. Because the samples were not randomly selected, further random serological surveillance in all age group animals is necessary both to estimate the prevalence of FMD in the country, and to confirm the serological results with serotype C and SAT-1.

Keywords: Foot-and-mouth disease virus; serotype; virus neutralisation test, NS ELISA, surveillance, validation.

Introduction

Foot-and-mouth disease (FMD) is a contagious disease of cloven-hoofed animals and has a great potential for causing severe economic losses and affects both domestic and wild Artiodactyl (even-toed hoofed animals) species. The disease is characterized by vesicles of the feet, buccal mucosa and, in females, the mammary glands. The causative agent of FMD is foot-and-mouth disease virus (FMDV), which is a small positive sense single-stranded RNA virus (approximately 8.4 kb) that belongs to the Aphthovirus genus of the family Picornaviridae [1]. There are seven serotypes of FMDV, namely, O, A, C, SAT-1, SAT-2, SAT-3 and Asia 1 that do not confer cross immunity to each other, and further numerous variants and lineages, described as topotypes [2].

FMD is endemic in Eritrea, but information on distribution over different regions and prevalence of antibodies against different serotypes is lacking. Foot-and-mouth disease vaccination is not practised in indigenous cattle, although indoor kept dairy breeds exotic to Africa (e.g. Holstein Frisians and crossbreeds) are sometimes vaccinated. Also countries neighbouring Eritrea are not vaccinating their traditionally kept indigenous cattle. To be able to develop a control strategy using vaccination this information is essential. From several outbreaks that occurred between 1996 and 2009, a limited number epithelial tissue samples were collected and sent to FAO/OIE World Reference Laboratory (WRL) for FMD, Pirbright, U.K. From these samples, FMDV serotypes O, A and SAT-2, were confirmed and genotyped. FMDV serotype O has been confirmed in the laboratory in 1996 and 2004; FMDV serotype A in 1997 to 1998 and in 2006 to 2009 and FMDV SAT-2 in 1998. FMDV serotype C, Asia 1, SAT-1 and SAT-3 have never been isolated in Eritrea. But from many outbreaks no samples have been submitted for serotyping. Serological studies are therefore needed to determine which serotypes have occurred in Eritrea. The aim of this first study was to assess the occurrence of FMDV serotypes in Eritrea using sera collected in 2009 shortly after clinical outbreaks.

Materials and Methods

Sera for validation of the virus neutralization test (VNT)

Serum samples (n=74 from Central Veterinary Institute, Lelystad, Netherlands) were collected from 70 experimentally vaccinated and/or infected cattle (n=36), sheep (n=14) and pigs (n=20), and 4 non-infected cattle (Table 1). In total 68 out of 70 FMD positive sera were obtained from cattle, sheep or pigs vaccinated and/or infected with a single serotype and 2 sera were obtained from cattle infected with 3 different FMDV serotypes (Table 1) consecutively one month apart. The sera were used to quantify the level of cross-neutralization between serotypes.

Collection of samples from Eritrea

In a cross-sectional study performed from September to December 2009 we collected sera from FMD unvaccinated indigenous cattle breeds (n=212) from villages that reported FMD outbreaks in three regions in Eritrea namely, Debub, Gash Barka and Maekel. These areas (Fig. 1) had active FMD outbreaks 2 weeks to 2 months earlier. At the time of sampling cattle with clinical signs and lesions, as well as cattle that had recovered from FMD were present. In each village approximately 15 samples were randomly collected.

Virus neutralization test (VNT)

The VNT detects neutralising antibodies directed against structural proteins, and is used as serotype specific serological test. Titres of neutralising antibodies of all sera were determined against FMDV strains A_{22}/IRQ/24/64, O_1 Manisa, C_1 Detmold, SAT-1/23/86 ZIM, SAT-2/Saudi/2000, and Asia 1 Shamir. The test was performed as described in OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, (www.oie.int) using BHK-21 cells. VNT titres $\geq$ 1.65 (^{10}log) were considered positive. The viruses used in the VNT were checked by the antigen detection ELISA [3]. In cases where a sample tested positive for multiple serotypes, the one(s) with the highest titre was considered to be positive. For statistical analysis titres < 0.3 were considered as 0.

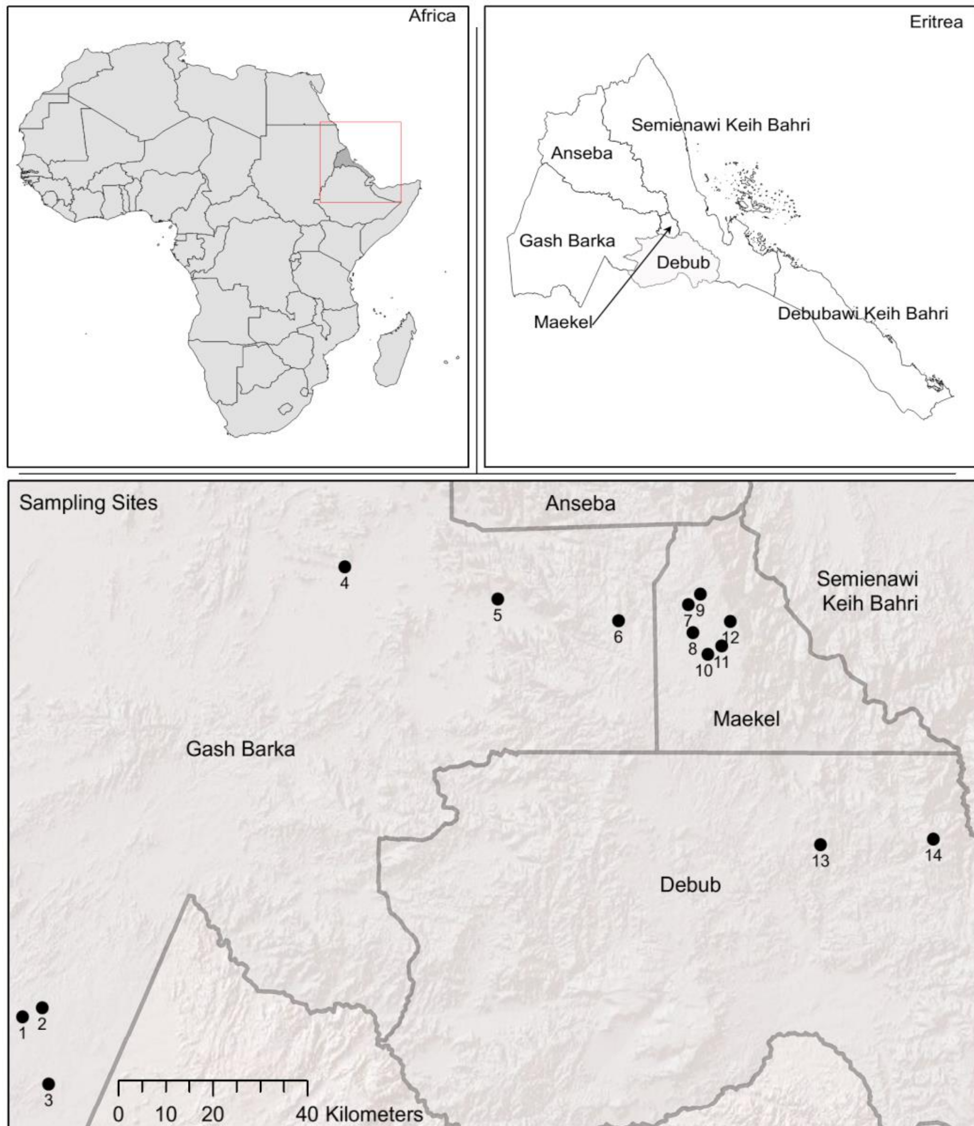

Figure 1. Sampling sites for field sera in Eritrea in 2009. From each site approximately 15 sera were collected shortly after clinical recognition of FMD

FMDV NS ELISA

The PrioCHECK® FMDV NS ELISA (Prionics ®) [4] is a blocking ELISA that detects antibodies directed against the non-structural 3ABC protein of FMDV. This ELISA detects FMDV infected animals independent of the serotype that causes the infection.

Serum samples were tested by PrioCHECK® FMDV NS ELISA kit and test results were interpreted as indicated by the producer, i.e.: < 50 percent inhibition (PI) negative to NS antibodies and ≥ 50 PI values as positive to NS antibodies.

Comparison of VNT and NS ELISA results

To compare the VNT and NS ELISA results we made 2 x 2 table and calculated both the kappa [5] for the agreement between VNT and NS ELISA results as well as the sensitivity and specificity of the NS ELISA under the assumption that the VNT is the gold standard test (Table 2) [6, 7].

Results

Validation of VNT from experimentally vaccinated and infected animals

Sera from animals (n=70) that had been vaccinated and/or infected with FMDV as well as negative control sera (n=4) were used to validate presence of possible cross reactions between the serotypes (Table 1). The four negative cattle sera were all negative for all 6 serotypes in the VNT (highest titre observed 1.35). Out of the 68 sera from animals infected and/or vaccinated with a single serotype 60 were positive in the VNT for the homologous serotype. In 3 (5%) out of these 60 sera a cross-neutralization titre ≥ 1.65 was detected. The sera from 2 cattle infected with serotype A, Asia1 and C, showed high titres (>3.3) against the serotypes used for infection but also a high cross-neutralising antibody titre for serotype O (titre 2.4).

Table 1 Characteristics of the sera used for validation of the virus neutralisation test (n=74)

Animal species	FMDV serotype	No. vaccinated and infected	Titre $\bar{X} \pm$ SD	No. only vaccinated	Titre $\bar{X} \pm$ SD	No. only infected	Titre $\bar{X} \pm$ SD	uninfected control sera	Total no. of sera
Cattle	O	4	2.18 ± 0.26	3	2.5 ± 0.09	11	2.26 ± 0.23	-	18
	A	14	1.92 ± 0.36	-	-	2	2.1 ± 0.42	-	16
	A, C & Asia1	-	-	-	-	2	2.5 ± 0	-	2
	Negative						0.13 ± 0.24	4	4
Sheep	O	5	2.28 ± 0.20	-	-	3	2.1 ± 0.3	-	8
	Asia 1	2	1.73 ± 0.53	2	1.88 ± 0.74	2	1.95 ± 0.42	-	6
Pigs	O	20	1.87 ± 0.50	-	-	-	-	-	20
Total		**45**		**5**		**20**		**4**	**74**

$\bar{X}$: Mean titre
SD: Standard deviation
Titres <0.3 were considered as zero

Table 2 Comparison of FMDV NS ELISA and virus neutralization test (VNT) resulting in a kappa of 0.27 with a 95% confidence interval of 0.15 – 0.4.

		VNT (assumed to be gold standard)		
		+	**-**	Total
NS ELISA	**+**	138	5	143
	-	51	18	69
	Total	189	23	**212**

FMD samples from Eritrea

Out of the 212 samples, 23 were negative in the VNT (titre <1.65). But 5 of these 23 sera were positive in the NS ELISA. In total 49 sera were positive for a single serotype, and 140 sera were positive for 2 to 5 serotypes. When considering the highest titre, 105 were positive for serotype O, 59 for serotype C, 22 for serotype A, 21 for SAT-1, 3 for SAT-2 and 2 for Asia 1 (Fig. 2). In total 21 sera had equally high titres against 2 or more serotypes, these were classified positive for all two or three serotypes. The distribution of the serotypes observed in the regions (Fig. 1) was not significantly different between the different regions (Fisher's exact test, P = 0.2).

We found 143 (67.0 %) out of 212 sera positive for FMDV non-structural protein antibodies by NS ELISA. Using the VNT as the gold standard test, the sensitivity and specificity of the FMDV NS ELISA was estimated to be 73 and 78 % respectively (Table 2). There was fair agreement between the two sets of test results (kappa 0.27 with a 95% confidence interval of 0.15 – 0.4). Out of the 143 sera positive by the NS ELISA 138 of these sera were also positive in one or more virus neutralization tests. In the 51 sera that were negative in the NS ELISA but positive by VNT, 24 were positive for type O, 22 for type C, eight for SAT-1 and three for type A. There was no significant difference between serotypes for NS positive results by Fisher's exact test (p = 0.17).

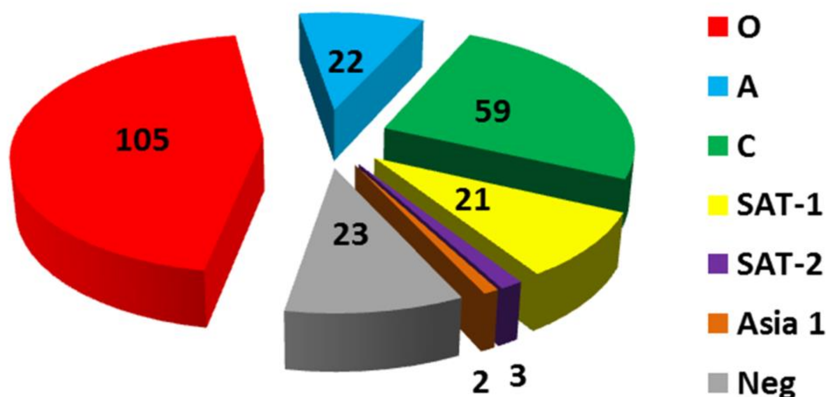

Figure 2. Virus neutralization test results on field sera from Eritrea. Only the serotype with the highest titre are indicated unless titres against two serotypes were equally high then both serotypes were considered positive.

Discussion

The objective of this study was to obtain information on the occurrence of FMDV serotypes in Eritrea. To be able to interpret the VNT results we tested 74 sera from animals used in animal experiments in a VNT. All 4 negative sera were negative at a cut-off of 1.65 for all 6 serotypes. In a previous study [8] testing 464 negative sera against serotype A, O and C also showed a maximum titre of 1.35. Therefore, we considered titres larger than of 1.65 as positive, which equal to the cut-off used in the World Reference Laboratory for FMD between doubtful and positive. At this cut off, we observed a specificity of 100%. In the 70 sera collected from vaccinated and/or infected animals 62 sera were positive, showing that using this cut-off the sensitivity is over 88%. In the 68 experimental sera obtained from animals vaccinated and/or infected with a single serotype only 5% had cross-reactions with another serotypes, and in all cases the highest titre was detected against the serotype used for vaccination and/or infection. The 2 sera obtained after multiple infections, with three serotypes were positive for neutralising antibodies for the serotypes used for infection. But both sera also showed a positive result for serotype O which was not used for infection. The titre against serotype O was, however, lower than the titres obtained against the serotypes

used for infection. By taking into account only the highest titre against any of the serotypes that was found in a serum, any effect of cross-reactions was excluded from the analysis. However, subsequent multiple serotype infections do occur and even simultaneous multiple infections cannot be excluded. The results on sera derived from experimental animals after multiple infections suggest that such animals may show a higher degree of cross-reactivity compared with animals infected with only one serotype. The distribution of neutralizing antibody titres in the 212 field serum samples, showed the highest number of positive reactions were found for serotype O followed by serotype C, SAT-1, A and SAT-2. Serotype O was the predominant serotype in our study. This is consistent with the findings of the World Reference Laboratory for FMD in Pirbright that found type O in 80 % of all the samples tested in 2010 and 60% in 2011 collected from all continents [9]. A similar high percentage of serotype O was also observed in isolates from North, West, Central and East Africa. Although the last isolated type O virus in Eritrea was in 2004.

A very striking result is the relatively high proportion (59 out of 212) of serotype C VNT positive sera (Fig. 2). FMD vaccine serotype A, O and SAT-2 is only used in dairy cows (less than 1% of the total cattle population) in urban areas, and the sera were not collected from these areas. So vaccination cannot explain the positive serotype C titres and therefore it should be assumed that they are caused by infection by serotype C FMD virus. But there have been no recent reports of type C circulation, neither in Africa, nor in other parts of the world, the last reported outbreaks in Africa where serotype C was isolated, were in East Africa in Kenya in 2004 [10] and in 2005 in Ethiopia [11]. It has been assumed that serotype C is disappearing in Africa [12]. The finding of antibodies against serotype C in Eritrea now suggests that serotype C might be circulating without being detected. Our findings on antibodies against serotype C are supported by a similar study carried out in Uganda [13], but need further confirmation. Serotype SAT-1 is endemic in East Africa, although, it has never been reported in Eritrea, the serological results, however, indicate that SAT-1 has been circulating in Eritrea. Serotype A is responsible for 22 of the positive VNT results out of 212 sera. Because this serotype has been isolated from the FMD outbreaks sampled in 2006 – 2009 we expected a higher positive percentage for serotype A (www.wrlfmd.org). Serotype SAT-2 was detected in 1998 in Eritrea, in our results only three sera were

positive for this serotype. This indicates that the serotype has not become established in the country.

The results found by serology do not match with the serotypes isolated in Eritrea. There are several possible explanations. Most likely under reporting of disease outbreaks and the limited submission of samples to diagnostic laboratories, causes bias and strains that circulate have been missed in the virological typing. But also the antigenic similarity between outbreak and test strain could have caused lower titres. However, a considerable mismatch was only present for serotype A, where we used A_{22}/IRQ/24/64 instead of the A/ERI/2/98 (approximately 25% nucleotide difference). For serotype O the strain used in the VNT did not deviate much from the field strain and for serotype SAT-2, we used a strain closely related to the Eritrea outbreak strain from 1998. For serotype C the nucleotide difference between VP1 of C/Oberbayern/GER/72 and C/ETH/6/2005 is approximately 15%, which is low; we assume that antigenic differences between European C strains and African C strains are also limited. For the other serotypes (C, SAT-1 and Asia 1) no FMDV has been isolated and typed in Eritrea, therefore the quality of the antigenic match cannot be determined. The mismatch between serotype A_{22}/IRQ/24/64 used in the VNT and the A type strains isolated in Eritrea would cause lower titres in the VNT against type A, but we assumed that type A positive samples would still produce a higher titre against A_{22}/IRQ/24/64 than to the other serotypes.

The high number of cross reactions observed in the VNT in the field sera matches the results observed in the validation sera from multiple infected animals. Therefore, the reactions against multiple serotypes observed in the field sera are most likely caused by reoccurring infections with different serotypes.

The NS ELISA results showed that 73% of the sera positive by VNT have antibodies against FMDV non-structural proteins. This percentage is lower than would be expected based on the validation of the NS ELISA in non-vaccinated cattle [14]. But analysis of the serotypes detected by VNT in sera negative by NS ELISA does not indicate that infections by one serotype are more likely missed than infections with another serotype (Table 3).

Table 3 FMDV virus neutralization test result and NS ELISA results on sera collected in Eritrea in herds sampled shortly after FMD was reported clinically. The table also identifies the number of double positive sera.

	NS ELISA		
Serotype based on the VNT result	Positive	Negative	total
A	14	2	16
A & O	2	1	3
A & SAT-1	1	0	1
A, O & SAT-1	1	0	1
Asia1	2	0	2
C	28	17	45
C & SAT-1	4	3	7
C & SAT-2	1	0	1
O	72	21	93
O & C	4	2	6
O & SAT-1	2	0	2
SAT-1	5	5	10
SAT-2	2	0	2
Negative	5	18	23
Total	143	69	212

The pilot study in sera collected after clinical detection of FMD outbreaks gives valuable insight in the occurrence of various FMDV serotypes. Because the sera are not a random sampling from the population, it still leaves several questions unanswered, it is therefore imperative that further studies should be undertaken. In such studies information about the age of randomly sampled animals, would possibly enable us to identify the timing of the occurrence of the different serotypes.

Acknowledgement

This work is supported by the International Atomic Energy Agency (IAEA), Vienna, Austria. The authors thank Ghebremichael G.Egziabhier (Asmara Veterinary Clinic) for assisting in the collection of samples in the field. We thank Froukje van Hemert-Kluitenberg for her assistance in the laboratory work.

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Chapter 4

Foot-and-mouth disease sero-prevalence in cattle in Eritrea

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(Submitted)

Abstract

Information about sero-prevalence of foot-and-mouth disease (FMD) and virus serotypes in Eritrea is unavailable, but are very important as it may guide the choice of intervention measures including vaccination to be implemented. We carried out a cross-sectional study in 2011 in Eritrea with a two-stage cluster design, sampling cattle in 155 villages with the objective of determining the sero-prevalence of FMD in four administrative regions of the country. We analysed cattle sera (n=2429) for FMD virus antibodies using the non-structural ELISA (NS ELISA) and virus neutralisation test (VNT). The overall sero-prevalence was 26% and 30% for the NS ELISA and VNT respectively. FMD virus serotype O (14%) and A (11%) were the most prevalent. Gash Barka showed the highest (39%) sero-prevalence both in NS-ELISA and VNT compared to the other three administrative regions. Strategic FMDV vaccination with type O and A (matching circulating strains) in combination of zoo-sanitary measures would be the best control option for Eritrea which could be started in areas where the disease is less endemic.

Keywords: FMD virus, serotypes, sero-prevalence, Eritrea, administrative regions, vaccination

Introduction

Foot-and-mouth disease (FMD) is an animal disease of socio-economic importance in Eritrea and in other parts of Africa. FMD affects cattle, swine, sheep, goats, water buffalo and more than 70 wildlife species [1]; causing loss of production and with high mortalities in the young animals [2, 3]. The virus which causes FMD is an aphthovirus of the family *Picornaviridae* [4, 5]. There are seven immunologically distinct serotypes of FMD viruses namely; A, O, C, SAT-1, SAT-2, SAT-3, and Asia1 [6, 7]. There is a variety of subtypes within each of these serotypes. Therefore, to ensure protection in the event of an outbreak, a specific vaccine is required, effective against the circulating viral field strains [8].

FMD is endemic in Eritrea [9] as well as in the neighbouring countries namely, Ethiopia [10, 11] and Sudan [12]. Within Eritrea the semi-nomadic and transhumance traditional livestock management systems, with unregulated livestock movements, are probably responsible for the spread of FMD virus (FMDV). Although outbreaks occur, vaccination is not practiced, except for a small segment of the dairy cross-bred cows in the urban and peri-urban areas, which are vaccinated occasionally at irregular intervals. The epidemiology of FMDV in Eritrea is not yet studied; although it is assumed that FMDV spreads among domestic animals, with probably little involvement of wildlife as the African buffalo is not present in Eritrea. In Eritrea, livestock species mainly consist of small ruminants (7.0 million) cattle (2 million) and camels (0.4 million).

Despite the extensive occurrence of FMD, clinical and laboratory investigation for identification and genotyping of the virus has never been exhaustive and complete, because there is no FMDV surveillance programme in place. Out of the six serotypes found in sub-Saharan Africa, only 3 serotypes (O, A and SAT-2) have been isolated and genotyped by the World Reference Laboratory for FMD (WRLFMD) since 1989 (Table 1).

Table 1. FMD virus serotypes identified in Eritrea, 1989-2013 after typing at WRLFMD, The Pirbright Institute, U.K.

Year	Species	Serotypes	Topotype	Genotype/Strain
1989	Pigs	O	Not done	-
1996	Cattle	O	Not done	-
1997-98	Cattle	A	Central Africa 2	V
1997-98	Cattle	SAT-2	VII	-
2004	Cattle	O	EA-3	
2006-09	Cattle	A	AFRICA	G-IV
2011	Cattle, pigs	O	EA-3	

In East Africa, serotype A, O, C, SAT-1, SAT-2 and SAT-3 (only from African buffalo in Uganda) have been isolated [13-15]. Outbreaks of SAT-3 in domestic cattle have only been recorded in southern Africa. In a study conducted in Nigeria, SAT-3 antibodies were found in sheep in 2008 and in cattle in 2009 [16]. However, no SAT-3 virus was isolated to conclude the presence of SAT-3 serotype in livestock in West, Central and East Africa.

To date, Eritrea has not yet implemented the global FAO/OIE progressive control pathway strategy [17] for the control of FMD. The first step in this progressive control pathway is the identification of risk factors for virus transmission based on a serological survey. In a previous serological study in Eritrea, serum samples were collected from villages that reported FMD outbreaks [9] and antibodies against type O, A and C were detected. In that study, samples were not collected randomly and were not tested for antibodies against SAT-3; therefore, neither conclusions on differences between geographic areas and livestock systems could not be drawn, nor definite conclusions on the absence of SAT-3. Previous serological studies in Africa showed that FMD prevalence in cattle was higher than in small ruminants [18, 19]. Therefore in this study, only cattle were sampled. The objective of the current study was to determine the FMD sero-prevalence in cattle for each serotype and to study differences regarding animal age, regions, and villages with and without livestock migration.

Materials and Methods

Negative and positive sera for validation of VNT

Cattle sera (n = 464) collected in 2000 at a Dutch slaughterhouse were used to determine the cut-off values in the VNT for each serotype. The sera were tested against the seven serotypes using strains; A/ERI/2/98, O1 Manisa, C_1 Detmold, SAT1/23/86 ZIM, SAT2/SAUDI/2000, SAT3/KNP/10/90 and Asia1 Shamir. To study cross reactions in the VNT post vaccination and/or infection, sera (n=76) were tested for all 7 serotypes. These sera included 4 negative controls, 2 sera of cattle infected subsequently one month apart with 3 different serotypes (A, C and Asia 1), and further sera from animal experiments (cattle, pigs and sheep) using serotypes A22/IRQ/24/64, O/NET/2001, O1 Manisa, O/Taiwan/97, C1 Detmold, and Asia1/TUR/11/2000.

General description of study areas

Eritrea is located in the Horn of Africa bordered by Sudan in the West, Ethiopia in the South, Djibouti in southeast and the Red Sea in the East. Approximately 86% of the total cattle population of the country is kept in 4 out of the 6 administrative regions of the country, namely, Gash Barka, Debub, Maekel and Anseba (Fig. 1, Table 2). The main production system is the traditional extensive type featuring extensive land use per animal, low productivity and long distance seasonal animal movement.

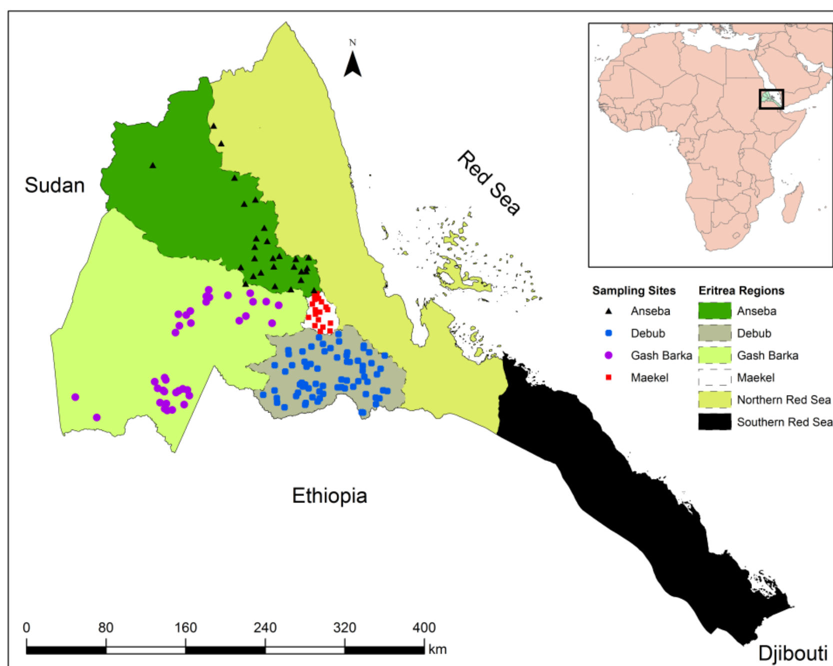

Figure 1. Distribution of sampled villages in the FMD Sero-surveillance study in the four administrative regions in Eritrea. The sampling villages were plotted by using ArcGIS ArcMap v10.0 (ESRI Inc.) geographical information system (GIS) software package.

The livestock species in these regions are predominantly small ruminants, cattle, camels and equine. The two remaining administrative regions (Northern Red Sea and Southern Red Sea) are located in the eastern coastal lowland which are dominantly arid and semi-arid areas and that are sparsely populated with cattle (approximately 3 - 6 cattle per km^2) and therefore not included in the study. The cattle densities in the study area were between 9 and 61 cattle per km^2 (Table 2).

Table 2. Description of the cattle population in Eritrea (based on 1997 census data)

Region	No. of villages	No. of villages sampled	Cattle population	Cattle density per km^2
Anseba	704	28	218 923	9
Debub	1469	71	490 093	61
Gash Barka	936	39	917 344	28
Maekel	104	17	40 505	31
North Red Sea	269		178 532	6
South Red Sea	94		82 060	3
Total	3576	155	1 927 457	16

Sampling methods and type of samples collected

In 2011, a cross-sectional study was used with a two-stage cluster design. The villages served as primary sampling units (clusters) and all cattle in the village were considered as one herd since as animals shared grazing land and watering points. The country as well as the regions was considered as strata. To detect 1% seropositive cattle with 95% confidence 298 sampling units were planned to be sampled, which is approximately 15 serum samples for each 100 000 cattle present. The 298 were divided over the 6 regions based on the cattle population in each region. For this study we only sampled 4 of the 6 regions, as from previous studies we knew that FMD prevalence in Northern red Sea and the Southern Red Sea regions were low. Within each region the villages were selected randomly. Within a village (primary sampling unit), 15 animals each over 1 year of age were randomly selected and bled. Stock owners were requested to provide age of the animal and other information such as migration of the herd. Whole blood samples were collected from cattle using 10 ml sterile plain vacutainer tubes, and allowed to clot at room temperature. Serum was decanted from the clotted blood into a sterile labelled cryovials and stored at -20°C.

Laboratory assays

(i) FMD NS ELISA

The FMD NS ELISA was performed at the National Veterinary Reference Laboratory in Asmara (Eritrea) using the PrioCHECK FMDV NS ELISA (Prionics) [20]. This ELISA detects FMDV infected animals independent of the serotype causing the infection. The test results were interpreted as indicated by the producer, i.e.: < 50 percent inhibition (PI) negative to NS antibodies and ≥ 50 PI values as positive to NS antibodies.

(ii) Virus neutralisation test (VNT)

The VNTs were performed at the Central Veterinary Institute (CVI) in Lelystad, the Netherlands. Neutralising antibody titres of all sera were determined against FMDV strains A/ERI/2/98, O_1 Manisa, C_1 Detmold, SAT1/23/86 ZIM, SAT2/SAUDI/2000, SAT3/KNP/10/90 and Asia1 Shamir. The VNT was performed as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [21]. Stock viruses for the VNT were grown in monolayers of BHK-21 cells, titrated and stored at -70 °C until use. The sera were inactivated at 56 °C for 30 minutes before testing. Dulbeco's modified Eagle's medium (DMEM) (Gibco/Invitrogen), supplemented with 5% foetal calf serum (FCS) and 2% antibiotics, was used for dilutions of the serum samples, virus and as medium for the cells. Serial two-fold dilutions of serum (50 µl) was mixed with 50 µl of virus (100 X the 50% tissue culture infective dose ($TCID_{50}$)) in flat-bottomed tissue culture grade microtitre plates (Greiner Bio-one) and the mixture was incubated for 1 hour at 37 °C and 5% CO_2, in a humidified atmosphere. After 1 hour 150 µl BHK-21 cell suspension was added. After 3 days of incubation, plates were washed and the monolayers were stained with 50 µl of amido black solution and the cytopathic effect (CPE) was read macroscopically. The endpoint titres of the serum samples were expressed as the logarithm (base 10) of the reciprocal of the last dilution of serum that neutralised 100 $TCID_{50}$ of the virus in 50% of the wells [22]. In the test, the virus concentration was determined and a test was considered correct if the amount of virus used was between 30 and 300 $TCID_{50}$ per well. The viruses used in the VNT were checked by the antigen detection ELISA [23].

(iii) Analysis of epithelial tissue and vesicular fluid

Widespread FMD outbreaks occurred in the sampled regions after sample collection for the sero-surveillance. From a specific outbreak area epithelial tissue and vesicular fluid (n=19) samples were collected from cattle (n=10) and pigs (n=9). The samples were split into two and half of them were stored in RNAlater solutions for RNA stabilization. These RNAlater samples were submitted to the CVI, Lelystad, The Netherlands for analysis. The other half of the same tissue samples was submitted to the WRLFMD, The Pirbright Institute, UK. These RNAlater samples were tested by the real-time RT-PCR [24] and from the positive samples the FMDV VP1 gene was sequenced. The samples with the lowest Ct value in the RT-PCR were tested by virus isolation on primary lamb kidney cells.

Data analysis

All VNT results were recorded in Microsoft Excel®. From the true negative sera collected in the Netherlands, we determined the titre at which 99% of the samples scored negative, using the Excel function "Percentile". This “threshold titre” was subtracted from the titres found in the field sera. The agreement between the NS ELISA result was compared to the VNT result by calculating the kappa coefficient [25]. In this analysis different cut-offs above the 99% percentile titre were used for the VNT. When a serum sample had a test result for one or more serotypes above the cut-off, the serum was considered positive by VNT. We selected the cut-off with the highest kappa. The serotype with the highest VNT result above the selected cut-off was considered as the serotype responsible for infection. If the highest response was found for 2 or more serotypes then both were considered responsible for infection [9].

The sero-prevalence based on the NS ELISA and VNT were determined for different regions and age groups using the survey library in R [26-28]. The VNT results were further analysed by mixed effects logistic regression, in which the VNT results (positive or negative) were used as result variable, village as random variable, and migration, region and age as possible explanatory variables. We started with a univariate analysis followed by a multivariate analysis using a forward selection process. The best model was selected using the AIC (Akaike's Information Criterion). The mixed effects logistic regression was performed in R [28, 29].

Results

Selection of 99 percentile in negative sera

In the 464 Dutch cattle sera, 8 were identified with antibodies against the serotypes used for vaccination in the Netherlands (although vaccination had stopped in 1991, in 2000 still 4% of the cattle slaughtered on the day the sera were collected were born before 1991), these sera were excluded from the analysis. The observed 99% percentile in the Dutch negative sera, were for serotypes A: 0.6, O: 0.9, C: 0.3, SAT-1: 0.9, SAT-2: 1.05, SAT-3: 1.5 and for serotype Asia1: 0.667 (log_{10}).

Selection of cut-off for VNT

We compared the results of the NS ELISA with the results of the VNT using the 2429 sera collected in Eritrea. For the VNT, we used different cut-offs. The highest agreement with a kappa of 0.44 was observed at a cut-off value 0.45 above the 99% percentile. The agreement with the NS ELISA at the selected cut-off was higher than with a VNT standard cut-off of 1.65 [21] for all serotypes. The comparison of the VNT and NS ELISA results at the selected cut-off is given in Table 3.

Table 3. Comparison of positive sera in VNT and NS ELISA.

		One or more serotype with a VNT result above 99 percentile + 0.45 log_{10}		Total
		+	-	
NS ELISA	+	365	190	555
	-	329	1545	1874
	Total	694	1735	2429

At the selected cut-off value, 100% specificity was observed in the true negative Dutch sera for serotype A, O, C, SAT-1, SAT-2, SAT-3 and 99.3% specificity for serotype Asia1.

Cross-reactions in the VNT

In the 76 sera from vaccination and/or infection experiments cross-reaction with other serotypes was observed. Out of these 4 sera samples were negative at the chosen cut-off. In the remaining 72 post infection sera 65 were identified correctly. In total 2 sera from O/TAW/97 vaccinated and infected pigs were scored negative. One serum sample from an O/TAW/97 vaccinated and infected pig scored the highest titre for both type O

and A, and one serum sample from an O/TAW/97 vaccinated and infected pig was identified as type A. In total 3 of the 8 Asia1 positive sera were identified as type A. The two serum samples from cattle infected with FMDV serotypes A, Asia1 and C were found positive also for O and SAT-3.

Samples collected in Eritrea.

The total number of sampled villages was 155, which was lower than intended, especially in Gash Barka where 144 villages were planned only 39 were sampled due to limitations in transport availability. In Anseba 28 out of the planned 33, in Debub 71 out of the planned 78 and in Maekel 17 out of the 6 planned sampling units were sampled. Therefore the probability for being sampled was different for different regions, which was taken into account by the software during analysis [27]. Details on the number of serum samples per region (total number 2429) are given in Table 4.

FMD NS ELISA

As shown in Table 3 a total of 155 of the 2 429 serum samples (26% in the four regions when taking sampling probability into account) were positive for non-structural protein antibodies by NS ELISA test. The Gash Barka region showed the highest number of NS ELISA positives (39%), followed by Maekel (31%), Anseba (15%) and Debub (14%) in that order (Table 4). When the serum samples were grouped according to age, the

Table 4. Sero-prevalence of FMD in the studied regions in Eritrea using NS ELISA and VNT. Taking the sampling probability into consideration [27].

Region	No. of animals sampled	% positive NS ELISA	95% CI	% positive VNT	95% CI
Anseba	420	15	11 - 20	27	21 - 34
Debub	1069	14	11 - 17	21	17 - 25
Gash Barka	649	39	31 - 46	39	32 - 46
Maekel	291	31	25 - 36	35	30 - 41
Total	2429	26	22 - 31	30	26 - 34

number of positives in the NS ELISA was found to increase with the age of cattle (Table 5).

VNT

The overall VNT sero-prevalence was 30% (Table 4 and 5). The highest sero-prevalence was in Gash Barka (39%) followed by Maekel (35%). Under the assumption that the highest titre above the cut-off was responsible for the infection, all sera tested showed positive neutralising antibody titres for all seven serotypes (Table 6). Out of the

Table 5. Positive NS ELISA and VNT results stratified for age. Taking the sampling probability into consideration [27]

Age (year)	No. of animals in age group	% positive NS ELISA	95% CI	% positive VNT	95% CI
1 - 2	445	15	11 - 19	15	11 - 20
2 - 3	375	21	16 - 26	26	20 - 32
3 - 4	395	24	18 - 29	26	21 - 32
4 - 5	345	23	17 - 29	28	22 - 35
5 - 6	238	32	25 - 39	35	28 - 41
6 - 7	261	35	27 - 44	42	36 - 48
7 - 8	192	41	28 - 53	47	35 - 60
8 - 9	105	45	31 - 60	55	43 - 67
≥9	73	47	34 - 60	46	27 - 64
Total	2429	26	22 - 31	30	26 - 34

total VNT sero-positive (n = 694) serum samples, 634 (91%) tested positive for a single serotype and the remaining 60 (9%) sera tested positive for multiple serotypes. The

Table 6. Percentage of VNT FMDV Serotyping. Taking the sampling probability into consideration.

Region	A	O	C	SAT-1	SAT-2	SAT-3	Asia1
Anseba	1.4	20	1.7	2.6	0.48	0.48	1.7
Debub	8.9	4.9	5.1	1.0	0.094	1.3	1.0
Gash Barka	14	21	4.6	0.46	1.5	1.1	0.46
Maekel	8.2	19	8.9	0.69	0.34	2.1	0.34
Total	11	14	4.7	0.87	0.82	1.1	0.79

overall highest sero-prevalence was found for serotype O (14%), followed by A (11%) and C (5%). For the other serotypes, the overall sero-prevalence was 1.1% or lower. Serotype O was highest in Gash Barka (21%) and Anseba (20%), followed by Maekel (19%) and Debub (4.9%). Serotype A was highest in Gash Barka (14%) followed by Debub (8.9%) and Maekel (8.2%) while serotype C was highest in Maekel (8.9%) followed by Debub (5.1%) (Table 6).

Table 7. Cattle positive by VNT grouped for age and serotype, taking the sampling probability into consideration [27].

Age (year)	No. of animals in age group	No. of Positive	Percentage							
			Total	A	O	C	SAT-1	SAT-2	SAT-3	Asia1
1 - 2	445	72	15	6.6	6.5	2.4	0.60	0.0	1.2	0.23
2 - 3	375	91	26	6.7	9.7	6.6	0.74	0.96	2.0	1.1
3 - 4	395	110	26	7.7	9.8	5.9	0.72	2.0	0.02	1.1
4 - 5	345	93	28	9.7	14	3.4	1.1	0.29	0.91	0.70
5 - 6	238	74	35	16	14	4.1	0.75	0.74	0.79	1.3
6 - 7	261	98	42	13	22	6.5	0.65	0.84	1.3	0.66
7 - 8	192	78	47	19	22	6.0	1.9	1.9	2.9	1.2
8 - 9	105	45	55	24	34	1.9	0.46	0.0	0.0	0.0
≥9	73	33	46	11	33	0.21	2.5	0.0	0.21	0.0
Total	2429	694	30	11	14	4.7	0.87	0.82	1.1	0.79

The VNT results in the different age groups are shown in Table 7. Out of 1874 NS ELISA negative serum samples 329 (18%) were VNT sero-positive (Table 3), of which 251 (76%) samples were from cattle greater than 2 years of age. Based on the fisher exact test the prevalence is significantly (<< 0.001) higher in the older animals. In these 329 serum samples the highest proportion was positive for serotype O (143, i.e. 43%) followed by type C (87, i.e. 26%). In the whole dataset, serotype O had the highest prevalence followed by serotype A.

Mixed effects logistic regression analysis

All villages had communal grazing, and shared watering points. Therefore, it was not possible to analyse differences for communal grazing and watering points. Univariate analysis showed significant differences for migration, region and age. The lowest AIC

was obtained with age as explanatory variable for the positive VNT results. In the multivariate analysis significant differences were only observed for age and regions and its interaction (Table 8). In Anseba and Debub there was no significant effect of age on the sero-prevalence, but there was in Maekel and Gash Barka.

Table 8. Odds ratios obtained from the multivariate mixed effects logistic regression model.

Variable		Odds ratio	95% CI	p-value
Intercept		----	---	<<0.001
Region				
	Debub	Reference		
	Anseba	1.3	0.65 – 2.5	0.5
	Maekel	0.9	0.44 – 1.7	0.7
	Gash Barka	0.7	0.39 – 1.2	0.2
Age		1.02	0.95 – 1.1	0.5
Age x Anseba		1.02	0.90 - 1.2	0.7
Age x Maekel		1.24	1.1 - 1.4	<<0.001
Age x Gash Barka		1.38	1.2 - 1.6	<<0.001

Analysis of epithelial tissue samples

The FMDV sequence analyses results obtained at CVI, Lelystad, The Netherlands showed serotype O topotype EA-3, and were exactly the same with the results obtained from the WRLFMD, The Pirbright Institute, UK, which are available at the Institute's website (www.wrlfmd.org). Virus isolation from the RNAlater samples was not possible.

Discussion

The aim of the current study was to determine the FMD sero-prevalence for each serotype in cattle in Eritrea and to study the differences between regions, age and villages with and without migration. To be able to interpret the results, we had to validate the VNT, as this was only performed in the laboratory for the European FMDV serotypes. In the VNT, we observed significant differences in the titres obtained with the different FMDV serotypes seen in negative Dutch cattle serum samples. For this reason, we based the cut-off in the VNT on the 99% percentile observed in these negative cattle sera as described by [30].

Under the assumption that NS ELISA positive cattle should also be positive for one of the serotypes in the VNT; we analysed the agreement between the overall VNT and NS ELISA results. The highest agreement was found at a titre of 0.45 $\log_{10}$ above the 99% percentile. The kappa (0.44) result indicates moderate agreement. Although, cross-reactions were observed in the 72 post vaccination and/or infection sera, in most cases, the highest VNT titre was found with the serotype used for vaccination and/or infection, suggesting that the criterion of using the highest response only is generally valid.

We selected 4 out of 6 administrative regions for this study. In previous studies 102 cattle from the Eastern coast administrative regions were used [9], and they proved to be serologically negative for FMD NS antibodies. These regions were therefore not included in this study. The overall FMD sero-prevalence in cattle was 23% (95% CI: 21 - 25%) based on the NS ELISA results. Using the defined cut-offs in the VNTs the sero-prevalence was slightly higher; 29% (95% CI: 27 - 30%). Tests that detect antibodies to non-structural proteins (NSPs) are less sensitive than tests based on structural proteins and may not detect animals with limited virus replication [31-35]. This statement is in agreement with our results. Moreover, titres to structural proteins may persist for the life of the animal, but antibodies to NSPs decline and become undetectable after some time [33]. In this cross-sectional study, 91% of the VNT positive sera were positive for just a single serotype but some sera reacted to multiple serotypes, which can be caused by cross-reactions or due to multiple infection.

Comparison of our results, with the sero-prevalence recorded in Ethiopia by others, we find higher sero-prevalence compared to one study by Mohamoud et al., [36] (15%) and a almost similar sero-prevalence in two other studies performed by Rufael et al., [37] (21%) and by Sahle [38] (27%). In Sudan, however, the sero-prevalence of FMD is much higher Habiela [18] (79%) which matches with the results observed in some villages in Eritrea, but not with the overall sero-prevalence. This shows that FMD is endemic in the region, and therefore when control is implemented it should be region based.

In this study, the Gash Barka administrative region has the highest sero-prevalence in NS ELISA and VNT (both 39%) compared to the other three administrative regions. Although Gash Barka has a high number of cattle, the density is lower compared to that in Debub. Therefore the cattle density cannot be the

explanation for the higher sero-prevalence in Gash Barka, but there are more nomadic and transhumance husbandry systems and also some important livestock markets that might explain the higher transmission. Maekel also has a cattle density lower than Debub, more or less comparable to Gash Barka. In Maekel there are terminal markets for live animals due to the fact that the capital city is located in this region. Gash Barka is a major supplier of cattle and small ruminants to Maekel livestock market, suggesting spread of FMDV during outbreaks by animals trekked from Gash Barka to the markets in Maekel. This probably explains that also in Maekel we find a high sero-prevalence.

This study shows that serotypes O (14%) and A (11%) are the dominant serotypes in Eritrea. The high prevalence for these 2 serotypes is logical in the light of the recent confirmed outbreaks of FMD in the country in 2009 and 2011 for both A and O serotypes respectively [9, 39]. On the other hand the sero-positivity found for serotypes C and SAT-1 was lower than in a previous study from reported FMD outbreak areas [9]. This difference is partly due to the fact that we now use a different cut-off. On the other hand 4.7% of the cattle are positive for serotype C, which confirms, the previous finding. But 87 out of the 118 type C positive sera were negative in the NS ELISA, this could be related to differences in the NS gene or an indication that the reactions for type C in the VNT are caused by other unknown factor. Further studies should be done on this type C serological reactions as they are also often combined with low reactions to other serotypes.

In this study, the number of cattle that are considered positive for antibodies against serotype SAT-1, SAT-2, SAT-3 and Asia1 are low compared to the number of cattle positive for antibodies for serotype O, A and C. Our validation showed high specificity for serotype SAT-1, SAT-2 and SAT-3 with no cross-reaction at the chosen cut-off. Because most SAT-3 isolates are from African buffalo and the African buffaloes are not present in Eritrea, it is highly unlikely that the FMD serotype SAT-3 is circulating in Eritrea. Consequently we concluded that virological verification by collecting epithelium and probang samples after outbreaks is necessary. For Asia1, we did not find 100% specificity in our validation; hence the few cattle with a titre against Asia1 are probably false positives.

In total 190 sera were NSP antibody positive and negative by VNT (Table 3). Because many of these sera had a relatively high NS response with a percentage inhibition of up to 97%, it is reasonable to assume that these cattle have been infected

with FMD virus. The fact that they are negative by VNT could be due to mismatch of test strains used in the VNT with strains circulating in Eritrea. Studies performed in Africa on the role of sheep and goats in FMDV transmission indicate that these species could play a role in FMD epidemiology [18, 40, 41]. It is important to carry out a study in Eritrea to understand the role that these species have on FMD epidemiology.

Note that shipping tissue samples for FMDV diagnosis is restricted by International Air Transport Association (IATA) rules and it is therefore often difficult to obtain them from endemic countries. In our study, we had the possibility to send tissue samples preserved on ice to the WRLFMD, The Pirbright Institute, United Kingdom and the same samples preserved in RNAlater to the CVI in Lelystad, the Netherlands. The results from both laboratories were the same showing that RNAlater preserves the RNA well for sequencing purposes. The virus isolation attempt shows that virus cannot be isolated, which is expected considering the low pH of RNAlater. RNAlater is therefore recommended for transporting FMD tissue samples from FMD endemic countries for sequencing purposes.

Within the progressive control pathway for FMD control, identifying the risk is the first step. Our results show that FMD is present in Eritrea more or less at a similar level as seen in neighbouring countries. Although there are differences in sero-prevalence between the 4 regions studied, there is a considerable amount of FMDV circulation in the sampled regions. This indicates that there is no scientific basis for differences in control measures in these 4 administrative regions. One of the options for FMD control in Eritrea would be the establishment of a protection zone (buffer zone) in the administrative regions found in the eastern lowlands of the Red Sea coast (assumed to have a very low sero-prevalence of FMDV) and allowing only properly vaccinated animals into this protection zone. This would assist in authorization to export cattle from this region. Nevertheless, this would immediately set the need for proper animal identification and movement restrictions within Eritrea. Another option is nationwide prophylactic vaccination. A study conducted in South Sudan indicated that the loss of production and mortality due to FMD infection was 11.5 times higher than the cost of vaccination [42], which suggest that this could be cost effective, but more studies on the economics of FMD vaccination are needed in Eritrea. Nevertheless this sero-surveillance study suggests that FMD vaccination with serotype O and A matching

with the field viruses would be feasible and could therefore be pursued for the control of the disease in Eritrea along the OIE/FAO progressive control pathway for FMD.

Acknowledgement

This study is funded by the International Atomic Energy Agency (IAEA), Vienna, Austria, and the Ministry of Economic Affairs project WOT-01-003-11 The Netherlands. The authors gratefully acknowledge to all animal health staff in the Ministry of Agriculture, Eritrea who have contributed to the collection of serum samples for their cooperation in this study and Gert-Jan Boender for his technical assistance to produce the sampling unit/village map of Eritrea. We are also grateful to Dr Amos Ssematimba for his contribution in editing and improving the manuscript.

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Chapter 5

Comparison of test methodologies for foot-and-mouth disease virus serotype A vaccine matching

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Clinical and Vaccine Immunology 2014:21(5):674-683,
DOI:10.1128/CVI.00034-14

Abstract

Vaccination has been one of the most important interventions in disease prevention and control. The impact of vaccination largely depends on the quality and suitability of the chosen vaccine. To determine the suitability of the vaccine strain, antigenic matching is usually studied by *in vitro* analysis. In this study, we performed three *in vitro* test methods to determine which one gives the lowest variability and the highest discriminatory capacity. Binary ethylenimine inactivated vaccines, prepared from 10 different FMD virus serotype A strains, were used to vaccinate cattle (5 animals for each strain). Antibody titres in serum samples of 3 weeks post-vaccination (WPV) were determined by virus neutralization, neutralization index and liquid phase-blocking ELISA. The titres were then used to calculate relationship coefficient ($r_{1)}$ values. These r_1-values were compared to the genetic lineage using receiver operating characteristic (ROC) analysis. In the two neutralization test methods the median titre observed against the test strains differed considerably, and the sera of vaccinated animals did not always show the highest titres against their respective homologous virus strains. When the titres were corrected for test strain effect (scaling) the variability (standard error of the mean per vaccinated group) increased because the results were on a different scale, but the discriminatory capacity improved. An ROC analysis of the r_1-value calculated on both observed and scaled titres showed that only r_1-values of the liquid-phase blocking ELISA gave a consistent statistically significant result. Under the conditions of the present study, the liquid-phase blocking ELISA showed less variation and still had a higher discriminatory capacity than the other tests.

Keywords: FMDV, vaccine matching, neutralization index test, VNT, liquid phase blocking ELISA, r_1-value, scaled titre

Introduction

Foot-and-mouth disease (FMD) is highly contagious infectious disease that affects all cloven-hoofed animals and is one of the most economically important diseases of livestock. The virus belongs to the genus *Aphthovirus*, in the family of the *Picornaviridae* [1]. It has seven different serotypes namely; O, A, C, Asia 1 and Southern African Territories (SAT) 1, 2 and 3, with multiple subtypes within each serotype [2]. FMD virus (FMDV) is a highly variable RNA virus [3-5], and in general, there is little or no cross-protection between serotypes and even between different strains of the same serotype [2, 6, 7]. Serotype A is considered to be the most antigenically and genetically diverse of the FMDV serotypes and new antigenic variants emerge frequently [8, 9].

Inactivated vaccine of FMDV is being used to control FMD. This type of vaccine is commonly used in many parts of the world namely, South America, Asia and Africa where FMD is endemic. Although the quality of the vaccine used is probably the most important factor for the success of a vaccination program, a reasonable antigenic match between the FMDV vaccine and the outbreak virus strains is also considered essential for the effectiveness of the vaccine [6]. Initially *in vivo* cross-protection studies were performed to test for antigenic differences. However, since serological tests became available, they are also being used for antigenic matching on the basis of the assumption/hypothesis that the level of protection is correlated to the antigenic match in the serological tests. In such serological tests the antibody titre of sera collected from vaccinated animals is determined against both the vaccine strain and field strain virus [6, 10]. The most used value to express antigenic match is the relationship coefficient, (r_1-value), which is the ratio of the titres obtained in serological tests using the heterologous strains and the vaccine strain [7, 10-12].

Serological test methods have been used to quantify antigenic differences between FMDV structural protein antibodies and thereby estimate the vaccine matching between a vaccine strain and a field isolate. Antigenic analysis of field isolates in relation to vaccine strains, based on virus neutralization tests (VNTs), plays a significant role in evaluating the suitability of existing vaccine strains [6, 10, 11], although significant variation has been reported with VNTs [13, 14]. The genetic diversity in the P1-coding region within the SAT serotypes are reflected in the antigenic

properties of these viruses; therefore, there are implications for the selection of vaccine strains that would provide the best vaccine match against emerging viruses [15]. In the antigenically diverse FMDV serotype A strains, predicting antigenic differences using genetic sequences alone does not provide reliable information for vaccine matching [16]. Therefore, others have added structural information on the location of the amino acid sequence in the virus to the sequence data and have shown that it was a powerful tool to predict antigenic relationships that has the potential to be applied to a variety of different infectious agents [17].

In studies in which cross-protection was related to cross-reactions in serology [10, 18, 19] the relationship between cross-protection and cross-reactions varies. In some studies, protection was observed when high potency (type A) vaccines were being used [10], even when the r_1-values were low (ranging from 0.04 to 0.23). In other studies, no cross-protection was detected after vaccination with A/Iran/05 vaccine and challenge with A/TUR/64/11 [18], while the $r_1$value of 0.1 fell within the range of the r_1-values found in the previous studies [10]. For the FMDV type O Manisa vaccine, a reasonable high r_1-value of 0.6 was observed against O Campos, but a vaccine containing 15 µg of O Manisa antigen protected in 99% of the cases against homologous challenge but only in 54% of the cases against a O Campos challenge [19]. This shows that a highly potent O Manisa vaccine did not protect sufficiently against a heterologous O Campos challenge even though the r_1-value indicated it should. This shows that r_1-values are not always consistent with the challenge results. Since protection against a challenge with FMD is well correlated with serology results [20], variation in r_1-value determination might have caused this inconsistency. Cross validation studies on r_1-value determination between laboratories [18, 21] showed huge variation between laboratories and techniques used. The exact cause of variation could not be established, as there were too many variables. A recent study [22] analysed the r_1-value of the VNT using ROC analysis. They showed that the r_1-value did not predict cross-protection. Other authors have linked genetic information to cross-reactions in serology [15-17] and have showed that the r_1-value can be predicted from surface exposed amino acid changes. These studies did not address the inherent variability of the r_1-value, and it is therefore important to have a more comprehensive view on the usefulness of r_1-values using results generated with various FMD strains in one

laboratory. One of the first questions is to identify which test to be used to test cross-reaction in serology.

In this study, we analysed the cross-reactions in serology using serum samples from cattle vaccinated with 10 different FMDV serotype A strain vaccines using three serological test methods, with the objective of determining which serological test method provides the least variation within a group of vaccinated cattle and provides the best discrimination between vaccines.

Materials and methods

FMDV serotype A strains

Different serotype A FMDV (n = 10) strains that were isolated in Africa, the Middle East, and Europe were selected from various genetic lineages (Table 1). The 10 strains were A/KEN/12/2005, A/ERI/2/98, A/SUD/2/84, A/ETH/13/2005, and A/MAU/1/2006, which were received from the OIE/FAO World Reference Laboratory for FMD (The Pirbright Institute, United Kingdom), and A10/Holland/42, A22/IRQ/24/64, A/TUR/20/2006, A/TUR/14/98, and A/IRN/2/97, obtained from Central Veterinary Institute (CVI), (Wageningen UR, Lelystad, The Netherlands). For control on the strain identity we sequenced the VP1 gene of the 10 FMDV serotype A strains and compared them to data in NCBI database.

Vaccine (antigen production)

The vaccines employed in this study were non-commercially produced at the CVI for this particular study. The vaccines were prepared from the aforementioned 10 different FMDV serotype A strains after growing them on monolayers of BHK-21 cells in 850-cm^2 roller bottles (Corning tissue culture treated, non-pyrogenic; Corning Incorporated, N.Y).The viruses were subsequently inactivated with 10 mM binary ethyleneimine (BEI) [23, 24].

The BEI was neutralized by 0.79% (wt/vol) sodium thiosulphate, and the inactivated antigens were concentrated with one cycle of 8.0% (wt/vol) polyethylene glycol (PEG) precipitation. The antigens were checked by standard VP1 sequencing [25]. The 146S antigen concentration was determined by quantitative sucrose density gradient analysis, as previously described [26, 27]. A known 146S FMDV antigen

reference standard was tested with each run. The aqueous vaccines were formulated using 2% aluminium hydroxide (Alhydrogel Ph. Eur. Brenntag Biosector) and 10% (wt/vol) saponin (Quil-A solution in [PBS]) as adjuvant. The vaccine payload was 10 µg of antigen per 2-ml dose and expected to have ≥3 the 50% protective dose (PD_{50}). The antigens were tested for the presence of live FMD virus both *in vitro* and *in vivo*.

TABLE 1 FMDV serotype A strains used in the study.

FMDV type A strain	Lineage / sub-lineage[a]	*Accession no. (VP1)[b]	Reference
A10/Holland/42	EURO-SA	AY593751	41
A22/IRQ/24/64	ASIA	AY593764	41
A/ERI/2/98	G-IV	EU919238	42
A/ETH/13/2005	G-II	FJ798145	43
A/IRN/2/97	Iran-96	KF152935	43
A/KEN/12/2005	G-I	KF112912	16
A/MAU/1/2006	G-VI	JF749842	44
A/SUD/2/84	G-IV	GU566067	45
A/TUR/20/2006	Iran-05	FG755116	46
A/TUR/14/98	Iran-96	DQ296537	47

[a]Ten strains came from 8 different lineages. Two lineages, Iran-96 and G-IV contained two strains. Within lineage Iran-96 both strains were 97.7% similar in VP1 on amino-acid level, within lineage G-IV both strains were 93.1% similar in VP1 on amino-acid level.
[b]Best matching VP1 sequence in NCBI

Vaccination of cattle

Vaccination was performed in 2010. Healthy unvaccinated Eritrean local indigenous zebu breed (*Bos indicus*) cattle, 10 to 18 months of age, were used for this study. The cattle used originated from regions known to be free from FMD without vaccination. Before purchase and prior to vaccination, all cattle were ear tagged and bled twice at a 2-week interval and tested for presence of anti-FMDV non-structural protein (NSP) antibodies using the FMD non-structural enzyme-linked immunosorbent assay (NS-ELISA) (PrioCHECK) [28]. Only cattle with a negative result in the FMD NS-ELISA were purchased. The cattle were housed in stables at the National Veterinary Laboratory, Asmara, Eritrea. The cattle were randomly allocated to 11 groups of 5 cattle. In total, we used 10 groups for the 10 different FMDV serotype A vaccines and 10 cattle as non-vaccinated controls to monitor incursions of FMD infection that would influence the experiment. The cattle in each vaccine group were vaccinated subcutaneously in the

middle of the cervical area. Blood samples were collected at 0, 7, 14, 21 and 28 days post vaccination for serology testing.

Rabbit and guinea pig antisera for liquid phase blocking ELISA (LPB-ELISA)

In total, 20 rabbits and 20 guinea pigs (two for each FMDV serotype A strain antigens) were immunized as described by Hamblin [29] using the same semi-purified PEG precipitated antigens as those used to vaccinate the cattle in this study.

The vaccine was prepared by mixing an FMDV serotype A antigen with an equal volume of Montanide ISA 206VG oil adjuvant to get a final concentration of 5 µg/ml. The rabbits were vaccinated subcutaneously and intramuscularly with 0.5 ml of vaccine on each site. The guinea pigs were vaccinated subcutaneously with 0.5 ml of vaccine. All animals were boosted 2 weeks post-vaccination (WPV) with the same protocol used for primary vaccination. The animals were bled 10 to 12 days after the booster vaccination by exsanguination under general anaesthesia.

FMDV NS ELISA

PrioCHECK® FMDV NS ELISA (Prionics) [28] is a blocking ELISA that detects antibodies directed against the non-structural 3ABC protein of FMDV. This ELISA detects FMDV-infected animals independently of the serotype causing the infection. Serum samples were tested by the PrioCHECK FMDV NS ELISA kit, and the test results were interpreted as indicated by the producer, i.e., < 50 percent inhibition (PI) negative to NS antibodies and ≥ 50 PI values was considered as positive to NS antibodies.

Virus neutralization test

A VNT was performed as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [7]. It was performed using BHK-21 cells in flat-bottomed tissue culture grade microtitre plates (Greiner Bio-one). Stock viruses for the VNT were grown in cell monolayers, titrated and stored at -70 °C until used. The experimental serum samples were inactivated at 56 °C for 30 min before testing. Dulbeco's modified Eagle's medium (DMEM) (Gibco/Invitrogen) supplemented with 5% foetal calf serum (FCS) and 2% antibiotics was used to dilute the sera and virus, and also as a medium for growing the cells. All 3-WPV sera were tested in duplicate by

mixing 50 µl of 2-fold dilutions of the serum with a 50- µl virus suspension containing 100 X the 50% tissue culture infective dose ($TCID_{50}$) FMDV. This mixture was incubated for 1 h at 37 °C and 5% CO_2, in a humidified atmosphere. After 1 h of incubation, 150 µl BHK-21 cell suspension was added to each well, and the plates were incubated for 3 days at 37 °C and 5% CO_2, in a humidified atmosphere. After 3 days, the monolayers were stained with 50 µl amido black solution (1 g/litre in water containing 88 mM sodium acetate, 10% glycerol, and 5.4% acetic acid), and the cytopathic effect (CPE) was read macroscopically. The endpoint titres of the serum samples, tested against all 10 vaccine strains, were expressed as the logarithm (base 10) of the reciprocal of the last dilution of serum that neutralized 100 X $TCID_{50}$ of the virus in 50% of the wells [30].

Serum samples collected at 0, 1, 2, 3 and 4 WPV were tested by VNT using the homologous test strain to see the response of neutralizing antibodies post-vaccination. For a comparison of tests, the 3-WPV sera were tested against all 10 virus strains used for vaccination. In the calculation of the mean titres, VNT titres with a titre of < 0.3 at the day of vaccination were considered 0. In the calculations of r_1-values and scaled titres, the titres below the detection limit (0.3) were excluded since no titre was measured.

Neutralization index test

In the neutralization index test (NIT), 50 µl of 10-fold dilutions of the virus (6 wells per dilution) were incubated with 50 µl of a fixed dilution of a 1:25 dilution of test serum for 1 h at 37 °C and 5% CO_2, in a humidified atmosphere. In each test, a titration mixed with 50 µl of dilution medium (DMEM with 2% foetal bovine serum and antibiotics) instead of the test serum was included. After 1 h, 150 µl BHK-21 cell suspension was added to each well, and the plates were incubated for 3 days at 37 °C and 5% CO_2, in a humidified atmosphere. After 3 days, the plates were washed and the monolayers were stained with 50 µl of amido black solution (see above), and the CPE was read macroscopically. The log_{10} titres of the virus with and without serum were calculated [30]. The neutralization index log_{10} titre was calculated by subtracting the virus log_{10} titre of the strain with test serum from the log_{10} titre of the strain without serum. All 3-WPV sera were tested against all 10 FMDV serotype A strains used for vaccination.

LPB-ELISA

The liquid-phase blocking ELISA (LPB-ELISA) was performed as described by Hamblin et al. [29]. Specific rabbit sera for coating and the guinea pig sera for detection were produced for each of the 10 strains. The rabbit and guinea pig antisera were partially purified by precipitation using saturated aluminium sulphate. The dilutions of coating antibody and detecting antibodies were optimized in the laboratory, as well as the rabbit anti-guinea pig conjugate (P0141; Dako).

Antigens were prepared from the 10 FMDV type A strains by growing them on monolayers of BHK-21 cells. After freeze-thawing the cell culture and centrifuging for 10 min at 250 x g, the unpurified supernatants were titrated, and the final dilution that gave an optical density (OD) at 450 nm of approximately 1.2 to 1.5 in the ELISA was used. ELISA plates (Corning Costar) were coated with a predetermined dilution of 100 µl/well rabbit anti-FMDV antibodies diluted in bicarbonate buffer (pH 9.6) and left overnight at room temperature. On the same day, in U-bottomed microtitre plates (dummy plate), each test serum sample was diluted in a 2-fold dilution series in duplicate wells, starting with a 1:5 dilution (60 µl/well) using phosphate-buffered saline containing 0.05% Tween 80, 0.5M NaCl and 5% FCS (ELISA buffer). Sixty microliters of a predetermined concentration of antigen, diluted in ELISA buffer, was added, mixed and incubated overnight at 4°C. The next day, the ELISA plates were washed 6 times with tap water containing 0.05% Tween 80. Next, 100 µl of test serum-antigen mixture was transferred from dummy plates into the corresponding wells of the rabbit serum-coated ELISA plates and incubated at 37 °C for 1 h. After washing, 100 µl of the guinea pig antiserum (in a predetermined dilution) homologous to the viral antigen used was added to each well and incubated at 37 °C for 1 h. The plates were washed, and 100 µl of a predetermined dilution of rabbit anti-guinea pig immunoglobulins conjugated with horseradish peroxidase was added to all wells and incubated for 1 h at 37 °C. The guinea pig antiserum and the rabbit anti-guinea pig conjugate were diluted in ELISA buffer. After washing, 100 µl of a substrate-chromogen solution (BioFX TMBS-1000-01) was added, and the plates were left to develop colour for 10 min at room temperature. The reaction was stopped by adding 100 µl 0.5 M sulphuric acid. The plates were read at 450 nm, and the $\log_{10}$ antibody titres were expressed as the $\log_{10}$ of the reciprocal of the final dilution of serum giving 50% of the mean OD value recorded in the maximum signal control wells (two wells with ELISA buffer instead of serum).

Data Analysis

(i) Mean titres

The mean titres were computed by adding the log_{10} titres and divided by the number of observations.

(ii) Scaled titre (correction for strain effect)

Scaled titres were used to minimize the variations observed between neutralization tests with different viruses. For each specified serotype A strain the titre was standardized using the following formula (observed titre – mean titre for all sera tested against a specified test strain) / (standard deviation of all sera tested against a specified test strain). Therefore, for each specified strain, the mean scaled titre will be 0, with a standard deviation of 1.0 [31].

(iii) Determination of r_1-value

A one-way antigenic relationship (r_1-value) for each of the 3-WPV serum samples was determined using VNT, NIT and LPB-ELISA titres, as described in the OIE manual [7] using the equation r_1 = (serum titre against heterologous virus) / (serum titre against homologous virus), where the heterologous virus is a field strain and the homologous virus is a vaccine strain.

To test whether the original and scaled titres of the sera from vaccinated cattle were able to discriminate between the various vaccine strains, we used an analysis of variance (ANOVA). In the ANOVA, we tested whether the titres induced by each vaccine were similar. If the ANOVA showed that one or more of the vaccine groups were significantly different, all possible pairwise comparisons were tested with a t-test using Holm's adjustment for multiple testing. A *p*-value of < 0.05 was considered statistically significant. Statistical analyses were carried out using R version 2.14 [32].

(iv) Receiver Operating Characteristic analysis

An ROC analysis was carried out to determine which serological technique and the r_1-value derived from that technique had the best sensitivity and specificity in determining differences between test strains. In the first analysis, we assumed that the two strains from lineage Iran-96 have the same antigenic make-up (gold standard for sensitivity

and specificity). In this case, the amino acid homology in VP1 was 97.5%. In the second, we assumed that strains within both the Iran-96 and the G-IV lineages were antigenically similar. In that case, the amino acid homology in VP1 was 93.1%. The ROC analysis was performed using the pROC library in R [33].

Results

FMD vaccine strains

The antigens used for vaccination came from 8 different lineages defined by the World Reference Laboratory in Pirbright (Table 1). In two lineages, we had two vaccine candidates. In lineage Iran-96, we selected A/IRN/2/79 and A/TUR/14/98, which are 97.7% similar in VP1 on amino-acid level.

In lineage G-IV, we selected A/ERI/2/98 and A/SUD/2/84, which are 93.1% similar in VP1 on amino-acid level. The identity on amino-acid level ranged from 84.3 - 91.4% for strains that did not belong to the same lineage. The VP1 sequences were compared to known sequences in the GenBank. For most strains, a match (99 - 100% nucleotide identity) was found, and these are shown in Table 1. For our A/KEN/12/2005 VP1 sequence only, the best match (93% nucleotide identity) was with A/KEN/22/2009 (GenBank accession no. KF112912). Both the *in vitro* and *in vivo* innocuity tests did not detect live FMDV in the BEI-inactivated antigens produced from these 10 strains.

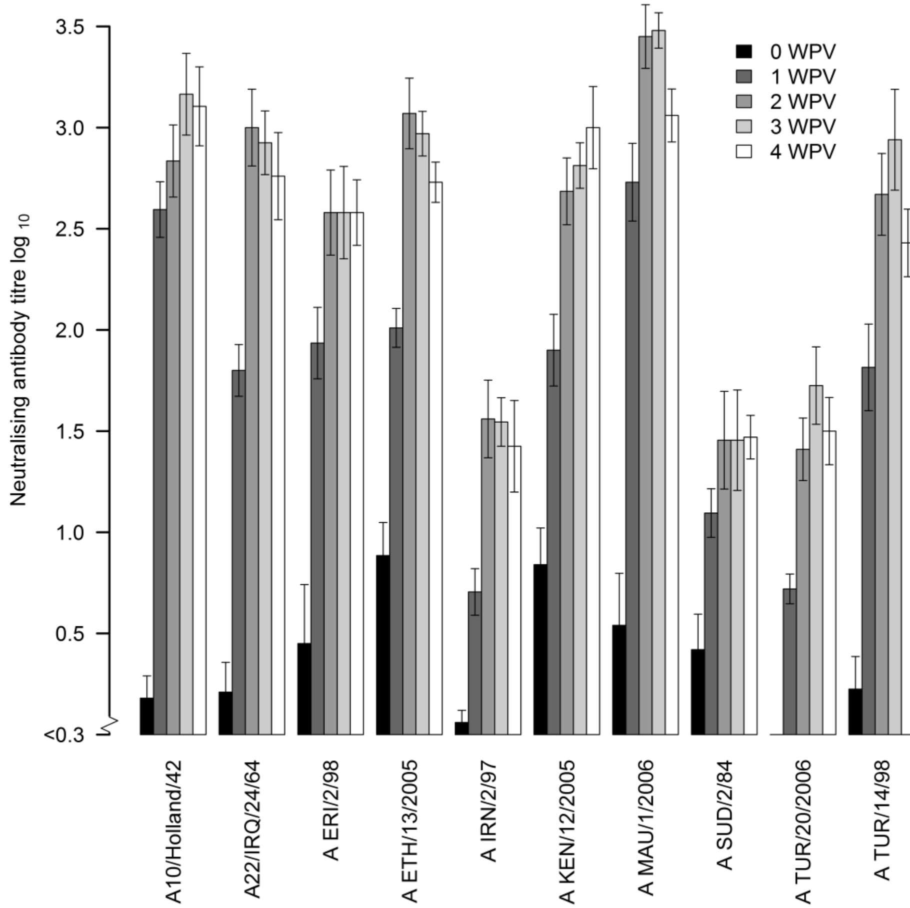

Figure 1. VNT homologous mean titres, including standard error of the mean values of FMDV serotype A strains post-vaccination responses in cattle at 0, 1, 2, 3 and 4 WPV

FMDV NS ELISA

All sera collected at the start of the experiment were negative in the Prionics NS ELISA [28]. In total, 7 cattle were positive in the NS ELISA post-vaccination, in 4 of them only at one time point post-vaccination (1, 3, or 4 WPV) in 2 cattle at two time points, and in one cow at 2, 3, and 4 WPV The maximum percentage inhibition observed was 58%.

Post-vaccination antibody response

Serum samples collected from all cattle at 1, 2, 3, and 4 were tested using VNT against the homologous vaccine test strain. In some cattle, a low reaction WPV was detected in the VNT before vaccination (Fig. 1), this was also seen in the sera of the control animals, especially with test strain A10/Holland/42. All vaccinated cattle responded to vaccination, with an increase in $\log_{10}$ antibody titre in the first week ranging from 0.3 (2-fold) to 3.0 (1000-fold), with a median of 1.1. At 2 – 4 WPV, the increase was ≥ 0.6 (4-fold) compared to the $\log_{10}$ titre before vaccination, with a median increase of 2.1 $\log_{10}$. The mean 3-WPV homologous $\log_{10}$ titre in the VNT ranged from 1.5 - 3.5 (Fig. 1). The mean VNT titre 3-WPV against the homologous and 9 heterologous virus test strains used in the VNT in the different vaccine groups from 1.6 for A/MAU/1/2006 to 2.0 for A/TUR/20/2006 with an overall mean titre of 1.8.

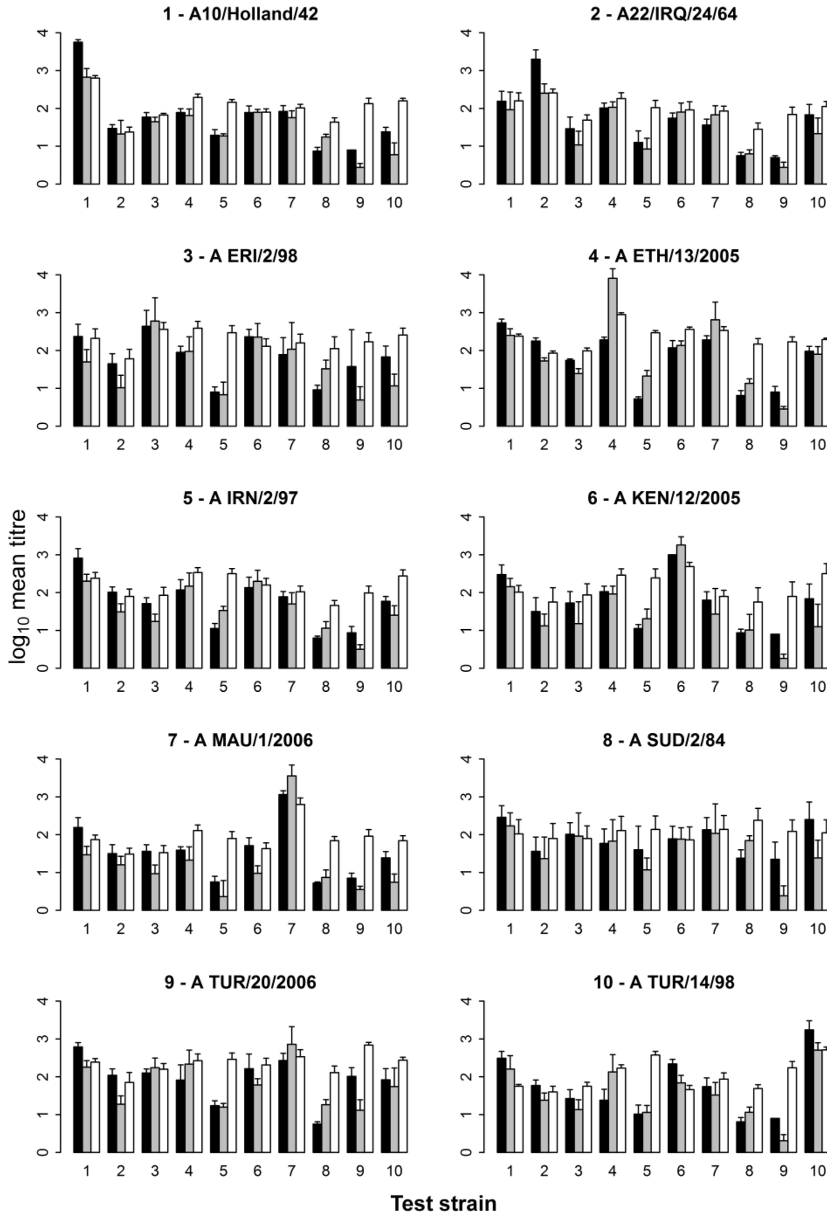

Figure 2. Mean titres and the standard error of the mean (SEM) values of the three serological methods, i.e., VNT (**black**), NIT (**gray**) and LPB-ELISA (**white**) for each vaccine and tested against the corresponding test strain (same number means the same strain). The individual graphs show the log_{10} mean titres and the standard error of the mean of the sera of each group of vaccinated cattle (5 cattle) tested against the 10 different FMDV type A test strains (x axis). The homologous responses are those for which the number of the test strain (x axis) is equal to the number in the title of a particular panel

Analysing differences between test strains using different serological test methods

All 3-WPV sera were tested and analysed by the three serological assays (VNT, NIT and LPB-ELISA) against the homologous and heterologous virus strains. The mean titres and standard error of the mean (SEM) were calculated for each vaccine (5 sera per vaccine) (Fig. 2). The correlation between VNT and NIT was higher (0.72) than correlation between NIT and LPB-ELISA (0.55) or VNT and LPB-ELISA (0.49). Some virus test strains were more easily neutralized than others. Consequently, the mean titre against the homologous strain is not always the highest one. With the test strains A/IRN/2/97, A/SUD/2/84 and A/TUR/20/2006, low responses were observed with all serum samples from vaccinated animals for both VNT and NIT, similar to the homologous strain shown in Fig. 1. Because of these inherent variations between the test strains, many sera were found where the homologous titre was lower than some of the heterologous ones. Figure 2 shows that the mean VNT (in 4 of 10 strains), the mean NIT (in 3 out of 10 strains) and the mean LPB-ELISA (in 2 out of 10 strains) homologous titres were not the highest.

Scaled titres (correction for strain effect)

Because some virus strains were more easily neutralized than others, as mentioned above, we used scaled titres to correct and standardize for the test strain effect. Using scaled titres the mean homologous response is in general the highest one (Fig. 3).
Table 2 shows the range of the standard error of the mean (SEM) determined for each group of 5 vaccinated cattle tested against each of the test strains for both the observed and scaled titres. Before scaling, the LPB-ELISA had the lowest maximum SEM. After scaling both NIT and LPB-ELISA have the lowest maximum SEM values.

r_1-value

One would expect the r_1-value to range between 0 and 1, but that was only observed in 78%, 92% and 96% for VNT, NIT and LPB-ELISA respectively (Table 3). The maximum r_1-values were 178, 1148 and 2.8 for VNT, NIT and LPB-ELISA respectively. After scaling, a higher proportion of r_1-values were found between 0 and 1 (Table 3).

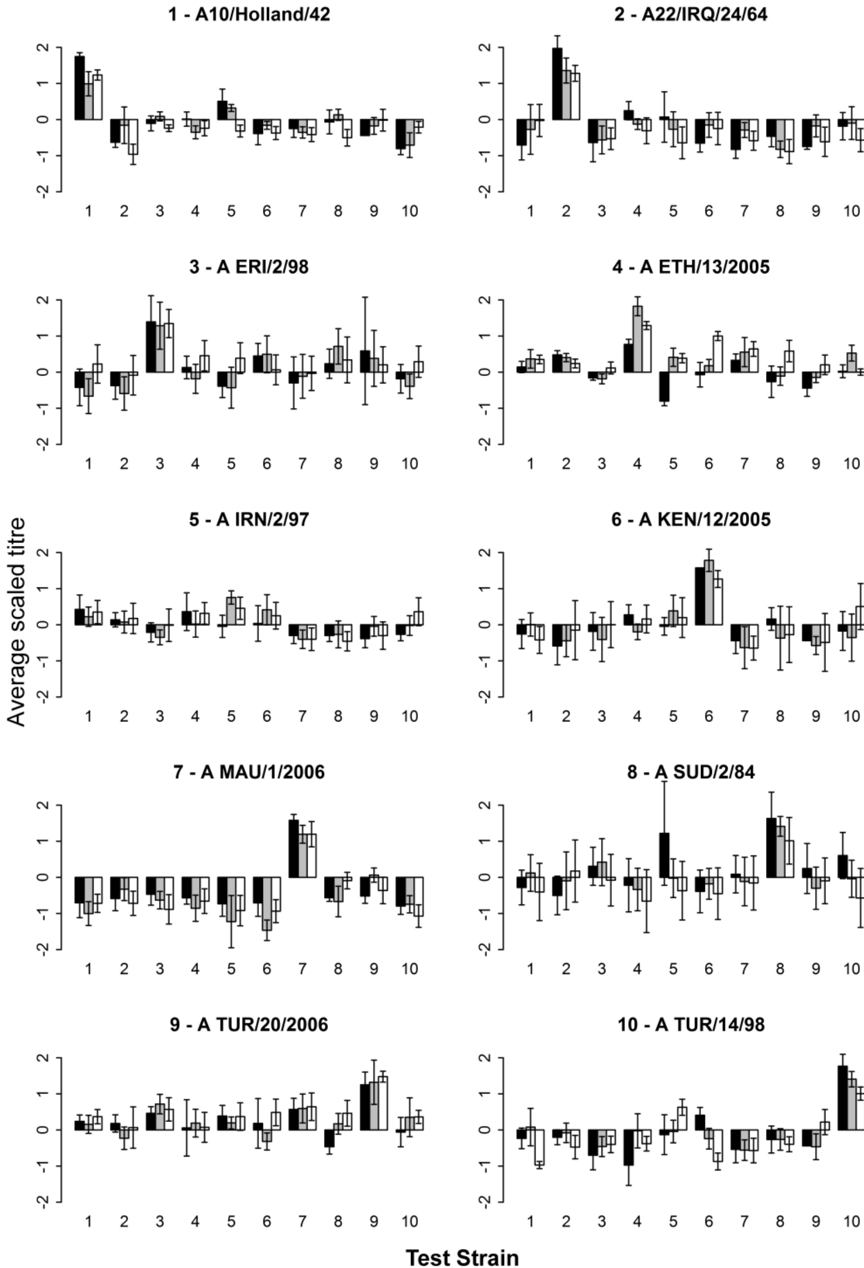

Figure 3. Mean scaled titres including the standard error of the mean of VNT (**black**), NIT (**gray**) and LPBE (**white**). The homologous responses are those for which the number of the test strain (x axis) is equal to the number in the main title of a particular panel.

Discriminating between vaccines

As stated above, the mean titres induced by the different vaccines were similar; therefore, we used an ANOVA to determine whether there are significant differences between vaccines. Using titres and scaled titres obtained by VNT, no significant differences were found between vaccines. Using titres and scaled titres obtained by NIT, a significant difference between the test strains was observed in the ANOVA and in 1 (based on titre) or 3 (based on scaled titres) out of the 45 (Table 4) possible pairwise comparisons. Using titres and scaled titres obtained by LPB-ELISA, a significant difference between test strains was observed in the ANOVA, and in 7 (based on titres) or 12 (based on scaled titres) out of the 45 possible pairwise comparisons (Table 4).

TABLE 2 Comparison of the 3 serological test methods using titres and scaled titres. Comparison of the range of SEM within groups of 5 cattle.

Test method	Range of SEM of observed titres	Range of SEM of Scaled titres
VNT	0 – 0.98	0 – 1.49
NIT	0.06 – 0.78	0.10 – 0.88
LPB-ELISA	0.04 – 0.40	0.09 – 0.87

ROC analysis

In the data set, we had 2 genetic lineages (Iran-96 and G-IV) that each contained 2 strains. In the ROC analysis, we compared the sensitivity and specificity of the r_1-values of the different test methods before and after scaling. In the first analysis, we assumed that only strains A/IRN/2/97 and A/TUR/14/98 were antigenically similar (Fig. 4A and B for titres and scaled titres, respectively).

TABLE 3 The percentage of r_1-values within the range 0 – 1 before and after scaling the titres.

Test	Before scaling titre	After scaling titre
VNT	78 %	93 %
NIT	83 %	94 %
LPBE	96 %	96 %

In the second analysis, we assumed that also strains A/ERI/2/98 and A/SUD/2/84 also were antigenically similar (Fig. 4C and D for titres and scaled titres, respectively). The 95% confidence interval of the area under the curve (AUC) of the ROC curve of the r_1-value based on the LPB-ELISA titres and scaled titres did not

include 0.5, hence showing a statistically significant result. However, for VNT and NIT and only when using the assumption that the strains in both lineages are antigenically similar, the AUC of the ROC curve of the r_1-values based on scaled NIT titres (AUC = 0.67; 95% confidence interval [CI], 0.56 to 0.79; Fig. 4D) had a 95% confidence interval that did not include 0.5. Therefore, the dashed line in Fig. 4D was the only statistically significant result for NIT in the ROC analysis.

Discussion

The study was performed to determine which serological test method provides the least variation within a group of vaccinated cattle and provides the best discrimination between vaccines. We selected 10 FMDV serotype A strains from 8 different lineages (Table 1). Sequencing of the VP1 region of the genome confirmed the identity of the strains used. Although Eritrea is not free from FMD, the cattle used in this study were obtained from a region where no outbreaks of FMD were recorded. All cattle were free of antibodies to non-structural proteins of FMD virus at the start of the study, implying that they had not been exposed to FMDV. Before vaccination the cattle were tested for neutralizing antibodies against 5 other serotypes (O, C, Asia 1, SAT-1 and SAT-2), with a negative result. Some cattle showed low antibody responses to NS protein post-vaccination, but no FMDV clinical signs were observed. The observed 58% inhibition is just above the cut-off of 50% in the test. It is probable the NS response was due to some remaining NS proteins present in the vaccine, as has been described before [34]. The low titres found before vaccination in some of the cattle in both VNT and LPB-ELISA is similar to the results reported for cattle in the Netherlands [35]. Using the 10 strains as vaccine antigens, we observed a good homologous neutralizing antibody response, with at least a 4-fold increase in titres at two WPV The small differences, with mean titres ranging from 1.6 - 2.0, were observed in the mean response of each group of vaccinated cattle tested against all test strains; this shows that the quality of the vaccines was similar. Therefore,, this experimental design in which cattle are vaccinated with vaccines containing a similar amount of antigen, offers an excellent opportunity to compare the different serological test methods.

TABLE 4 P values of the pairwise t test results from the serological tests in which the ANOVA gave a significant difference between vaccine strains.

Vaccine Strain	**Vaccine Strain**								
	A_{10}/Holland/42	A_{22}/IRQ/24/64	A/ERI/2/98	A/ETH/13/2005	A/IRN/2/97	A/KEN/12/2005	A/MAU/1/2006	A/SUD/2/84	A/TUR/20/2006
Observed NIT titres									
A_{22}/IRQ/24/64	1								
A/ERI/2/98	1	1							
A/ETH/13/2005	1	0.672	1						
A/IRN/2/97	1	1	1	1					
A/KEN/12/2005	1	1	1	1	1				
A/MAU/1/2006	1	1	1	***0.006***	1	1			
A/SUD/2/84	1	1	1	1	1	1	1		
A/TUR/20/2006	1	1	1	1	1	1	0.056	1	
A/TUR/14/98	1	1	1	1	1	1	1	1	1
Scaled NIT titres									
A_{22}/IRQ/24/64	1								
A/ERI/2/98	1	1							
A/ETH/13/2005	0.967	0.274	1						
A/IRN/2/97	1	1	1	1					
A/KEN/12/2005	1	1	1	0.828	1				
A/MAU/1/2006	0.261	0.938	0.064	***0.000***	0.074	0.680			
A/SUD/2/84	1	1	1	1	1	1	***0.034***		
A/TUR/20/2006	1	0.682	1	1	1	1	***0.000***	1	
A/TUR/14/98	1	1	1	0.740	1	1	0.373	1	1
Observed LPB-ELISA titres									
A_{22}/IRQ/24/64	1								
A/ERI/2/98	0.975	0.087							
A/ETH/13/2005	0.122	***0.005***	1						
A/IRN/2/97	1	1	1	1					
A/KEN/12/2005	1	1	1	1	1				
A/MAU/1/2006	1	1	***0.008***	***0.000***	0.333	0.690			
A/SUD/2/84	1	1	1	0.135	1	1	1		
A/TUR/20/2006	0.110	***0.005***	1	1	1	1	***0.000***	0.121	
A/TUR/14/98	1	1	0.299	***0.027***	1	1	1	1	***0.024***
Scaled LPB-ELISA titres									
A_{22}/IRQ/24/64	1								
A/ERI/2/98	0.365	***0.032***							
A/ETH/13/2005	***0.033***	***0.001***	1						
A/IRN/2/97	1	1	1	0.810					
A/KEN/12/2005	1	1	1	0.718	1				
A/MAU/1/2006	1	1	***0.001***	***0.000***	0.09	0.300			
A/SUD/2/84	1	1	0.349	***0.030***	1	1	1		
A/TUR/20/2006	***0.039***	***0.002***	1	1	0.814	0.717	***0.000***	***0.035***	
A/TUR/14/98	1	1	0.152	***0.011***	1	1	1	1	***0.014***

The major difficulty in interpreting the results with both VNT and NIT was the difference in the level of neutralization observed using different virus test strains. Some virus test strains like A10/Holland/42 were neutralized easily, with high titres in the homologous and heterologous serum samples. For this reason, we also evaluated scaled titres.

Even though a difference in titre level was observed, the mean response within the groups of 5 vaccinated cattle was consistent, resulting in a small range of observed SEMs in all the 3 test methods. The range of observed SEM, however, was lowest in the LPB-ELISA, showing that the smallest amount of variation is observed with the LPB-ELISA results. Lower variation in the LPB-ELISA compared to the VNT has also been reported by Van Maanen and Terpstra [36]. After scaling the range of the SEM observed in the groups of cattle increased, but the lowest maximum SEM was still found using the LPB-ELISA, but after scaling, the results obtained with the NIT were similar.

The titre differences between test strains resulted in r_1-values that were often higher than one. This was mainly observed with the seven test strains that had high homologous neutralizing antibody titres. After scaling, lower r_1-values were calculated; this is consistent with the fact that the strains were obtained from 8 distinct genetic lineages, from which we assume that they are also antigenically different. Using the LPB-ELISA more significant differences between vaccines could be observed by ANOVA, followed by pairwise t tests. The four strains from 2 genetic lineages were still similar in the pairwise t-test (Table 4). Not only were the LPB-ELISA results more capable to distinguish between vaccines, they are also more reproducible, as they are not influenced by variations in tissue culture susceptibility. Although neutralizing antibodies are often considered in relation to protection, LPB-ELISA titres are also correlated with protection [36, 37]. Therefore, LPB-ELISA seems to perform better than the neutralization tests to discriminate between vaccines.

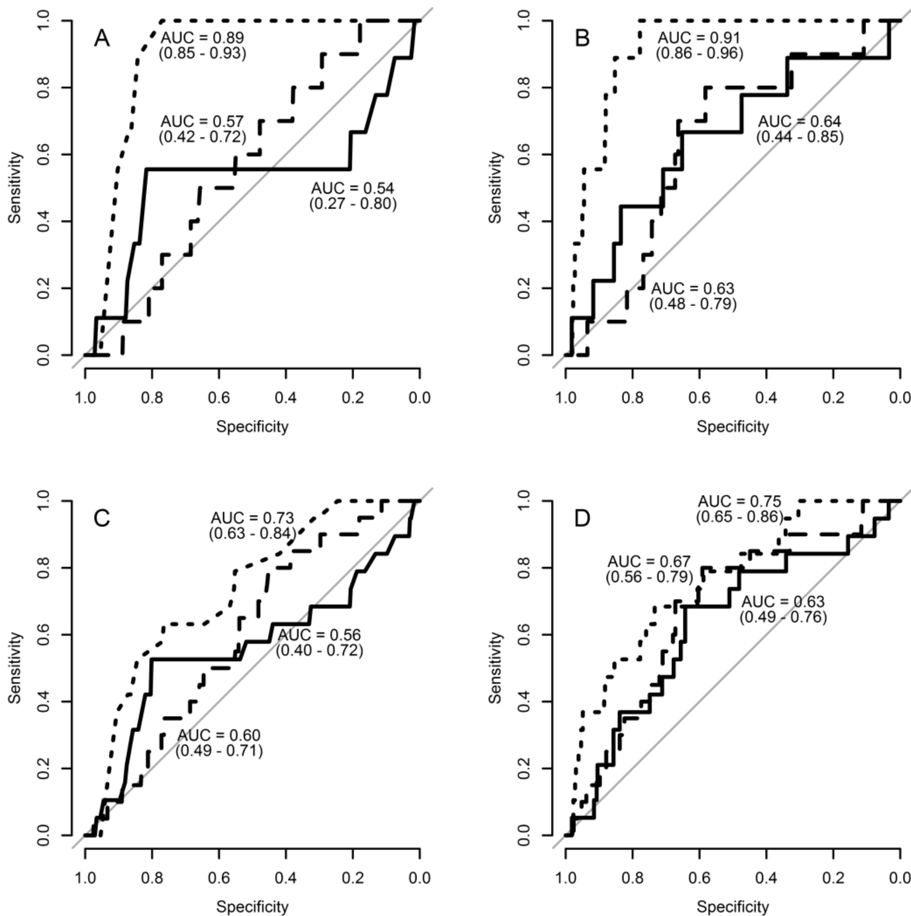

Figure 4. ROC curves of the r_1-value titres of the VNT (solid line), NIT (dashed line) and LPB-ELISA (dotted line). In the graphs, the area under the curve (AUC) is given, with the 95% confidence interval, in the parenthesis. (A and B) strains A/IRN/2/97 and A/TUR/14/98 are assumed antigenically the same, using the observed titres (A) and scaled titres (B). (C and D) strains A/IRN/2/97 and A/TUR/14/98 as well as A/ERI/2/98 and A/SUD/2/84 are assumed to be antigenically the same, using the observed titres (C) and scaled titres (D).

To further build on the assumption that genetically related strains should also be antigenically related, we did an ROC analysis using the $r_1$values derived from the different tests before and after scaling. An ROC curve with an AUC of 0.5 would indicate that the test does not differentiate the differences defined by the gold standard (in our case, the sequence data). Before scaling only the r_1-values based on titres from the LPB-ELISA had an AUC of the ROC curve that was significantly different from 0.5.

Therefore, without scaling the titres, the LPB-ELISA is the only test that can discriminate between strains that are genetically different, and that are therefore also assumed to be antigenically different. After scaling, the AUC of the ROC curve of the NIT, when assuming the strains in both the Iran-96 and the G-IV lineage were similar, and was significantly different from 0.5. This shows that scaling can help improve the NIT to discriminate strains antigenically, but the LPB-ELISA is the best test for this.

In our analysis, we used the r_1-value as a measurement of the antigenic relationship, but the reason we do the analysis is to see whether a vaccine can protect against infection. Previous studies have shown that there is a strong relationship between antibody response and protection [36-38]. The precision of this relationship can be improved slightly by including other immunological techniques [39, 40]. However, the degree of the titre that relates with protection is not the same in different strains. Therefore, the use of an r_1-value to estimate protection against challenge is not logical, because the difference in titre needed for protection is not taken into consideration. Hence, if the objective is to choose a vaccine that will best protect against infection with a field isolate, it is better to look for the vaccine that induces the highest titre against the field virus strain instead of looking at the r_1-value. In a recent study that analysed the serological results of cross-protection tests [22], it was shown that the r_1-value of a serum sample from a vaccinated and heterologous-challenged cow did not predict protection, but the serum titre against the challenge strain in the VNT did. In fact it was shown that analysis of the humoral immune response using IgG_1 and IgG_2 ELISAs even improved this prediction [22]. This also indicates that VNT or ELISA titres are probably more important in a prediction of protection than is the r_1-value.

As stated before, the cattle serum samples used in this study are unique, as they were produced using the same amount of antigen in each vaccine batch. These cattle sera can be very valuable for further research on antigenic differences of new serotype A FMDV isolates, as they cover a large part of the genetic differences observed in Europe, Middle East and Africa. We conclude that the LPB-ELISA, under the conditions in the present study, is the best test for detecting antigenic differences between FMDV strains. The smaller the variability within a vaccinated group of animals, the greater is its discriminatory capacity in the neutralization tests. However, one should realize that in our study, we used strain specific guinea-pig and rabbit

antibodies. Nevertheless, neutralizing antibody tests also have their place, as VNT titres are closely related to protection [36, 38], but in that case the use of r_1-values is not feasible, and the suitability of a vaccine strain should be evaluated on the titre against the outbreak strain. The higher the titre, the better suited the vaccine.

Acknowledgements

This work was supported by the International Atomic Energy agency (IAEA) grant C6/ERI/07010, Vienna, Austria. The production of the guinea pig and rabbit antisera was supported by the EuFMD.
We thank The Pirbright Institute, United Kingdom, for providing FMDV type A strains. We also thank Tsghe Kfleyesus, Ghebremichael G.Egziabhier and the late Tesfu Yemane (animal health staff at MoA, Asmara, Eritrea) for assisting in sample collection during animal experiments. We also thank Boonstra Christine for sequencing the FMDV serotype A strains, and the FAO Representative Office in Eritrea for providing internet access during the study period.

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Chapter 6

No significant differences in the breadth of the foot-and-mouth disease serotype A vaccine induced antibody responses in cattle, using different adjuvants, mixed antigens and different routes of administration

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Vaccine 2014: 32(41):5330-5336

DOI:10.1016/j.vaccine.2014.07.033

Abstract

Inactivated whole virus foot-and-mouth disease (FMD) vaccines are used worldwide for protection against FMD, but not all vaccines induce protection against all genetic variants of the same FMD virus serotype. The aim of this study is to investigate whether the “breadth” of the antibody response against different strains of the same FMD virus serotype in cattle could be improved by using a different adjuvant, a mix of antigens and/or different routes of administration. To this end, six groups of five cattle were vaccinated with different FMD virus serotype A strain vaccines formulated with Montanide ISA 206 VG adjuvant. Antibody responses for homologous and heterologous cross-reactivity against a panel of 10 different FMD virus serotype A strains were tested by a liquid-phase blocking ELISA. Results of cattle vaccinated with ISA 206 VG adjuvanted vaccine were compared with results obtained in a previous study using aluminium hydroxide-saponin adjuvant. No significant effect of adjuvant on the breadth of the antibody response was observed, neither for mixing of antigens nor for the route of administration (subcutaneous vs. intradermal). Comparison of antigen payload, however, increased both homologous and heterologous titres; a 10-fold higher antigen dose resulted in approximately four times higher titres against all tested strains. Our study shows that breadth of the antibody response depends mainly on the vaccine strain; we therefore propose that, for vaccine preparation, only FMD virus strains are selected that, among other important characteristics, will induce a wide antibody response to different field strains.

Keywords: Foot-and-mouth disease virus; Vaccine; Adjuvant; Mixing antigen; Administration route; Antigen payload; Breadth

Introduction

Foot-and-mouth disease (FMD) is an economically important disease of cloven-hoofed animals. In FMD endemic countries, vaccination of susceptible animals is the best option in the disease control programme. Match between vaccine and outbreak strain is considered very important for a successful FMD vaccination control programme [1-4]. In a previous study [5], it was shown that FMD virus (FMDV) serotype A vaccines all induced a high homologous antibody response but the heterologous antibody response differed. Vaccine strains inducing a high homologous and low heterologous response were considered as narrow antibody inducers while those vaccine strains that induced good antibody titres to both homologous and heterologous strains tested were considered as a better breadth of antibody inducers. In vivo cross-protection studies are very costly and not animal friendly. Many studies have shown that there is a strong relation between FMDV antibody response and protection [6, 7]. Therefore, cross-reactions in serological tests are often used as indicators of the cross-protective capacity of a vaccine [8].

For a vaccine, it would be an asset if it would induce protection against different subtypes within a serotype. In our previous study we showed that the choice of antigen influences the breadth of the antibody response. Inactivated FMDV antigen is poorly immunogenic and it is therefore necessary to employ adjuvants to enhance the immune response [8]. In the past, adjuvants used in FMD vaccines were optimised mainly using the antibody titre against the homologous virus as a criterion and rarely the response to heterologous strains of the same serotype [9], so information on the influence of adjuvant on the breadth of the antibody response is missing [10, 11]. Another potential option to increase the breadth the antibody response is the mixing of different antigens, which is normally performed to protect against different serotypes. It might also increase the breadth of the antibody response within a serotype. A third potential option for increasing the breadth of the antibody response is by changing the administration route. In a study, it was shown that intradermal (ID) vaccination (with Al(OH)3-saponin as adjuvant) of cattle with trivalent vaccine induced more often a titre against two serotypes not included in the vaccine compared to cattle that were subcutaneously (S/C) vaccinated [12]. Furthermore, it has been shown that protection increases with increasing antigen payload in an FMD vaccine [13, 14] and also a higher

antigen dose will improve cross-protection [14]. In the present study, serological cross-reactions were examined to 10 different serotype A strains, not the cross-protection.

As there are several potential options to increase the breadth of the antibody response we wanted to investigate whether the formulation, antigen composition, antigen payload and administration route of a FMD vaccine had any influence on the breadth of the antibody response in cattle.

Materials and methods

FMDV serotype A strains

Different serotype A FMDV (n = 10) strains that were isolated in Africa, the Middle East, and Europe were selected from various genetic lineages [5]. The 10 strains were A/SUD/2/84, A/ERI/2/98, A/KEN/12/2005, A/ETH/13/2005, and A/MAU/1/2006, which were received from the OIE/FAO World Reference Laboratory for FMD (The Pirbright Institute, United Kingdom), and A10/Holland/42, A22/IRQ/24/64, A/IRN/2/97, A/TUR/14/98 and A/TUR/20/2006, obtained from Central Veterinary Institute (CVI), (Wageningen UR, Lelystad, The Netherlands). For this study strains A/MAU/1/2006 and A/KEN/12/2005 were selected because of their high homologous and low heterologous titres observed in a previous experiment. They were consequently marked as strains with a narrow response. In the same experiment, strain A/TUR/20/2006 showed similar titres against homologous virus and various heterologous viruses and was therefore marked as a strain with a broader response [5].

Vaccine (antigen production)

The antigens used in this study were noncommercially produced at the CVI, for these particular studies [5]. The aqueous vaccines used in the previous study [5] were formulated on-site using 2% aluminium hydroxide (Alhydrogel Ph. Eur. Brenntag Biosector, Frederiksund, Denmark) and 10% (wt/vol) saponin (Quil-A solution in phosphate buffered saline) as adjuvant. The oil vaccines used in this study were formulated from the same antigen batch, which was kept frozen till formulation. Formulation was performed on-site with the ready-to-use oil adjuvant Montanide ISA 206 VG (Seppic, France), according to the producer’s instructions. In both types of vaccines the total antigen payload was 10 µg per 2 ml dose. The mixed antigens were

made by combining A/MAU/1/2006 and A/KEN/12/2005 5 µg each, in a final volume of 2 ml per dose, formulated with Montanide ISA 206 VG.

Vaccination

The vaccination with $Al(OH)_3$-saponin adjuvanted vaccine was performed in 2010 and the experiment has been reported before [5], and the vaccination with the Montanide ISA 206 VG vaccine in 2011. The cattle used in both experiments were unvaccinated, Eritrean local indigenous zebu breed (Bos indicus), 10-18 months of age. Before buying the cattle they were bled twice and the serum samples were tested for presence of anti-FMDV nonstructural protein (NSP) antibodies using the FMD nonstructural enzyme-linked immunosorbent assay (NS-ELISA) (PrioCHECK, Schlielieren-Zurich, Switzerland) [15]. Only cattle with a negative response were acquired. The cattle were housed in stables at the National Veterinary Laboratory, Asmara, Eritrea. These animals were brought from regions known to be free from FMD without vaccination. The cattle for the Montanide ISA 206 VG vaccine were randomly allocated to 6 groups of 5 cattle plus 1 group of 2 animals, serving as non-vaccinated controls. Four groups of cattle (Groups 1M-4M, Table 1) were vaccinated subcutaneously with 2 ml of vaccine in the middle of the neck. The remaining two groups (Groups 5M and 6M, Table 1) were vaccinated with 0.2 ml of vaccine using either the subcutaneous (SC) or the intradermal (ID) route. The SC vaccination was done using a standard needle (20 G, 1½"; 0.9 mm diameter, 38 mm length). The ID vaccination was done using a short needle (26 G 3/8"; 0.45 mm diameter, 3–4 mm length) bevel edge outwards and graduated syringe, inserted obliquely into the deeper layers of the skin. The 0.2 ml dose was injected and confirmed by palpating a small pea-like swelling at the injection site. Both SC and ID vaccinations were injected in the middle of the neck. Cattle were bled at 0, 7, 14, 21 and 28 days post vaccination (DPV) for serology. The ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines [16] were followed in this report.

FMDV NS ELISA

Prior and after vaccination, all sera were tested for presence of anti-FMDV NSP antibodies using FMD NS-ELISA (PrioCHECK) [15] according to the producer's instructions.

Table 1. Composition of the experimental groups, 5 cattle per group.

Group	Route	Adjuvant	Dose	FMDV strain
1A	SC	$Al(OH)_3$-saponin	10 µg in 2 ml vaccine	A/TUR/20/2006
2A	SC	$Al(OH)_3$-saponin	10 µg in 2 ml vaccine	A/MAU/1/2006
3A	SC	$Al(OH)_3$-saponin	10 µg in 2 ml vaccine	A/KEN/12/2005
1M	SC	Montanide ISA 206 VG	10 µg in 2 ml vaccine	A/TUR/20/2006
2M	SC	Montanide ISA 206 VG	10 µg in 2 ml vaccine	A/MAU/1/2006
3M	SC	Montanide ISA 206 VG	10 µg in 2 ml vaccine	A/KEN/12/2005
4M	SC	Montanide ISA 206 VG	5 µg for each antigen in 2 ml vaccine	A/MAU/1/2006 and A/KEN/12/2005
5M	SC	Montanide ISA 206 VG	1 µg in 0.2 ml vaccine	A/MAU/1/2006
6M	ID	Montanide ISA 206 VG	1 µg in 0.2 ml vaccine	A/MAU/1/2006

LPB-ELISA

The liquid-phase blocking ELISA (LPB-ELISA) was performed as described before [5]. Specific rabbit sera for coating and the guinea pig sera for detection were produced for 10 FMDV type A test strains [5]. Antigens used in the LPB-ELISA were prepared from the 10 FMDV type A test strains as described before [5]. The antibody titres were expressed as the log10 of the reciprocal of the final dilution of serum giving 50% of the mean OD value recorded in the maximum signal control wells (two wells with ELISA buffer instead of serum). For the calculations sera negative at a 1/10 dilution (titre <1.0) were assigned a titre of 0.7.

Data analysis

For each vaccine group, the mean titre and the standard error of the mean (SEM) were calculated and plotted. The response was considered broader if the homologous titre remained the same and some or all heterologous titres were higher. To test differences between the vaccine groups, we used a multi-way ANOVA. In the ANOVA, the LPB-ELISA titre was used as response variable. Adjuvant, Vaccine strain, administration route, antigen payload and test strain were used as explanatory variables. We used a forward selection strategy using the AIC (Akaike's information criterion) for selection. The difference between models was tested using the likelihood ratio test. All statistical

analyses were performed using R version 3.02 [17] and for significance $p \leq 0.05$ was used.

Results

Post-vaccination antibody response

All animals used in this study were negative for anti-FMDV NSP antibodies. Moreover, after vaccination no antibody response to the NS protein was detected. In some animals, vaccinated with a full dose of Montanide ISA 206 VG adjuvanted vaccine, a local tissue reaction was observed with a big lump (>10 cm) formed at the injection site. This reaction was not observed in our first experiment using the $Al(OH)_3$-saponin formulated vaccine [5]. The highest mean homologous antibody titres were found in Group 1M (A/TUR/20/2006) and 3M (A/KEN/12/2005), reaching levels up to 2.95 and 2.88 at 28 and 21 DPV respectively (Fig. 1). At 7 DPV the homologous response of the Montanide ISA 206 VG adjuvanted vaccines were higher compared to the homologous response of the Al $(OH)_3$-saponin vaccines (Fig 1).

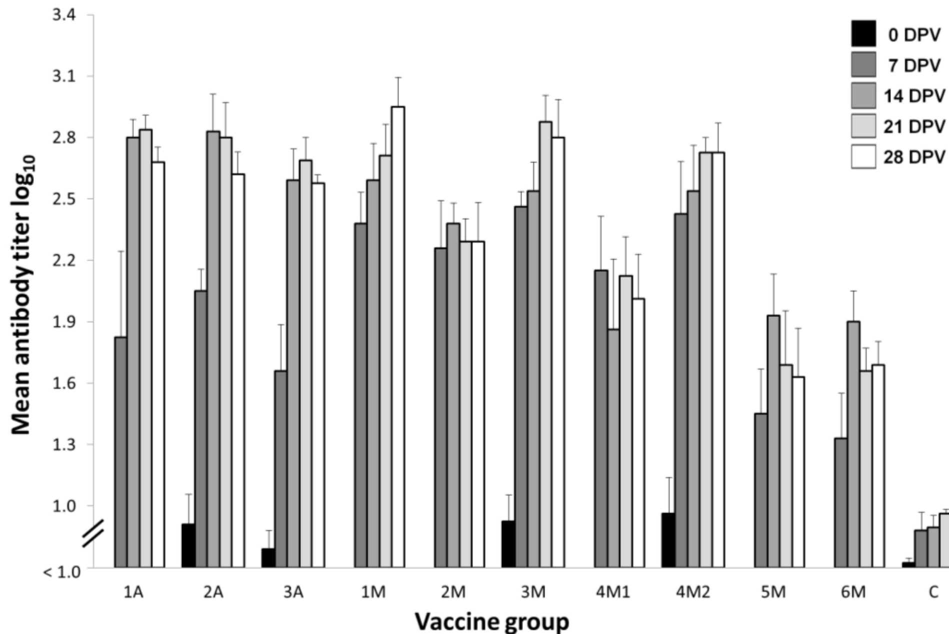

Figure 1. Mean homologous antibody titres (±SEM) observed in the LPB-ELISA from cattle sera vaccinated with $Al(OH)_3$-saponin and Montanide ISA 206 VG formulated vaccine at different time points after vaccination. Groups 1A: A/TUR/20/2006, 2A: A/MAU/1/2006, 3A: A/KEN/12/2005 formulated with $Al(OH)_3$-saponin and Groups 1M: A/TUR/20/2006, 2M: A/MAU/1/2006, 3M: A/KEN/12/2005, Groups 4M1: mixed vaccine containing A/MAU/1/2006 and A/KEN/12/2005 tested against A/MAU/1/2006 strain, 4M2: same mixed vaccine tested against A/KEN/12/2005 strain, Group 5M: A/MAU/1/2006 for antigen payload (1µg/dose), Group 6M: A/MAU/1/2006 intradermal route, C: Controls (for these the mean and SEM against all 10 test strains was used).

Comparison of vaccine adjuvants ($Al(OH)_3$-saponin and Montanide ISA 206 VG)

The 21 DPV mean homologous titres induced by the $Al(OH)_3$-saponin and Montanide ISA 206 VG formulated A/KEN/12/2005, A/MAU/1/2006 and A/TUR/20/2006 vaccines are shown in Table 2. The overall mean homologous titre induced was the same for the three vaccine strains (mean titre 2.7) using both adjuvants. The heterologous vaccine induced titres were in the multi-way ANOVA significantly correlated with the test strain ($p \ll 0.001$), but no significant difference was found in heterologous titres induced by the two different adjuvants (Table 2). The differences between different adjuvants were variable; the mean homologous titre for A/MAU/1/2006 were higher when using $Al(OH)_3$-saponin adjuvant, and the mean homologous titre for A/KEN/12/2005 and A/TUR/20/2006 were higher when using ISA 206 VG adjuvant (Table 2). In the

heterologous response the differences were the other way around, so no consistent difference in response when comparing $Al(OH)_3$-saponin and ISA 206 VG adjuvants.

Table 2. Mean titres induced by the various $Al(OH)_3$-saponin and Montanide ISA 206 VG adjuvants.

Vaccine strain	Homologous titres				Heterologous titres			
	$Al(OH)_3$-saponin		ISA 206 VG		$Al(OH)_3$-saponin		ISA 206 VG	
	Mean titre	SEM	Mean titre	SEM	Mean titre	SEM	Mean titre	SEM
A/KEN/12/2005	2.7	0.1	3.0	0.2	2.0	0.1	1.9	0.09
A/MAU/1/2006	2.8	0.2	2.3	0.1	1.7	0.06	2.0	0.05
A/TUR/20/2006	2.5	0.4	2.8	0.2	2.1	0.09	1.9	0.07

Comparison of the mixed and single antigen FMD vaccines

The LPB-ELISA titres, obtained with the bivalent (mixed) A/KEN/12/2005 and A/MAU/1/2006 vaccine as well as the monovalent vaccines are shown in Fig. 2. The mean homologous LPB-ELISA titres for the monovalent vaccines were 3.0 (SEM = 0.2) and 2.3 (SEM = 0.1) for A/KEN/12/2005 and A/MAU/1/2006 vaccine respectively. The mixed A/KEN/12/2005 and A/MAU/1/2006 vaccine had a mean LPB-ELISA titre of 2.4 (SEM = 0.4) and 1.9 (SEM = 0.3) using the test strains of A/KEN/12/2005 and A/MAU/1/2006 respectively. Therefore, for both monovalent A/KEN/12/2005 and A/MAU/1/2006 vaccines, the homologous antibody had a tendency to be higher compared to the bivalent vaccine response, but they were not significantly different.

The mean LPB-ELISA titres against the other 8 heterologous strains were 1.9 (SEM = 0.1), 2.0 (SEM = 0.05) and 1.9 (SEM = 0.09) for the monovalent A/KEN/12/2005 and A/MAU/1/2006 vaccines and the bivalent mixed A/KEN/12/2005 and A/MAU/1/2006 vaccine respectively. Therefore, the difference in titres induced by the three different vaccines was not significant. In the multi-way ANOVA the variation in titres was significantly explained (p = 0.02) by the test strains, but no significant difference between the various vaccines.

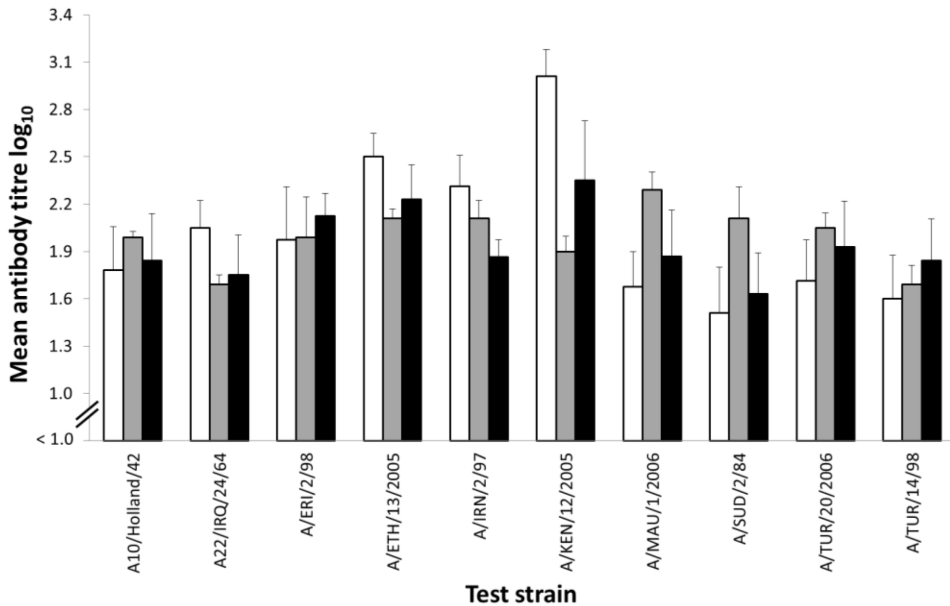

Figure 2: Comparison of mean antibody titres (± SEM) of 21 DPV sera induced by ISA 206 VG adjuvanted vaccine containing A/KEN/12/2005 (white bars) or A/MAU/1/2006 (grey bars) or mixed antigens (A/MAU/1/2006 and A/KEN/12/2005) (black bars). All sera were tested by LPB-ELISA.

Comparison of antigen payload using the A/MAU/12/2005 vaccine

The mean homologous LPB-ELISA titres, were 1.7 (SEM = 0.2) and 2.3 (SEM = 0.1) in cattle vaccinated with 1 and 10 µg antigen respectively. These homologous titres did not differ significantly ($p = 0.07$). The analysis of the heterologous sera showed that LPB-ELISA titres obtained with the A/MAU/1/2006 containing 10 µg of antigen per dose were significantly ($p \ll 0.001$) higher (0.6 log10) than the vaccine containing 1 µg per dose for all test strains used (Fig. 3).

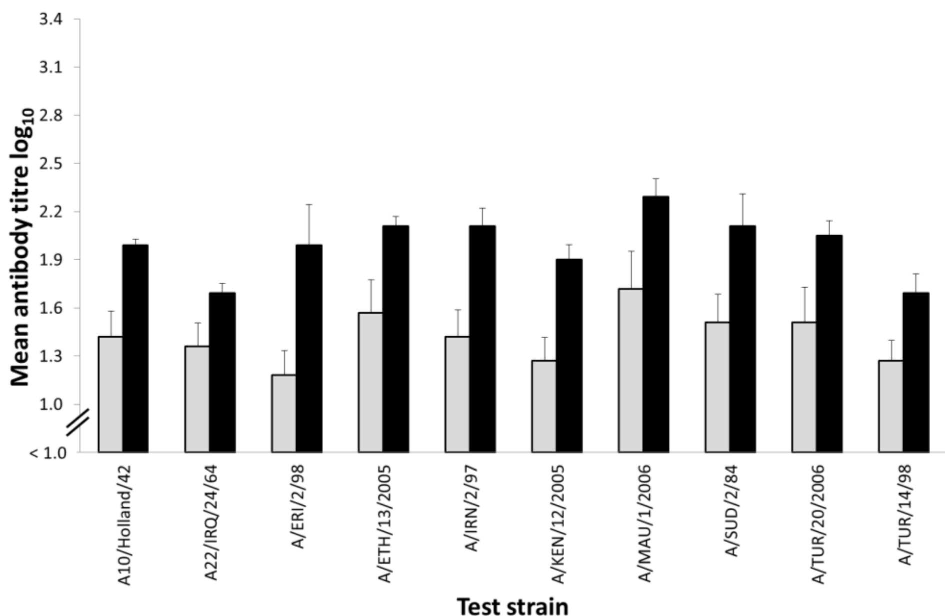

Figure 3. Comparison of mean antibody titres (± SEM) of 21 DPV sera using different antigen payloads. Sera of cattle vaccinated with A/MAU/1/2006 (1 µg/dose) (grey bars) and A/MAU/1/2006 (10 µg/dose) (black bars) were tested by the LPB-ELISA.

Comparison of vaccine administration routes

The mean homologous LPB-ELISA titres against A/MAU/1/2006, with the vaccine containing 1 µg of A/MAU/1/2006 antigen per dose were 1.7 for both the subcutaneous (SEM = 0.2) as well as intradermal (SEM = 0.1) route. Therefore, no significant differences in homologous titres observed between subcutaneous and intradermal vaccination (Fig. 4). The mean LPB-ELISA titres against all heterologous strains was higher (1.4, SEM = 0.05) when using the subcutaneous route compared to the intradermal route (1.2, SEM = 0.05). The differences between subcutaneous and intradermal vaccination varied for different strains. In Fig. 4 we ordered the strains according to this difference. In the multi-way ANOVA, however, the difference in response, was significant only for route ($p = 0.004$) not for the test strain used.

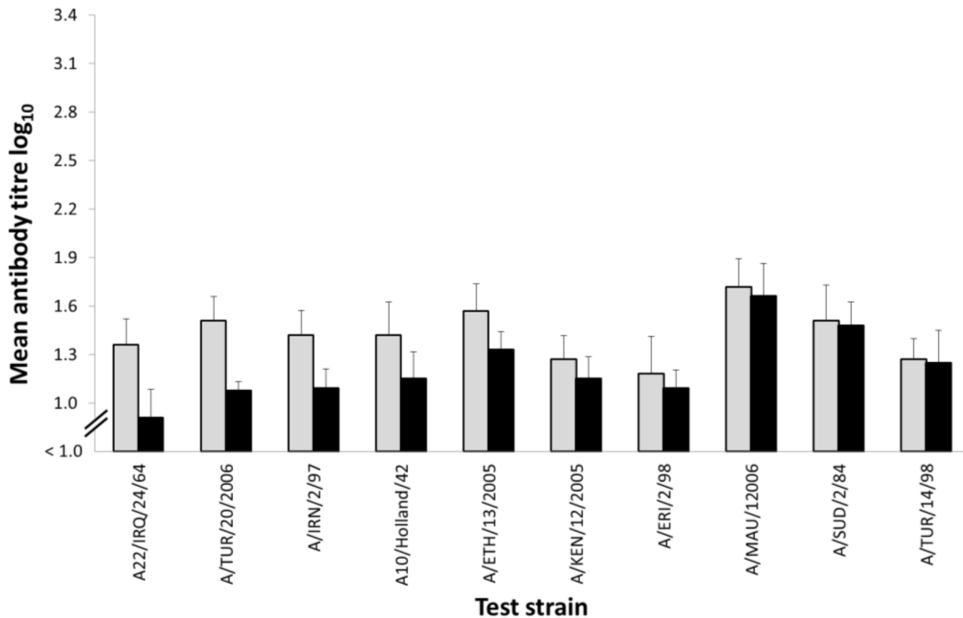

Figure 4. Mean antibody titres (± SEM) of 21 DPV sera determined by the LPB-ELISA, comparing administration routes; SC (grey bars) versus ID (black bars). In both routes the cattle were vaccinated with 1 µg per 0.2 ml dose. The strains are ordered based on the difference in response.

Discussion

The aim of this study was to investigate whether the breadth of a vaccine induced antibody response against FMDV in cattle could be influenced by comparing: (i) two adjuvants, i.e. Montanide ISA 206 VG and $Al(OH)_3$-saponin, (ii) a vaccine containing two different antigens (bivalent) with a single antigen (monovalent) vaccine, (iii) antigen payload, and (iv) the subcutaneous route with the intradermal administration route. The cattle used in this study all had negative results in the FMD NS ELISA before and after vaccination. A quick and strong homologous antibody response was observed in all vaccinated animals. The post-vaccination tissue reactions observed on the injection site in some cattle vaccinated with Montanide ISA 206 VG formulated vaccines was not observed in the previous study [5] in which cattle were vaccinated with the $Al(OH)_3$-saponin adjuvanted vaccine. Similar tissue reactions after vaccination with oil emulsion vaccines have been reported before [18-20].

In our study, we compared the homologous and heterologous responses between two or three different treatments. If the homologous response was the same and the heterologous response was higher, then it was considered as an improvement in the breadth of the immune response. No significant differences were found when comparing $Al(OH)_3$-saponin with Montanide ISA 206 VG formulated vaccines neither in the height of the homologous antibody response nor in the breadth of the heterologous antibody response. The difference in LPB-ELISA titres was only explained by the difference in test strain and not by the adjuvant or vaccine used. Therefore, based on these results there is no reason to prefer one of the adjuvants over the other. This is supported by previous observations made on comparison of these two adjuvants in cattle or sheep [9, 21-26], although there have also been a number of reports that claim superiority of oil-based adjuvants to aqueous ($Al(OH)_3$-saponin) vaccines in cattle, pigs and goats [20, 25, 27]. For pigs however, the difference between $Al(OH)_3$-saponin and oil adjuvant was clear [8, 22]. For ruminants, the differences, if detected, are probably based on the specific formulation and not on the difference between oil-based and aqueous adjuvants.

In the present study, the mixing of two serotype A antigens with a narrow response did not show an effect on the heterologous antibody response to the eight strains not included in the vaccines when compared to a vaccine based on the single antigen. For example, adding A/KEN/12/2005 antigen to the A/MAU/1/2006 vaccine improved the response to A/KEN/12/2005 but the titres against more heterologous strains did not change significantly. In the FMD DISCONVAC project (unpublished results), it was reported that mixing of A/IND/7/82 and A/IND/17/82 improved the serological response against A/RAJ/21/96 but in that study they measured the serological response only against one other strain. We examined the improvement of antibody response against eight other strains. We observed that the bivalent vaccine in comparison with one monovalent vaccine in some strains improved the antibody response but we found the opposite when comparing it with the other monovalent vaccine. This suggests that more than one other strain should be used to observe the effect on the breadth of the antibody response. Our previous study showed that some vaccine strains have a wider antibody response than others [5]. Based on these results, it is better to select vaccine strains with a wider antibody response instead of mixing antigens with a narrow response.

Cattle vaccinated with an antigen payload of 10 µg per dose formulated with Montanide ISA 206 VG developed approximately 4-fold, higher in both homologous and heterologous LPB-ELISA antibody titres when compared to the vaccine containing 1 µg per dose. The difference was only significant in the comparison of heterologous LPB-ELISA antibody titres, probably due to a lack of power when comparing the homologous titres. We do not consider this as an increase in the breadth of the response because the improvement in the homologous titre was similar. The LPB-ELISA titre (1.7) induced by the vaccine containing only 1 µg of antigen could be sufficient for protection when compared with earlier publications on the correlation between LPB-ELISA titre and protection [9, 28, 29]. But, in studies done in South-America [30], higher LPB-ELISA titres were needed for protection. Our results confirm other studies that have shown that higher payloads induce higher antibody responses [31]. In our studies we used 10 µg of antigen to make sure that we could measure the antibody response to heterologous strains. Based on the LPB-ELISA titres an amount of antigen lower than 10 µg could still protect cattle, but this need to be confirmed by cattle protection tests.

Comparison of the two administration routes (SC and ID) in cattle vaccinated with Montanide ISA 206 VG formulated vaccines did not show a significant effect on the LPB-ELISA titres. Although the serological response was lower when the vaccine was applied intradermally, in previous studies in pigs, a low antibody response after ID vaccination was also seen [32], but the pigs were still protected against a challenge. Therefore, it is likely that the relation between antibody response and protection is different for different administration routes [32]. Therefore, this non-significant lower response after ID vaccination does not mean that the vaccine applied via this route would not protect, this has to be tested by challenging the animals. In a recent study, vaccine has shown good protection against homologous challenge after intradermal vaccination in cattle, even when using lower doses [33]. The antibody response in our study was lower when using intradermal vaccination when comparing it with subcutaneous vaccination. Based on the definition this would mean a decrease in the breadth of the antibody response when using intradermal vaccination. But we did not challenge, so the cattle might have been protected as the relation between antibody response and protection can be different when using different application routes [32].

In our study we used LPB-ELISA titres because our previous study [5] showed that these titres are more reproducible and allow better discrimination between vaccine strains compared to the neutralising titres. Although in many studies r_1-values are used for comparison of vaccine strains we chose to analyse only the titres, because r_1-values are not normally distributed and therefore not valid for such an analysis. Furthermore, protection is better related with antibody response than with r_1-value [34]. We compared the titres of cattle vaccination studies using $Al(OH)_3$-saponin and Montanide ISA 206 GV adjuvants that were not performed at the same time. Performing the experiments 1 year apart could have led to a systematic difference between the experiments, which would then result in the wrong conclusion that the response when using $Al(OH)_3$-saponin was different from Montanide ISA 206 VG. We did, however, not find differences between $Al(OH)_3$-saponin and Montanide ISA 206 VG, and therefore believe that the comparison is valid.

In our study we used indigenous zebu breed. The breed might have an influence on the immune response to specific epitopes, but we test for the breadth in a polyvalent response was similar between different cows in two different experiments, therefore we assume that our findings can be extrapolated to all cattle, and probably to all FMDV susceptible species.

The breadth of the antibody response of FMD vaccine is considered important as outbreaks are mostly caused by strains that are not homologous to the vaccine strains. In this study, we have found no significant effect of choice of adjuvant, mixing of antigens, and/or route of administration on the breadth of the antibody response. Therefore, in addition to other criteria, like e.g. growth in cell culture and antigen stability, the breadth of the serological response of a vaccine strain should be included in the process of vaccine strain selection.

Acknowledgements

This work was funded by the International Atomic Energy agency (IAEA); grant C6/ERI/07010, Vienna, Austria as well as the Ministry of Economic Affairs in the Netherlands; grant WOT-01-003-11. The production of the guinea-pig and rabbit antisera was supported by the EuFMD.

We thank WRLFMD, The Pirbright Institute, UK for providing FMDV type A strains. We are grateful to Tsghe Kfleyesus, Gebremichael G. Egziabhier and the late Tesfu Yemane, Laboratory and Animal Health staff, Ministry of Agriculture, Asmara, Eritrea for assisting in sample collection during animal experiment. We are indebted to the FAO, Representative Office in Eritrea for providing internet access during the study period, and are grateful to Dr Amos Ssematimba for his contribution in editing the manuscript.

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Chapter 7

General Discussion

Introduction:

Foot-and-mouth disease virus (FMDV) was the first animal pathogen to be identified as a virus, and today, more than a century later, it remains at the forefront of major animal diseases. It is a very contagious disease and affects cloven-hoofed animals, mostly cattle, swine, sheep and goats [1-4]. Despite mortality rates being generally low (estimated 5%) FMD severely decreases livestock productivity and trade [5-7]. It is considered an economically very important disease of farm animals. Among other control measures, vaccination is one that should be considered. The genetic variability and antigenic diversity of FMD virus, however, has implications for vaccine design and disease control [8]. Therefore, before the use of a vaccine, its quality and matching capacity need to be carefully evaluated and assessed. To be able to advice on the best control measures in Africa, information on transmission of FMDV, circulating FMDV strains in Africa and how to match vaccines to circulating strains is essential. In this thesis these issues are addressed.

FMDV transmission and serotype/topotype distribution in Africa

In Africa, there is a lack of data on FMDV transmission and FMDV distribution. Some countries do not report or submit samples to diagnostic and reference laboratories during outbreaks of FMDV. In Africa, there were no comprehensive region based studies done on the role of animal husbandry and trade practices on FMDV transmission. The role and involvement of wildlife in the epidemiology of FMDV, however, had been studied in the southern region of Africa [9-16], but only on a limited level in other parts of Africa. We therefore reviewed the literature on the role of animal husbandry, trade and wildlife on the distribution and transmission of FMDV in Africa (Chapter 2). The outcome of this review study indicated that, the distribution of FMDV serotype/topotype in Africa can be explained by the animal husbandry system and trade practices and occurrence of infected African buffalo. In North, West, Central and East Africa, livestock movement is one of the main risk factors involved in the transmission of FMDV [17]. The role of African buffalo in FMDV SAT serotypes transmission to livestock has been recorded in southern Africa; however, it remains unclear in the other buffalo inhabited areas of Africa. The role of other wildlife in the transmission of FMDV is most likely limited, like the role of deer in Europe [18].

Because the animal movement from endemic areas to FMDV free countries is very restricted, the risk of FMDV transmission to disease free countries is most likely through indirect transmission of the virus mainly in animal products such as uncooked meat and meat products, from which left-overs are fed to animals.

In the review process (Chapter 2) it was clear that there was insufficient serological data in many African countries. We therefore carried out two separate sero-surveillance studies (Chapter 3 and 4) in Eritrea (East Africa) to quantify the sero-prevalence of the different FMDV serotypes. The samples in Chapter 3 were selectively collected from areas where FMDV outbreaks had occurred. A validation of virus neutralisation test (VNT) was carried out using sera from experimental infected animals to check for the presence of possible cross-reactions (Chapter 3). Based on this validation study, we showed that the highest VNT titre was most likely caused by the most recent infection. It was shown that in this sample set serotypes O, A, C and SAT-1 were the most frequently found serotypes in the VNT. Because the study in Chapter 3 was based on selectively sampled sera, we performed a random cross-sectional study (Chapter 4). In this study we observed that some of the FMDV strains were more easily neutralised than others. To standardise the VNT tests for the different serotypes on the specificity of the tests, we evaluated the specificity on over 450 negative serum samples collected in the Netherlands. For each serotype we selected a cut-off 0.45 log10 higher than the cut-off that would provide 99% specificity. Again we observed a reasonably high sero-prevalence against type O (14%), A (11%) and C (4.7%). The overall sero-prevalence in the VNT was 30% which was slightly higher than the sero-prevalence in the NS ELISA (26%). The percentage positive cattle were similar to the few reported studies in Ethiopia [19], but lower than the sero-prevalence reported in Sudan [20]. But it shows that the region is similar and therefore control should be implemented in a region-wide approach.

Vaccine antibody response and vaccine strain selection

Vaccination is widely used to control, eradicate and prevent FMDV [21, 22]. In FMDV endemic settings, vaccination remains an option as part of an effective control strategy for FMD and the decision to use vaccination is a national responsibility. Conventional vaccines against FMDV are based on chemically inactivated viruses [23, 24]. As antigenic diversity of FMDV is a major concern for FMDV control, vaccine matching and

selection are important areas that need to be assessed for easy and rapid selection of vaccine strains in an outbreak situation [25].

In this thesis, we performed two cattle vaccination experiments. In the first trial, our research question was to identify how antigenic differences can best be covered using serological test methods. This study (in chapter 5) provided a quantitative insight on the homologous and heterologous vaccine antibody response in cattle against 10 different vaccine strains. We used three serological test methods to measure antibody titres, the standard VNT with a fixed amount of virus and a variable amount of serum, the neutralisation index test (NIT) with a variable amount of virus and a fixed amount of serum and the Liquid Phase Blocking ELISA (LPB-ELISA). We determined the antigenic relationship using the relationship coefficient (r_1-value), which has been widely used for vaccine matching determined by ELISA and VNT [26-28]. In our study, results of the r_1-values were not consistent in the tests used and were difficult to interpret. Under the assumption that genetically linked strains would also be antigenically linked, the LPB-ELISA outperformed both neutralisation tests. Both the VNT and LPB-ELISA serological test methods have been used in several studies to study the relation between antibody response and protection in vaccine potency tests [28-32]. Those studies show that higher antibody titres correspond with a higher probability of protection. Studies done by Brehm [28] and Brito [26] indicated that the r_1-value was also found to be an inaccurate indicator of cross-protection. Furthermore, no quantitative information on the sensitivity (Se) and specificity (Sp) of the r_1-value is available in the peer-reviewed literature [26]. This shows that although the r_1-value for vaccine matching has some merits, protection also depends on the height of the titre which as well depends on the quality of the vaccine. Protection against FMDV is often associated with the induction of high levels of neutralising antibodies in serum [30]. Therefore, a vaccine should not only be selected on the basis of r_1-values but also on the height of the antibody response. In this study (chapter 5), the LPB-ELISA was found to show less variation and had a discriminatory capacity greater than the other tests.

In the second cattle experimental trial (chapter 6), our research hypothesis was first, to answer if the use of Montanide ISA 206 VG adjuvant in the vaccine would change the breadth of cross-reaction when compared to that observed with the $Al(OH)_3$-saponin adjuvanted vaccines, used in chapter 5. Second, does mixing of two antigens of the same serotype strains formulated with Montanide ISA 206 VG improve

the breadth of the cross-reactive antibody response when compared to using a single antigen vaccine? Third, can the intradermal (ID) administration route broaden the cross-reaction antibody response when compared to subcutaneous (SC) route in cattle? And finally, the effect of antigen payload on the cross-reactive antibody response was determined in this animal experiment. We found no significant effect of adjuvant, mixing of antigens and route of administration on improvement of the breadth of the cross-reactive antibody response in the LPB-ELISA test. However, a higher payload (10 µg) per dose resulted in higher homologous and heterologous LPB-ELISA antibody titres when compared to lower payload (1 µg) per dose, and thus had a positive effect on cross reactive antibody responses. The selection of a vaccine adjuvant should be based on analysis of the potential benefit of the adjuvant in enhancing the immune response of the vaccine. On the basis of our results, both adjuvants can equally well be used in an FMD vaccine for cattle. Previous studies done showed similar results on comparison of these two adjuvants in cattle [24, 33-35]. In our studies, a bivalent vaccine containing two antigens with a narrow immune response did not show a broadening effect on cross-protective activity. The reason for this could be the origin of the test strains used. These strains had significant genetic and antigenic variations within the same serotype A virus. There are no studies on the comparison of bivalent versus monovalent vaccines in cattle in literature. In a recent report [27], an improved immune response has been shown using a bivalent vaccine compared with heterologous monovalent vaccines tested serologically against one test strain. In our study we tested the antibody response against eight test strains. This indicates that the broadening effect of a mixed antigen vaccine antibody response needs to be tested against a number of test strains. We therefore propose that for FMD vaccine strain selection purposes, strains need to be selected, which induce a cross-reactive antibody response within the serotype, but without giving in on other important characteristics such as good growth in tissue culture.

Concluding remarks

Control of FMDV in Africa is challenging. It will need social economic backing, but our studies show that FMDV type O and A are most prominent in Africa in the areas where buffalo are absent, although incursions of SAT-2 into these regions have been observed. The prevalence of the disease is not high, control should be possible.

Control by vaccination seems the best option, as control of animal movement will be difficult. Use of broadly cross-protective vaccines for FMD is recommended as variant viruses could be introduced from neighbouring regions. As is shown in this thesis high homologous and heterologous antibody titres can be induced in primo vaccinated cattle with vaccines containing a high antigen content which has been kept stable till the moment of vaccination.

Not only a good vaccine quality and matching antigens are needed, but also a good delivery system and effective vaccination coverage and frequency of vaccination. This should go, along with active participatory support from the farmers, governmental decision makers and manufacturers of vaccine to make FMD control a success. An all-inclusive region-based FMDV control strategy along the OIE/FAO progressive control pathway for FMDV control in Africa, when implemented in a well-coordinated manner, would effectively reduce the occurrence and transmission of FMDV. In the long run, these efforts would improve national and regional economies and food security and protect livelihoods.

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Nederlandse samenvatting

Curriculum vitae

List of publications

Acknowledgements

Nederlandse samenvatting

Mond-en-klauwzeer virus (MKZV) is het eerste dierlijke pathogeen waarvan is aangetoond dat het een virus is. Nu 100 jaar later is MKZV nog steeds een belangrijk pathogeen. Er zijn 7 verschillende serotypen van MKZV, maar binnen de serotypen zijn ook nog vele varianten. MKZV is een zeer besmettelijke virus dat infecties veroorzaakt in evenhoevige dieren. Het virus veroorzaakt grote economische schade als het runderen, schapen, geiten, varkens of waterbuffels infecteert. Om deze reden wordt het virus bestreden, vaak met behulp van vaccinatie. Het virus kan echter genetisch snel veranderen wat bij vaccinatie kan resulteren in selectie van varianten die niet kunnen worden bestreden met het gebruikte vaccin. Om MKZV bestrijding in Afrika mogelijk te maken is meer informatie nodig over welke stammen waar in Afrika voorkomen en op welke manier het best virusstammen kunnen worden geselecteerd om adequate vaccins te kunnen produceren.

Het onderzoek beschreven in dit proefschrift draagt bij aan drie belangrijke aspecten; ten eerste welke stammen komen voor in Afrika, ten tweede welke testen zijn het meest geschikt voor vaccin selectie en ten derde kan de antilichaam respons van het vaccin worden verbeterd door wijziging in vaccinsamenstelling, vaccindosis of van de toedieningswijze. In hoofdstuk 2 wordt een overzicht gegeven van de stammen die zijn aangetroffen in verschillende delen van Afrika en de rol die handel, dierhouderij en het wild kunnen spelen bij de verspreiding van MKZV. Veel MKZV stammen hebben slechts een beperkt verspreidingsgebied. SAT serotypen (SAT-1, SAT-2 en SAT-3) komen hoofdzakelijk in het zuiden van Afrika voor in gebieden waar de Afrikaanse buffel voorkomt. In het zuiden van Afrika is contact met geïnfecteerde buffels de belangrijkste bron van infectie. In Noord, West, Centraal en Oost Afrika worden vooral de serotypen O en A aangetroffen, hoewel SAT-2 ook regelmatig naar het noorden verspreidt. In Noord, West, Centraal en Oost Afrika lijkt de verspreiding van MKZV gerelateerd te zijn aan de verplaatsing van dieren als gevolg van de nomadische levenswijze die voorkomt in dit deel van Afrika en de handel van levende dieren. De rol die de Afrikaanse buffel speelt in de verspreiding van MKZ buiten het zuiden van Afrika lijkt niet groot, maar is onvoldoende onderzocht. Er zijn weinig aanwijzingen dat andere gevoelige wilde evenhoevigen een belangrijke rol spelen in de verspreiding van MKZV. In hoofdstuk 3 en 4 is onderzocht of de detectie van antilichamen tegen de 7

verschillende serotypen MKZV het beeld gevonden in hoofdstuk 2 bevestigen. In eerste instantie zijn serum monsters verzameld in gebieden waar in 2009 MKZV uitbraken waren gediagnosticeerd. In deze selecte set van sera (hoofdstuk 3) werden hoofdzakelijk antilichamen aangetroffen tegen type O en A, en niet veel tegen type C en SAT-1. Om dit resultaat te bevestigen zijn er in 2011 aselect 2429 rundersera verzameld uit 4 van de 6 regio's in Eritrea (hoofdstuk 4). Ook nu wordt het hoogste aantal sera gevonden met antilichamen tegen type O (14%), A (11%) en C (4.7%). Verdere analyse laat zien dat het aantal serologisch positieve runderen in sommige regio's sterk is gerelateerd aan de leeftijd en niet afhankelijk is van het feit of er migratie plaatsvindt in het dorp.

In hoofdstuk 5 wordt een dierexperiment beschreven waarbij gekeken is welke testen het meest geschikt zijn om verschillen tussen MKZV stammen aan te tonen. In 50 runderen (plus 10 controle runderen) zijn 10 verschillende MKZV type A stammen als vaccin gebruikt. De sera verzameld 3 weken na vaccinatie zijn getest in zowel een virus neutralisatie test (VNT), een neutralisatie index test (NIT) als ook in de liquid phase blocking ELISA (LPBE). Voor alle drie de testen is op basis van elk serum de relatie tussen de verschillende stammen, de zo geheten r1 waarde, bepaald. De r1 waarde is de titer van het serum tegen een heterologe stam gedeeld door de titer tegen de homologe stam. Met behulp van Receiver Operating Characteristic curves is gekeken welke test methode de genetische verschillen en overeenkomsten tussen de vaccinstammen het best verklaard. De LPBE was daarbij het best in staat om verschillen tussen de stammen aan te tonen en liet minder variatie zien tussen de dieren gevaccineerd met hetzelfde vaccin.

In hoofdstuk 5 werd ook gezien dat sommige stammen relatief weinig kruisreactie gaven met andere stammen en andere stammen juist veel kruisreacties. In hoofdstuk 6 is gekeken of de mate van kruisreactie afhankelijk is van het gebruikte adjuvantia, het mengen van antigenen, de route van vaccinatie (subcutaan of intradermaal) of van de vaccin dosis. Nog de samenstelling van het vaccin of de wijze van vaccinatie hadden invloed op de mate van kruisreacties. Alleen de hoogte van de vaccindosis had effect op de hoogte van de kruis reagerende antilichamen. Hoe hoger de vaccindosis hoe hoger de antilichaamrespons en daarmee ook de mate van kruisreactie. Gebaseerd op de resultaten in hoofdstuk 5 en 6 kan worden

geconcludeerd dat het belangrijk kan zijn om een vaccinstam te kiezen die veel kruisreactie geeft met andere stammen.

Hoewel bestrijding van MKZV in Afrika niet makkelijk zal zijn, vanwege de socio-economische omstandigheden, laat ons onderzoek zien dat bestrijding niet onmogelijk is. De gevonden prevalentie is in sommige gebieden laag, waardoor het starten van bestrijding in die gebieden tot de mogelijkheden behoort. De vaccinatie studies laten zien dat er voldoende hoge titers, ook tegen heterologe stammen, worden geïnduceerd door vaccins die met een hoge dosis vers antigeen worden bereid. Als gekozen kan worden voor een stam met een brede respons tegen veel virussen zal vaccinatie zeker kunnen bijdragen aan de bestrijding (onder de voorwaarde dat er een goede kwaliteitscontrole wordt uitgevoerd). Als gekozen wordt voor het bestrijden van MKZV in Afrika dan zal dit vanwege het feit dat dieren vaak over grenzen worden verplaatst in meerdere landen tegelijk moeten worden geïmplementeerd, waarbij de OIE/FAO “progressive control pathway” als richtlijn moet worden gebruikt.

About the Author (Curriculum Vitae)

Tesfaalem Tekleghiorghis Sebhatu was born on 7 March 1960 in Nielto, Eritrea. In 1986 he graduated and received his degree in Veterinary Medicine (DVM) from Addis Ababa University, Faculty of Veterinary Medicine, Debre-Zeit, Ethiopia. He worked for the Veterinary Services, Ministry of Agriculture as Veterinary Officer in Semhar administrative region in 1987, as head of the Veterinary Services in 1988, as Pan African Rinderpest Campaign (PARC) Coordinator, and head of the regional veterinary diagnostic laboratory for Eritrea before its independence from 1988 to 1991. After the independence of Eritrea in 1991, he continued to work as director of the National Veterinary Laboratory. He got a scholarship for a post-graduate study in 1996 and obtained his MSc degree in Veterinary Microbiology 1997 from the University of London, Royal Veterinary College, UK. He went back to Eritrea and continued to work as director of the National Veterinary Laboratory until he started his PhD study in March 2009 on foot-and-mouth disease virus at Central Veterinary Institute, Part of the Wageningen University and Research in Lelystad, the Netherlands. His PhD study was affiliated to Utrecht University, the Netherlands.

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Acknowledgements

First and foremost, I would like to express my deepest gratitude to my PhD supervisors; Dr Aldo Dekker and Professor dr Rob J.M. Moormann. Without your guidance and a great level of patience, this project would have not been possible to be successful. The good advice, support and friendship of my supervisor, Dr Aldo Dekker, has been invaluable on both an academic and a personal level, for which I am extremely grateful.

Above all, I would like to thank the Ministry of Agriculture; Government of Eritrea for giving me the opportunity to do the PhD study, and special thanks goes to H.E. Arefaine Berhe, Minister for Agriculture and Tekleab Msghna for their full support and encouragement.

I would like to acknowledge the financial support of the International Atomic Energy Agency (IAEA), Vienna, Austria, particularly in the award of my PhD Research Fellowship that provided the necessary financial support for this research. I would also like to thank the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale, Teramo, Italy for supporting air transport costs of serum samples from Eritrea to The Netherlands.

I am indebted to my colleagues in the Ministry of Agriculture, Eritrea, Efrem G., Tsghe K., Ghebremichael G., Mekonen G, the late Tesfu Y., and Dr Uqubazghi K. who supported in sample collection. My appreciation and thanks goes to Dr Iyasu G.tatyos, Dr Tsegay T., Dr Yonas W., Dawit B. and Ibrahim (Wekiro) who supported me in the animal experiment in Eritrea.

Of course, this project would not have been possible without the participation of Klaas and Froukje. Klaas is always on my side in the academic and social discussions making my life easier and feel at home. Thanks Klaas again and again. I am indebted to colleagues at CVI, FMD laboratory in Lelystad, Christine, Elma, Sjaak, Phaedra and Berni for their technical assistance in the lab and for getting together during social events.

I am also grateful to Riks Maas, Jan Boonstra, Jantine, Eric, Ruth, Daniel, Gert-Jan and Thomas for their support and advice. I also thank to the Central Veterinary Institute, Part of Wageningen UR who gave me the financial support to print the thesis. A special thanks goes to Alganesh Tsegaye (USA) was always on my side with her

moral support and encouragement and will never forget the unforgettable deeds. Algu, betam amesegnalehu.

Petra Kuiper, Willem and Paula I thank you so much for all the support at the CVI, Lelystad. Last, but by no means least, I thank my friends Weldegebriel I. and his wife Yrgalem, Mrs Antonia van des Dool (Mam), Klaas and Mari for their support, encouragement and throughout my stay made me feel at home. I cannot find words to express my gratitude to friends in Lelystad, Betty and Philip; Jose and Marieke and Andro for their moral support, encouragement and social events. Amongst my fellow friends and colleagues especially Amos Ssematimba and Jose Gonzalez did contributed in promoting a stimulating and welcoming academic and social environment will stand in my memories for ever. I would like to thank my fellow PhD students Bram, Francesca S., Francesca B. Akis, Helena, Vijay, Jide, Viviana and Carla. Thanks to all for those their names unintentionally not mentioned that in one way or the other have motivated me during my study period. Special thanks go to Jose Gonzales and Abubeker Abdulalim for their help in editing this thesis.

I am indebted to my family Semere, Hermon, Meron, Samuel, Mhret, Mizan, Rahel, Aron, Eyob, Maekele, Hiwet and Bruk who supported morally and encouraged me during my study period. Finally, to my family who have been greatly affected by my absence: my dear Eden Maekele is a pillar in my life and my children who missed me a lot Yosief, Fieruth, Michael and Saron. Finally, I am forever indebted to Eden Maekele for her understanding, endless patience and encouragement. I am eternally grateful for your presence. Without your patience and tolerance my PhD study would not have been possible.