The use of milk for the surveillance of foot-and-mouth disease

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Abstract

Effective surveillance of foot-and-mouth disease (FMD) is of the utmost importance in order to understand the disease risks and implement effective control strategies. Epidemiological data obtained for FMD is mostly obtained through recognition and reporting of clinical cases by farmers which has several limitations. For example, under-reporting of disease is common, due to deficiencies in veterinary infrastructure, the effort involved for sample collection, or the repercussions of control measures for farmers. Diagnostic sample types, usually vesicular epithelium and fluid, are invasive and labour intensive to obtain, and can only be collected from acutely infected animals. Therefore, animals with sub-clinical FMD infection (particularly those in vaccinated herds) may not be identified but may still contribute to disease transmission. It is likely, therefore, that the true prevalence of FMD is not accurately known in parts of the world where the disease is endemic. Consequently, the requirement exists for a simple approach for FMD surveillance that does not rely on farmer reporting. Milk is a non-invasive sample type routinely collected from dairy farms and has been utilised for the surveillance of a number of other diseases. Despite numerous publications suggesting the potential of milk as a valuable sample type for foot-and-mouth disease (FMD) surveillance, empirical studies have mainly focused on the risk of transmission via milk, or the detection of FMD virus (FMDV) in milk from individual animals. This thesis aimed to expand on previous studies to determine the utility of milk and its limitations for the surveillance of FMD at the individual and herd level, using data collected from experimental and field studies. A highly sensitive and specific high-throughput RNA extraction and real-time rRT-PCR was optimised and utilised for FMDV RNA genome identification throughout the project. Using this method, it was demonstrated that FMDV RNA genome could be detected up to 28 days post infection using milk samples collected from individual cows. Further analysis using serotyping or lineage-specific rRT-PCR assays and VP1 sequencing of milk samples collected from individual cows in northern Tanzania highlighted the use of milk as a suitable alternative to more invasive sample types such as epithelium. Additionally, storage and shipment condition simulations performed demonstrated good stability of FMDV RNA genome within milk samples. Following these experiments, the potential use of pooled milk for herd–level FMD surveillance was investigated. Two proof-of-concept pilot studies were

performed comparing the rRT-PCR results of pooled milk samples collected from both a large-scale dairy farm in Saudi Arabia and milk pooling facilities supplied by smallholder dairy farms in Kenya, with farmer reports of clinical disease. Results supported laboratory limit of detection studies, demonstrating that FMDV could be detected from milk pools of up 10,000 litres, even when there were low numbers of clinical cases. Furthermore, both studies suggested the detection of subclinical infection in milk samples, where disease was not reported. Data from pilot studies performed in this thesis therefore support the use of milk as a simple, non-invasive approach for herd-level FMD surveillance. Further field studies are required to determine the full utility of this method before it may be implemented for targeted/risk-based surveillance alongside existing surveillance systems to facilitate improved knowledge of FMD epidemiology, or for use in FMD contingency plans.

Declaration

I declare that this thesis and the research contained within it is my own work unless otherwise stated, and no part of it has been submitted as part of any other degree or qualification.

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Armson, B., Wadsworth, J., Kibona, T., Mshanga, D., Fowler, V. L., Knowles, N. J., Mioulet, V., Reeve, R., King, D. P., Bachanek-Bankowska, K., Lembo, T. (2019) 'Opportunities for enhanced surveillance of foot-and-mouth disease in endemic settings using milk samples', Transboundary and Emerging Diseases, 66(3), pp. 1405–1410. doi: 10.1111/tbed.13146.

Nyaguthii, D. M., **Armson, B**., Kitala, P. M., Sanz-Bernardo, B., Di Nardo, A., Lyons, N. A. (2019) 'Knowledge and risk factors for foot-and-mouth disease among small-scale dairy farmers in an endemic setting', Veterinary Research. BioMed Central, 50(1), p. 33. doi: 10.1186/s13567-019-0652-0.

Armson, B., Di Nardo, A., Nyaguthii, D. M., Sanz-Bernardo, B., Kitala, P. M., Chepkwony, E., Mioulet, V., King, D. P., Lyons, N. A. (2020) 'Utilising milk from pooling facilities as a novel approach for foot-and-mouth disease surveillance', Transboundary and Emerging Diseases, doi: 10.1111/tbed.13487.

Armson, B., Gubbins, S., Mioulet, V., Qasim, I. A., King, D. P., Lyons, N. A. Foot-and-mouth disease surveillance using pooled milk on large-scale dairy farms in endemic settings. Submitted to Frontiers in Veterinary Science, January 2020.

Abbreviations

APHA	Animal and Plant Health Agency
AU-IBAR	African Union – Inter African Bureau for Animal Resources
BBSRC	Biotechnology and Biological Sciences Research Council
BFS	British Field Strain
BTy	Bovine Thyroid
BVDV	Bovine Viral Diarrhoea Virus
CI	Confidence Interval
CPE	Cytopathic Effect
C_T	Cycle Threshold
Cy5	Cyanine5
DEFRA	Department for Environment, Food and Rural Affairs
DNA	Deoxyribonucleic Acid
DPC	Days Post Challenge
DPI	Days Post Infection
EA	East Africa
ELISA	Enzyme-Linked Immunosorbent Assay
EuFMD	European Commission for the Control of Foot-and-Mouth Disease
FAM	6-Fluorescein Amidite
FAO	Food and Agriculture Organization of the United Nations
FMD(V)	Foot-and-Mouth Disease Virus
GPS	Global Positioning System
IC	Internal Control
IDE	Integrated Development Environment
IP	Infected Premise
JOE	4′,5′-dichloro-2′,7′-dimethoxy-6-carboxyfluorescein
KEN	Kenya
LAMP	Loop-mediated isothermal AMPlification
LFD	Lateral-Flow Device
Log_x	Logarithm to the power of x
NSP	Non-Structural Protein
OIE	World Organisation for Animal Health
OP	Oesophageal-Pharyngeal
OR	Odds Ratio
PCP-FMD	Progressive Control Pathway for FMD

PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRT-PCR	Real-Time Reverse Transcription Polymerase Chain Reaction
RT	Room Temperature
S	Survey
SAT	Southern African Territories
SAU	Saudi Arabia
Se	Sensitivity
Sp	Specificity
SP	Structural protein
TAMRA	Carboxytetramethylrhodamine
TAN	Tanzania
$TCID_{50}$	50% Tissue Culture Infectious Dose
TPI	The Pirbright Institute
UKG	United Kingdom
US	United States (of America)
UTR	Untranslated Region
VI	Virus Isolation
VP	Virus Protein
WHO	World Health Organization
WRLFMD	World Reference Laboratory for Foot-and-Mouth Disease

Glossary of Terms

Analytical sensitivity	The lowest concentration at which detection is possible. Also referred to as the 'limit of detection'.
Analytical specificity	The ability of an assay to distinguish between the target analyte and others that may be contained in the sample.
Clinical incidence	The proportion of diseased individuals in a population in a specified period of time.
Convalescent	Recovery from disease.
Cycle threshold (C_T)	The run cycle at which the amplification plot crosses the threshold, meaning there is a significant level of fluorescence.
Diagnostic sensitivity	The proportion of known positive samples to be correctly identified as positive.
Diagnostic specificity	The proportion of known negative samples to be correctly identified as negative.
Disease prevalence	The proportion of a population affected by a disease at any given time.
Gold standard	A benchmark method against which another is measured.
Lineage	A group of viruses with a genetic evolutionary relationship
Milk pool	Milk collected from more than one animal combined into one volume.
Serotype	Subtypes of viruses that are immunologically distinct as determined by serological tests (Samuel and Knowles, 2001).
Sub-clinical infection	The presence of infection with no observed clinical signs.
Topotype	Geographically distinct groups of FMDV isolates within a serotype (Samuel and Knowles, 2001)
Virus pool	An ecological niche of FMD viruses (Bachanek-Bankowska et al., 2018)

CHAPTER 1

Introduction:

A review of diagnostic approaches for the surveillance of foot-and-mouth disease

1.1 Summary

A significant challenge for the world's expanding population is sustainable food production. In order to manage this in the long term, animal health and welfare are key priorities. Infectious diseases have a considerable impact on global animal production and human poverty, and threats to food security are further increased by the international trade of animals and their products. Foot-and-mouth disease (FMD) is a contagious viral livestock disease of great economic importance with high costs for prevention and control. In order to quantify disease burden, inform control efforts and reduce disease impacts, effective surveillance is essential. However, in many regions where the disease is endemic, several limitations of current surveillance systems exist. Surveillance is often dependent upon the recognition and reporting of clinical cases by farmers, occasionally supplemented by targeted case finding or serological surveys. The capacity for undertaking outbreak investigation and collecting clinical specimens in countries with limited resources is also often restricted. Additionally, as clinical samples are usually only collected from acutely infected animals, subclinical infection may not be represented. This chapter explores these limitations in more detail, and highlights the potential use of milk as a cost-effective, non-invasive alternative for FMD surveillance, with the aim of reducing the bias observed when relying on sample vesicular epithelium or blood, and consequently aiming to improve the understanding of the epidemiology of FMD in a region.

1.2 Foot-and mouth disease

1.2.1 Overview of disease

Foot-and-mouth disease (FMD) is a highly contagious, transboundary disease of cloven-hooved mammals. Clinical signs include high body temperature, excessive salivation, and the formation of vesicles on the feet, in and around the mouth and nose (Kitching, 2002; Alexandersen et al., 2003; Jamal and Belsham, 2013). Although the disease has a low mortality rate, outbreaks of the disease in endemic regions are frequent, generally involving the infection of large numbers of animals, and therefore have a high impact (Onono et al., 2013). Additionally, the consequences of an outbreak in a normally disease-free country can be high, due to the rapid spread of infection through a naïve population. Consequently, FMD has an estimated annual global impact of US $11 billion (90% range: US $6.5 – 21 billion) (Knight-Jones and Rushton, 2013).

1.2.2 Causative virus

The disease is caused by FMD virus (FMDV), a single stranded positive sense RNA virus, that belongs to the genus *Aphthovirus* within the family *Picornaviridae* (Grubman and Baxt, 2004). The virus particle is 25-30nm in diameter, containing a single copy of the FMDV genome approximately 8400 nucleotides (nt) in length, surrounded by a icosahedral shaped protein capsid composed of 60 capsomers (Acharya et al., 1989). Each capsomer consists of four structural virus proteins (VP). VP1, VP2 and VP3 which are exposed on the surface of the virus, are encoded by the genes 1D, 1B and 1C respectively. VP4 is located internally and is encoded by the 1A gene. The non-structural proteins (NPs) are encoded by the 2A, 2B, 2C, 3A, 3B, 3CPro, 3Dpol and Lpro genes which control replication and maturation of the FMDV virus (Figure 1.1) (Domingo et al., 2003; Sangula et al., 2010; Jamal and Belsham, 2013; Kamel et al., 2019).

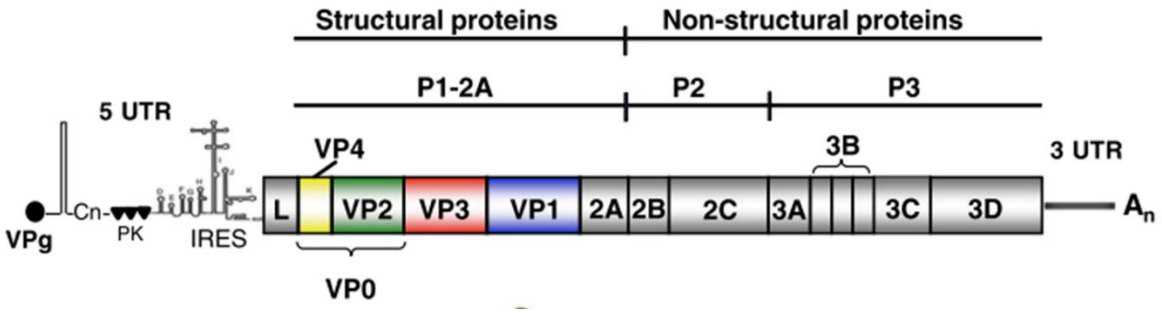

Figure 1.1 The structure of the FMD virus genome. Adapted from Jamal and Belsham (2013).

1.2.3 Geographical distribution of FMD

There are seven immunologically distinct serotypes of FMDV; O, A, C, Asia 1, and Southern African Territories (SAT) 1, 2 and 3 (Robson et al., 1977). The serotypes are not evenly distributed spatially, and there is a tendency for genetically related viruses within a serotype to appear in the same geographical area, for reasons such as animal movement and trade patterns. Globally, the distribution of FMDV is divided into seven geographic pools, based on nucleotide sequence analyses (World Organisation for Animal Health (OIE), 2019)(Figure 1.2). Pools 1, 2 and 3 include serotypes O, A, and Asia 1 and cover East and Southeast Asia, South Asia, and West Asia respectively; Pool 4 includes serotypes O, A, SAT 1, 2 (and 3) in East Africa; Pool 5 is restricted to West Africa (serotypes O, A, SAT 1 and 2); Pool 6 includes serotypes SAT 1, 2 and 3 in Southern Africa; and pool 7 in South America (O and A) (Paton *et al.* 2009). Serotype C is not included in any of the pools since it has not been identified since 2004 (Sangula et al., 2011).

Within each serotype, different topotypes exist in the respective areas, which are geographically distinct groups of FMDV isolates. For example in pool 4 (East Africa), at least 5 of the 7 serotypes are known to be in circulation. Within pool 4, serotype O comprises four different eastern African (EA) topotypes including EA-1, EA-2, EA-3 and EA-4, and serotype A includes lineages G-VII and G-I from the AFRICA topotype (Brito et al., 2015). Notably, the latter was identified in Tanzania in 2014, but had not been detected for over 30 years previously (Kasanga et al., 2015). SAT 1 includes topotype IX and I (NWZ), and SAT 2 topotypes IV, XIII, and VII, with some of these topotypes

being detected for the first time in the past ten years (Legesse et al., 2013; Brito et al., 2015). In the Middle East (pool 3) at least 3 of the 7 serotypes circulate, and again, several different topotypes also exist within these serotypes. These include but are not limited to: for serotype O: the PanAsia, PanAsia-2 and Ind-2001d lineages of the ME-SA topotype; for serotype A: the Iran-05, and Iran-96 lineages of the ASIA topotype and; for serotype ASIA 1: the Sindh-08 lineage of the ASIA topotype (Knowles and Samuel, 2003; Knowles et al., 2016).

Additionally, incursions of new serotypes and/or topotypes that are not normally found in that region frequently occur. Examples of such incursions include the 2015 emergence of the A/ASIA/G-VII lineage (which is endemic in Indian sub-continent) into Saudi Arabia, Iran, Armenia, Israel, and Turkey (Bachanek-Bankowska et al., 2018), and the expansion of the distribution of the O/ME-SA/Ind-2001 lineage into the Middle East and North Africa since 2013 (Valdazo-González et al., 2014; Knowles et al., 2015). Additionally, the role of wildlife in maintaining circulation of different FMDV serotypes is unclear. For example, African buffalo (*Syncerus caffer)* are known to be maintenance hosts for serotypes SAT 1, SAT 2 and SAT 3 in East Africa, and may contribute to increasing the level of antigenic diversity, with the potential to trigger new outbreaks in livestock (Vosloo et al., 1996; Casey et al., 2013). Outbreaks are reported through observation of clinical animals and diagnosis either in country or by international reference laboratories. However, there is a need to obtain a more thorough understanding of the exact serotypes/topotypes circulating in a region, to better understand the complex epidemiology of FMDV to improve effective control of FMDV outbreaks in endemic areas (Picado et al., 2011; Kasanga et al., 2012; Kasanga et al., 2015).

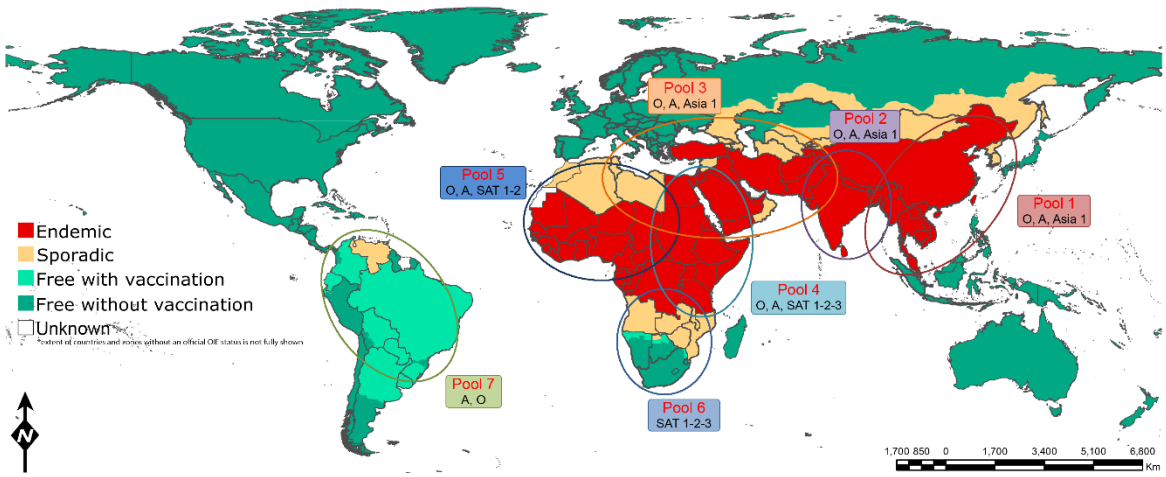

Figure 1.2 The geographical distribution of FMDV into the seven virus pools based on nucleotide sequence analyses, 2019. Colour coding displays the OIE FMD classification status of each country. (The extent of countries and zones without an official OIE status is not fully shown)(World Organisation for Animal Health (OIE), 2019). Courtesy of Dr Antonello Di Nardo (The Pirbright Institute, UK).

1.2.4 Impact of FMD

FMD has a large global impact of approximately US $11 billion (90% range: US $6.5 – 21 billion). The consequences of outbreaks in FMD free countries and zones can result in losses of over US $1.5 billion per year (Reid et al., 2001; Knight-Jones and Rushton, 2013; Hall et al., 2013). However, FMD has the most dramatic impact in endemic countries where approximately three-quarters of the world's livestock population reside. These are often low and middle income countries that lack the resources and infrastructure to eliminate the disease, and therefore also pose a significant threat to disease-free countries (Knight-Jones and Rushton, 2013; Knight-Jones et al., 2016).

1.2.4.1 Direct losses

FMD costs are a result of direct losses and indirect losses. The direct impacts of FMD are not typically due to animal mortality which is generally low, but due to production losses. These may include, but are not limited to, the suppression of fertility and livestock growth rates, the utility of draught animals due to lameness, delay in the sale of livestock or their products, and a reduction in milk yield (discussed further in section

1.5.4), which affects consumption for both calves and humans (Rufael et al., 2008; Knight-Jones and Rushton, 2013; Young et al., 2013; Ansari-Lari et al., 2017; Chaters et al., 2018).

1.2.4.2 Indirect losses

Indirect losses are due to the reaction to disease and include additional costs, forgone earnings and wider consequences. Additional costs may include control measures such as vaccination and culling, movement and trade restrictions, surveillance and the cost of diagnostic tests (Knight-Jones and Rushton, 2013; Robinson et al., 2011). For example, the UK 2001 outbreak resulted in losses of over £8 billion, and other than over £1.3 billion for additional costs, included £4.5-£5.4 billion in foregone tourism revenue and £1.4 billion in compensation to farmers (House of Commons, 2002; Knight-Jones and Rushton, 2013). In FMD endemic regions, the application of control measures may also have negative consequences such as the construction of fences that may damage wildlife habitats and behaviours. Additionally, if control measures are not effective, farmers may lose trust and become reluctant to comply in the future (Knight-Jones et al., 2016). Even in FMD free regions, costs are still incurred to maintain this free status, including potential vaccination schemes, import controls to prevent new disease introductions, maintenance of vaccine banks and supporting scientific research (Knight-Jones and Rushton, 2013).

1.3 Control of foot-and-mouth disease

1.3.1 Epidemiological patterns of FMD

Foot-and-mouth disease is not evenly distributed around the world. It is prevalent in most developing countries, and circulates in approximately 77% of the global livestock population (Rushton et al., 2012). To date FMD has been observed in every region of the world known to contain livestock, apart from New Zealand (Poonsuk et al., 2018).

The World Organisation for Animal Health (OIE) has classified FMD as a listed disease according to the Terrestrial Animal Health Code (OIE, 2019a). Consequently countries are listed according to their official FMD status into either: (i) FMD-free country or zone without vaccination (NV); (ii) FMD-free country or zone with vaccination (WV); or (iii) FMD endemic (see Figure 1.2). Any unclassified member state is assumed to be endemic. Member countries can apply for disease-free status by providing evidence that FMD has been eliminated for at least 12 months (OIE, 2019a).

Currently, 68 countries are classified as FMD free without vaccination, and 2 countries as FMD free with vaccination. Additionally, some countries have FMD free zones, but they are yet to provide evidence for virus elimination throughout the whole country. Often the country may also have zones with different classifications, i.e. some zones free WV and some free NV, for example in Argentina and Brazil (OIE, 2019b).

1.3.2 Control measures

Effective control strategies are imperative if the burden of disease is to be reduced, or if disease-free status is desired. Control measures can include vaccination, culling, restriction of animal movements, and removing contact of livestock with potentially infectious wildlife such as African buffalo (*Syncerus caffer*) (Rweyemamu, 1984; Paton et al., 2009; Picado et al., 2011; Kasanga et al., 2012; Namatovu et al., 2013). Implementation of the most effective control measures may be challenging due to variations in global livestock management practices, and consequently different regions require a tailored approach. For example, movement restrictions are difficult to enforce in areas where livestock management practices are based on pastoralism, such as in East Africa (Di Nardo et al., 2011; Brito et al., 2015).

Several types of FMD vaccines exist. Conventional inactivated vaccines are the most commonly used, and are produced by amplifying the live virus of interest on baby hamster kidney-21 (BHK-21) cells, then inactivating the amplified virus with binary ethyleneimine (BEI) to remove non-structural proteins (NSP). An appropriate oil-based adjuvant or aqueous-based adjuvant such as aluminium hydroxide/saponin is also added. These vaccines vary in potency, they are normally either 3 or 6 times the 50% protective dose (PD_{50}), and can be prepared against one, or multiple serotypes/topotypes (Doel, 1996; Cao et al., 2016; Kamel et al., 2019). Although these vaccines can be highly efficacious, additional frequent boosters are required, and production requires expensive bio-containment facilities. Additionally, decreased vaccine efficacy may be impacted by low capsid stability which is reliant on the maintenance of a cold chain, low vaccine potency, incorrect dosing, and poorly purified vaccines (Parida, 2009; OIE, 2017a). To overcome some of these limitations, newer vaccine types have also been developed including live-attenuated, DNA, peptide and live viral vector vaccines. For example, most of these do not require biocontainment facilities for production, and are able to differentiate between infected and vaccinated animals, which can often be a problem with poorly purified inactivated vaccines (Kamel et al., 2019).

However, although efficacious vaccines for FMD are available, many endemic countries do not have effective vaccination campaigns, due to a lack of incentives and resources, poor veterinary services or problems with cold chain maintenance (Smith et al., 2014). In many regions, it is also unclear exactly which serotypes/subtypes are circulating, with many exhibiting high antigenic variation (for example in pool 4), and the possibility of the emergence of new variant viruses. Additionally, infection or vaccination with one serotype/topotype may not protect against another (Parida, 2009). Therefore there is a requirement for vaccine strains to be carefully selected based on epidemiological data collected from a region, and the ongoing monitoring of vaccine efficacy due to virus evolution. Also, even though vaccines may fully or partially protect animals from acute clinical infection, animals may still present mild or subclinical infection (no clinical signs), leading to an epidemiological threat from carrier animals (Hutber et al., 1999; Parida, 2009; Rodriguez and Gay, 2011; Lloyd-Jones et al., 2017).

Effective control of FMD relies on successful co-operation between stakeholders including livestock owners, animal health workers, FMD experts and government personnel (Roberts and Fosgate, 2018). Additionally, thorough surveillance and accurate and timely identification of current and emerging field strains is required to better understand and predict patterns of viral circulation and to inform and improve vaccine selection, especially in endemic regions such as East Africa (Paton et al., 2009; Jamal and Belsham, 2013). This would allow a region to progress through the early stages of the Progressive Control Pathway for Foot and Mouth Disease (PCP-FMD) (Food and Agriculture Organization of the United Nations (FAO), 2011). This is a tool developed by the FAO to facilitate and assist FMD endemic countries to increase the level of FMD control so that countries/regions may apply for FMD free status, with or without vaccination, through a set of FMD control activity stages. These include active monitoring of virus circulation to understand the epidemiology of FMD, applying specific control measures in order to reduce the burden of FMD, and monitoring of outcomes (see Figure 1.3) (Food and Agriculture Organization (FAO), 2011; Jamal and Belsham, 2013).

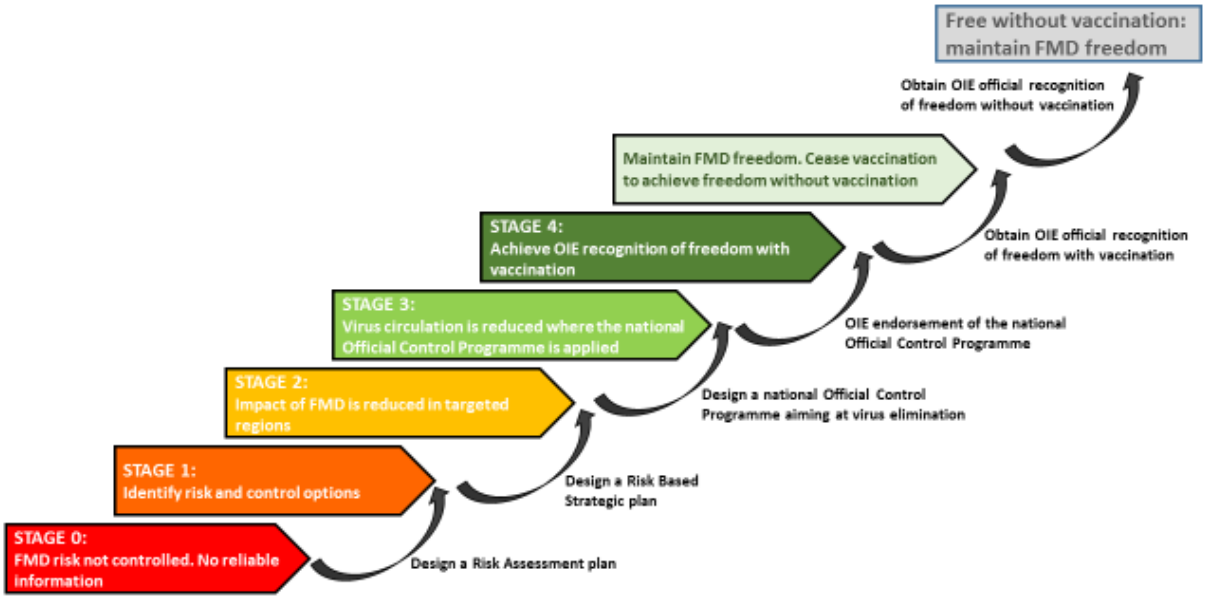

Figure 1.3 The Progressive Control Pathway for Foot-and-Mouth Disease (PCP-FMD). Adapted from the Food and Agriculture Organization (FAO) (2018).

1.4 Diagnosis of foot-and-mouth disease

1.4.1 Clinical signs

Diagnosis of FMD is primarily based on the observation of clinical signs. These become apparent after a short incubation period, typically between 2 – 6 days, and include a high temperature, excessive salivation, and the formation of vesicles on the oral mucosa, nose, teats, and the inter-digital spaces and coronary bands of the feet (Figure 1.4). Additionally, there may be fever, depression, lameness, mastitis and reduced milk production. Pigs often suffer from severe clinical disease, while in small ruminants the signs are generally more subtle, or even unapparent (Alexandersen et al., 2003). The clinical signs of FMD are often indistinguishable from those of other vesicular diseases such as swine vesicular disease (SVD), vesicular stomatitis (VS), vesicular exanthema of swine, and the signs associated with Seneca Valley virus-1 infection (SVV-1) (Nardelli et al., 1968; Gelberg and Lewis, 1982; Rodriquez and Nichol, 1999; Singh et al., 2012). Therefore, rapid and accurate detection of disease is imperative to confirm the disease causing agent, and to initiate the implementation of control processes. Data collected from clinical samples may assist in evaluating the epidemiological situation of FMDV in a region, detecting the presence of virus, identifying circulating serotypes and variants within serotypes, identifying vaccine strain candidates, and monitoring the effectiveness of control strategies (Paton et al., 2009; Jamal and Belsham, 2013).

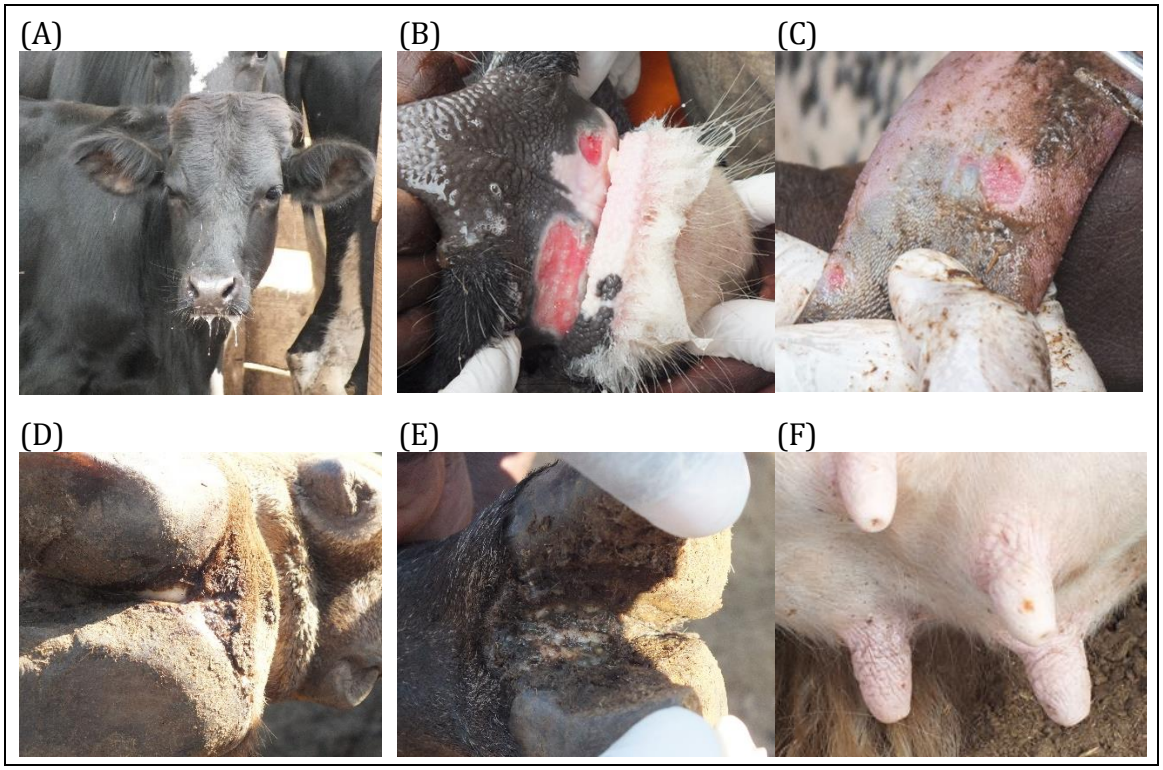

Figure 1.4 Clinical signs of foot-and-mouth disease, including excessive salivation (A), and vesicles on the gums (B), tongue (C), inter-digital spaces of the feet (D and E), and teats (F). Photographs taken by Bryony Armson.

1.4.2 Sample collection

A range of sample types may be collected for the diagnosis of FMD, with vesicular epithelium or fluid being favoured due to the high concentrations of virus, although are only available for collection during the acute stage of the disease (Figure 1.5)(Alexandersen et al., 2001; Ferris et al., 2006). Therefore, alternatives are required when infection is present but lesion material cannot be collected, for example on suspected farms before clinical signs become apparent ('pre-clinical' stage), during convalescence, in mild cases such as in small ruminants, or in 'sub-clinical' cases where no lesions are apparent.

Other sample types submitted to reference laboratories for confirmatory diagnostics include blood, oesophageal–pharyngeal (OP) fluid and oral, nasal or lesion swabs (OIE, 2018). The detection of FMD virus in blood samples is limited to the acute viremia stage of disease, although FMDV-specific antibodies may be detected for much longer periods (>1 year)(Alexandersen et al., 2003; Elnekave et al., 2015). Additionally, FMD

virus/viral RNA may be detected in oral or lesion swabs for up to at least 14 days post infection (Alexandersen et al., 2003; Stenfeldt, Lohse and Belsham, 2013), and in 'carrier animals', in OP fluid for up to 3 years in cattle (Stenfeldt et al., 2013) or 5 years in buffalo (Condy et al., 1985).

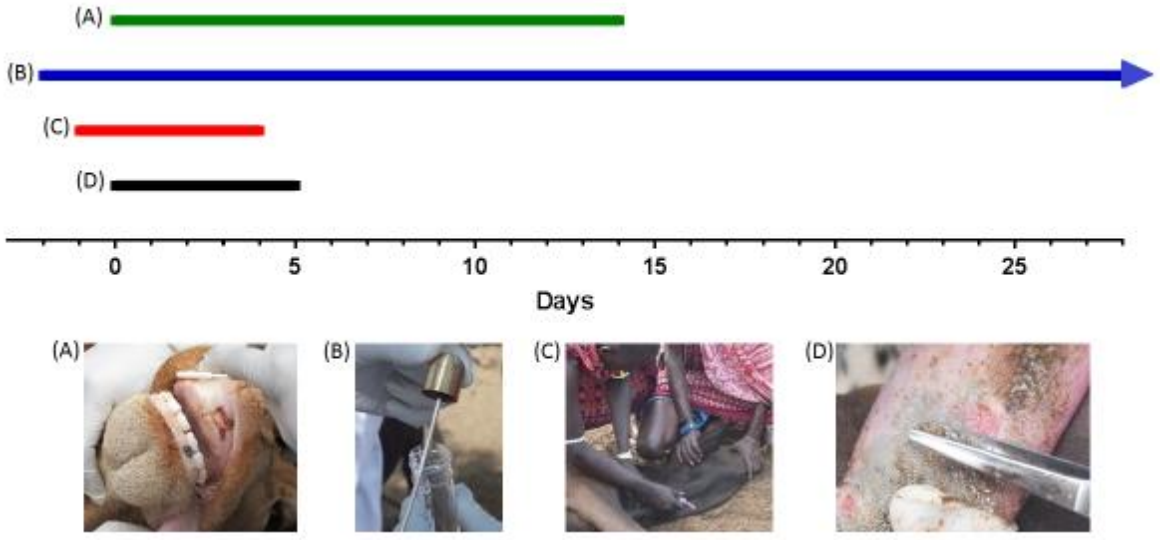

Figure 1.5 Approximate clinical window of FMD virus detection from different sample types: oral swab (A), OP fluid (B), blood (C), and vesicular epithelium (D). Day 0 indicates the day vesicular lesions are first noticed. Based on data from Alexandersen et al., 2003, King et al., 2012 and Stenfeldt et al., 2013. Oral swab photograph courtesy of Emma Howson, 2016.

1.4.3 Laboratory diagnosis

Clinical samples collected from suspect cases are usually transported for routine diagnostic testing to local laboratories or centralised reference laboratories containing high containment facilities that are equipped for handling infectious pathogens. In the laboratory, detection of current or previous FMDV infection can be carried out using virological, molecular and serological tests according to the OIE Terrestrial Manual (OIE, 2018) which are explained in further detail below.

1.4.3.1 Detection of live FMD virus

Conventional detection of live virus is by virus isolation (Snowdon, 1966), usually from vesicular epithelial tissue or fluid, although this method can be used with other sample

types. Briefly, the sample is removed from the transport media (ideally composed of equal amounts of glycerol and 0.04 M phosphate buffer to maintain a pH of 7.2 – 7.6), then a 10% epithelial suspension is prepared by grinding the sample with sterile sand in a pestle and mortar and clarified in a centrifuge. This suspension is inoculated onto a primary or established cell-line such as primary bovine (calf) thyroid (BTY) cells or IB-RS-2 cells respectively, and the cell cultures examined for cytopathic effect (CPE) for up to 96 hours (OIE, 2018). Other susceptible cell lines include Baby Hamster Kidney-21 (BHK-21) cells, lamb kidney cells, swine kidney cells (SK6), fetal goat cells (ZZ-R) and bovine kidney cells (LFBK-$\alpha_V\beta_6$)(Kasza et al., 1972; Ferris et al., 2006; Brehm et al., 2009; LaRocco et al., 2013). This method is highly sensitive, and the resulting cell culture isolates may be utilised further, for example for vaccine matching. However, testing by this method can take up to four days to report a result, and it does not provide a definitive diagnosis of FMD, as CPE may be caused by other vesicular viruses. Therefore, in order to confirm infection of the sample with FMDV, additional diagnostic tests are required, such as the detection of FMD virus genome by molecular methods.

1.4.3.2 Detection of FMD virus genome

Reverse transcription polymerase chain reaction (RT-PCR) assays have been developed with a much greater sensitivity, and detect viral genomes (or fragments) instead of intact viral antigens and/or live virus. This detection method can be used on several sample types, including those that may be partially degraded and no longer infectious. Additionally, they can be easily automated, and produce relatively rapid results (<4 hours) (Reid et al., 1998; Reid et al., 2003; Shaw et al., 2007).

The viral RNA must be extracted from the sample so that it may be used as a template for the RT-PCR assay. Additionally, this process inactivates the potentially infected sample, and can remove potential PCR inhibitors, such as RNases, proteins and lipids from a wide range of sample types (Wilson, 1997). A number of methods have been evaluated for the extraction of FMDV RNA. Many commercial kits or reagents include a lysis solution that contains guanidine isothiocyanate, which disrupts the cells and cell components by denaturing proteins, whilst maintaining the integrity of the RNA. For example, the use of Trizol® reagent produces high yields of RNA (Simms et al., 1993),

however samples are extracted manually and this method requires the use of potentially dangerous chloroform. Consequently, safer, higher throughput protocols have more recently been employed, for example using magnetic bead-based kits such as the MagMAX™-96 Viral RNA Isolation Kit (Applied Biosystems™, UK), and the LSI MagVet™ Universal Isolation Kit (Thermo Fisher Scientific, Loughborough, UK). RNA extraction using these kits may be performed manually, or in combination with automated extraction robots such as the KingFisher magnetic particle processors (Thermo Fisher Scientific), allowing for the processing of up to 96 samples in under 30 minutes (Figure 1.6). Alternatively, silica-based spin column kits such as the QIAamp Viral RNA Mini Kit (Qiagen, Germany) may also be used in combination with automated extraction robots, and use silica matrices to bind nucleic acid, however due to the potential for clogging the filter, only limited sample types can be processed with this method.

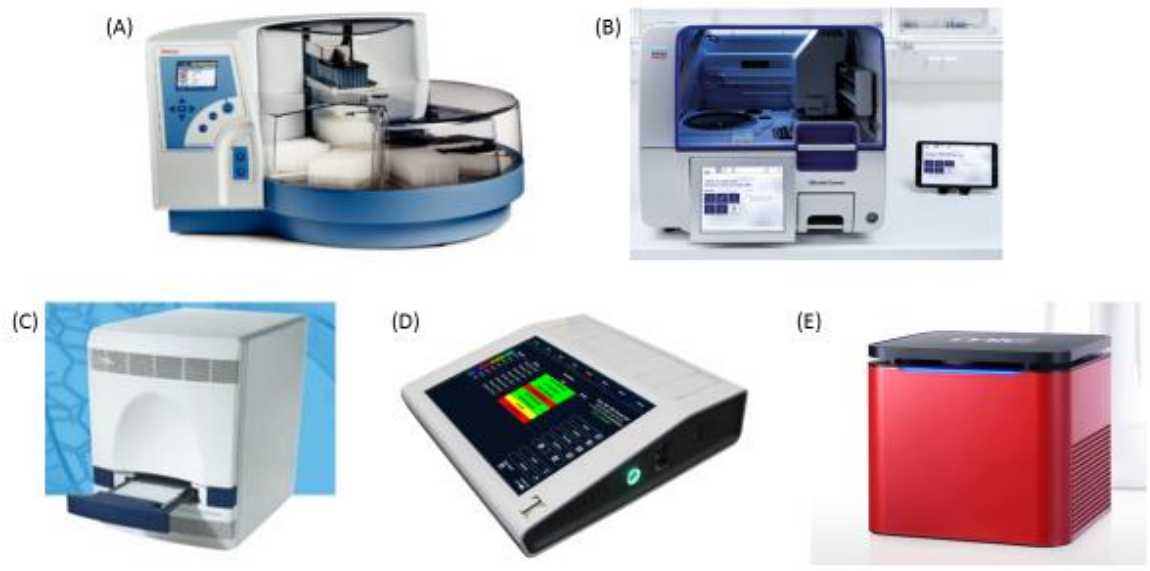

Figure 1.6 Examples of automated RNA extraction and real-time RT-PCR technologies. For RNA extraction: (A) - The KingFisher magnetic particle processor (Thermo Fisher Scientific) and (B) - QIAcube Connect (Qiagen). For laboratory rRT-PCR: (C) - Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument. rRT-PCR technology for field settings: (D) - T-COR 8™ Real-time PCR Thermocycler (Tetracore, Inc) and (E) – Mic qPCR cycler (Bio Molecular Systems).

After the nucleic acid has been isolated from the sample, RT-PCR can be performed to determine the presence and quantity of FMDV RNA in the sample. Conventional RT-PCR methods involve three separate steps: (i) conversion of the RNA into single-stranded complementary DNA (cDNA) using a reverse transcriptase enzyme, (ii) amplification of template cDNA by PCR and (iii) the examination of PCR product by agarose gel electrophoresis (Reid et al., 1998). The process of PCR involves the denaturation (melting) of the double stranded DNA into single strands by heat, to which two oligonucleotide primers are annealed that are complementary to sequences on the target gene. The presence of a thermostable enzyme (e.g. *Taq* DNA polymerase) allows extension of the primers in opposite directions by DNA synthesis. As these primers are located apart from each other on the target gene, two complementary strands are consequently generated. Repetition of this process results in an exponential increase in the number of copies of the specific nucleic acid (see Figure 1.7) (Guatelli et al., 1989; Holland et al., 1991).

More recent developments to reduce the number of user dependent steps has resulted in real-time (r), one-step fluorogenic RT-PCR assay procedures that can be automated, with an increased throughput of samples (Reid et al., 2003). These methods are modified versions of the RT-PCR process described above, which allow the results to be monitored in real-time. A number of different chemistries exist although the most commonly used for FMDV detection is the addition of a fluorogenic TaqMan® probe specific to the target gene that is dual-labelled with a reporter (e.g. FAM) and quencher dye (e.g TAMRA). During the extension phase of the PCR reaction, the *Taq* polymerase enzyme cleaves the probe by nuclease degradation, and fluorescence is emitted when the two dyes are physically separated (Figure 1.7) (Heid et al., 1996; Didenko, 2001; Johansson, 2006). The increase in fluorescence intensity is proportional to the number of copies of nucleic acid produced, and can be visualised using a number of systems including the Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument (Figure 1.6), the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Ltd.), and the AriaMx qPCR System (Agilent Technologies Inc.).

To ensure the detection of all serotypes/topotypes of FMDV by (r)RT-PCR, highly conserved regions of the genome must be targeted. Conventional and real-time RT-PCR assays have been developed using primers based on sequences from the 1D and 2A/2B

region (Amaral-Doel et al., 1993), the 3D polymerase (Callahan et al., 2002; Nishi et al., 2019) and the internal ribosomal entry site located in the 5'untranslated region (UTR) (Reid et al., 2002) (see Figure 1.1). The 5'UTR and 3D FMDV rRT-PCR assays are recommended by the OIE, are highly specific, and are as sensitive as the 'gold standard' method of virus isolation in cell culture (Shaw et al., 2004; OIE, 2018).

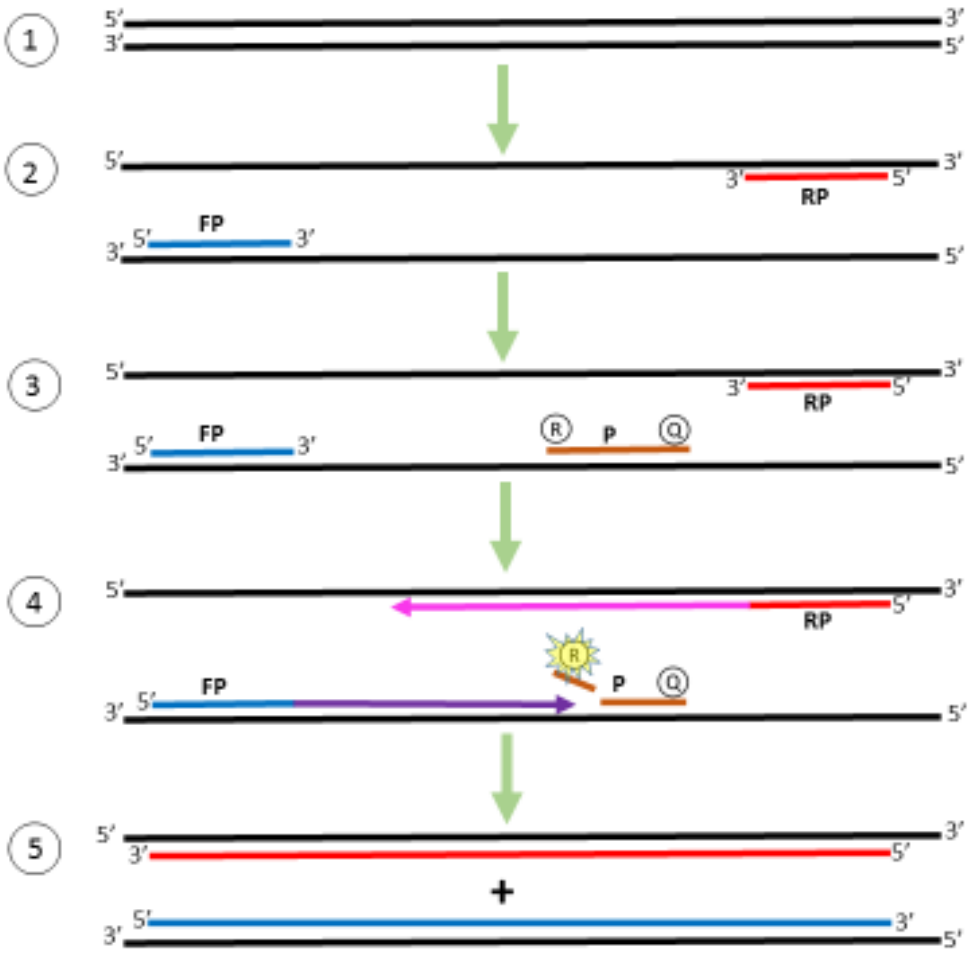

Figure 1.7 Schematic of the real-time polymerase chain reaction (PCR) process. (1-2): double stranded DNA is denatured into 2 separate strands by heat. (2-3): The forward primer (FP), reverse primer (RP) and fluorogenic probe (P) are annealed to the template. (4): primer extension causes physical separation of the reporter (R) and quencher (Q) dyes on the probe causing fluorescence to be emitted. (5): after primer extension there are 2 sets of double stranded DNA and the process begins again with an exponential increase in the number of copies of specific nucleic acid. Figure adapted from Guatelli, Gingeras and Richman (1989) and the ThermoFisher Scientific website.

1.4.3.3 Identification of the FMDV serotype/strain

Identification of the serotype responsible for a particular outbreak is of the utmost importance to inform vaccine selection and for tracing outbreaks. The antigen detection ELISA (enzyme-linked immunosorbent assay) (Ferris and Dawson, 1988) can identify the serotype of the causal FMDV and can be performed in 4-5 hours. However, the analytical sensitivity is often limited, and the test is only suitable for epithelium tissue/fluid and cell culture derived FMDV material. With the advent of molecular technology, various authors have demonstrated the utility of typing of FMDV by conventional RT-PCR assays (Callens and De Clercq, 1997; Giridharan et al., 2005; Bao et al., 2008; Saeed et al., 2011; Liu et al., 2018). However, due to the heterogeneity of FMDVs in different areas of the world, assays tailored to circulating virus strains are required (Jamal and Belsham, 2015). Recently, rRT-PCR assays for the detection of strains specific to a particular region, for example the Middle East (Reid et al., 2014; Knowles et al., 2015; Saduakassova et al., 2017), West Eurasia (Jamal and Belsham, 2015), North (Ahmed et al., 2012) and East Africa (Bachanek-Bankowska et al., 2016) have been developed. Most of these characterisation rRT-PCR assays are designed to target the VP1 coding region, as it is the least conserved region of the FMDV genome, with a high degree of sequence variation (Baxt et al., 1984; Wittwer and City, 1989; Jamal and Belsham, 2013). In order for these to be continually sensitive and specific, sequences of circulating strains must be assessed and if necessary, primer sets adapted (Jamal and Belsham, 2015; Bachanek-Bankowska et al., 2016).

1.4.3.4 Virus sequencing

Nucleotide sequence analysis is also used for the characterisation of circulating FMDV strains. Most of the nucleotide sequences of FMDV published are of the VP1 coding region using Sanger sequencing (Sanger et al., 1977). VP1 sequencing is frequently used to deduce evolutionary dynamics, genetic and epidemiological relationships, in the tracing of outbreaks and monitoring the transboundary movements of the disease (Wittwer and City, 1989; Marquardt and Adam, 1990; Knowles and Samuel, 2003; Kasambula et al., 2012; Logan et al., 2014). However, the VP1 region represents less than ten percent of the full genome (i.e., 639/8300 nucleotides), and can only identify

the predominant consensus sequence (Cottam et al., 2008; Logan et al., 2014). With recent technological advances, whole genome sequencing (WGS) has become much more accessible and has been used for high resolution molecular epidemiological studies, such as investigating the transmission pathways of the 2007 UK FMDV epidemic (Cottam et al., 2008). WGS tools have been refined with the development of next-generation sequencing (NGS) (Marston et al., 2013; Gilchrist et al., 2015). Recently, a NGS protocol has been adapted to sequencing the whole genome of FMDV (Logan et al., 2014). NGS offers a rapid, robust, and high throughput method for the generation of high resolution viral genome sequences enabling the identification of minority variants (viral swarm structure) beyond the consensus level of which would usually only identify the most frequently appearing strains (Wright et al., 2011; Orton et al., 2013; Logan et al., 2014; King et al., 2016).

1.4.3.5 Detection of FMDV antibody

Natural infection induces antibodies to both FMD viral structural proteins (SP) and non-structural proteins (NSP) (Paton et al., 2009; Sørensen et al., 1998), and these can be detected using serological tests. The virus neutralisation test (VNT) (Karber, 1931) and the liquid phase blocking ELISA (LPBE) (Hamblin et al., 1986) can detect serotype specific anti-SP antibodies. However, the VNT takes several days to perform, and requires the use of live virus and therefore must be carried out in specialised biocontainment laboratories.

Vaccination however, induces only anti-SP antibodies (providing the vaccines in use are purified to remove NSPs) and therefore tests which can detect anti-NSP antibodies can be used as differentiation of infected from vaccinated animals (DIVA) tests (Shen et al., 1999; Paton et al., 2006; Uttenthal et al., 2010). Tests based on anti-NSP antibodies can detect infection with all serotypes, however they are unable to identify with which serotype/topotype the animal has been infected with, due to the conserved nature of the NSP coding region (Grubman and Baxt, 2004). Additionally, many regions use poorly purified vaccines that may still contain NSP, and so differentiation is not so effective. Anti-NSP antibodies may persist for a long time (Elnekave et al., 2015), and as anti-NSP antibody ELISA testing is inherently retrospective these tests can therefore

only identify whether the animal has previously been infected with FMDV. Consequently, unlike virological methods, serological tests are unable to determine the current infectious status of an animal.

1.4.4 Methods to reduce time to result for FMD diagnosis

Rapid and accurate diagnosis is essential for surveillance and effective control of FMD. Although the more recently developed laboratory methodologies described above have been designed to reduce the time to result and minimise user intervention, samples from suspect cases still need to be transported to centralised reference laboratories for routine testing, as many countries lack the infrastructure to be able to carry out their own diagnosis (Fowler et al., 2014; Niedbalski, 2016; Howson et al., 2017). Transport of samples to these centralised facilities often involves lengthy travel times, including costly international shipments which can delay result reporting and critical decision making for days or even weeks after clinical signs have been observed. Also, transport times mean that results may not only be delayed, but also not as reliable if sample degradation could have taken place if a cold chain was not maintained (Fowler et al., 2014; Howson et al., 2017).

To overcome these difficulties, simple to use technologies that can be deployed either in the field or in local laboratories exist so that countries are able to improve their capacity for surveillance. Various portable platforms have been developed to enable local FMDV detection including the lateral-flow device (LFD)(Ferris et al., 2009) and closed tube tests that have been adapted for the use of thermostable lyophilised reagents such as the Enigma® Field Laboratory (FL) (Madi et al., 2012; Howson et al., 2017), and the T-COR 8™ (Tetracore, Inc)(Howson et al., 2018) (see Figure 1.6). Furthermore, serotype specific assays have been adapted for use with some of these technologies, allowing for rapid identification of the serotype either in local laboratories or at the point of sample collection (Chen et al., 2011; Howson et al., 2018).

1.5 FMD surveillance

1.5.1 Limitations of established FMD surveillance systems

Surveillance activities are designed to improve the epidemiological understanding of disease in a population of interest, and can be useful for informing disease prevention and control strategies (Falzon et al., 2019). In order to improve knowledge of the epidemiology of FMD in endemic regions, efficient, cost-effective surveillance systems are essential. Data collected through passive surveillance from clinical samples may assist in evaluating the epidemiological situation of FMDV in a region (Paton et al., 2009; Jamal and Belsham, 2013). However, although approximately 77% of the global FMD susceptible livestock population reside in FMD endemic regions (e.g. see Figure 1.8 for the global distribution of cattle), few clinical samples are sent to national reference laboratories for diagnostic testing (Rushton et al., 2012; Namatovu et al., 2013; Robinson et al., 2014). For example in 2018, only 442 clinical samples from 25 countries were received in the WRLFMD for FMD diagnosis (see Table 1.1), yet it is estimated that FMD affects 32 million livestock units (LSU), although it could be up to 79 million, globally per year (1 LSU = 1 cow, 3.3 pigs, or 10 sheep or goats) (Sumption et al., 2008; Knight-Jones and Rushton, 2013). Consequently it is evident that an incomplete global picture of the epidemiology of the disease exists based on established surveillance methods.

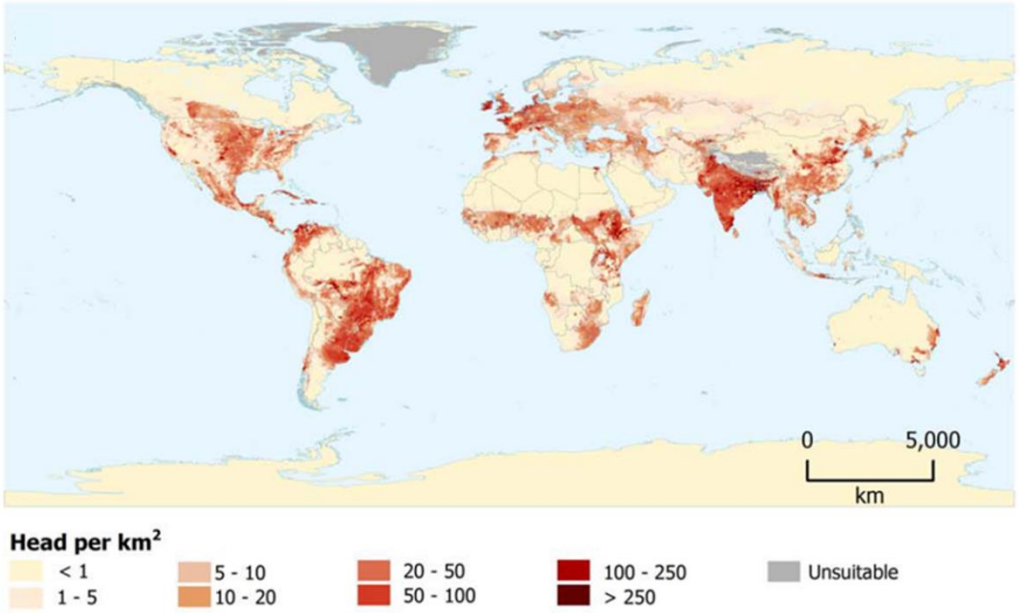

Figure 1.8 The global distribution of cattle. Reproduced from Robinson et al. (2014).

Table 1.1 Summary of samples collected and received to WRLFMD (January to December 2018). Adapted from WRLFMD (2018a, 2018b, 2018c, 2018d)

Country	Number of samples	Virus isolation/ELISA							
		O	A	C	SAT 1	SAT 2	SAT 3	ASIA 1	NVD*
Hong Kong SAR of PRC	27	7	-	-	-	-	-	-	16
Israel	14	8	6	-	-	-	-	-	-
Kenya	28	6	3	-	1	1	-	-	17
Mongolia	24	17	1	-	-	-	-	-	7
Nepal	18	3	-	-	-	-	-	8	7
Palestinian Autonomous Territories	12	12	-	-	-	-	-	-	-
Swaziland	3	-	-	-	-	-	-	-	3
Afghanistan	22	3	1	-	-	-	-	1	17
Bhutan	11	4	3	-	-	-	-	-	4
Ethiopia	28	11	7	-	-	1	-	-	9
Iran	25	11	9	-	-	-	-	4	1
South Korea	5	-	2	-	-	-	-	-	3
Sri Lanka	16	9	-	-	-	-	-	-	7
Vietnam	40	20	13	-	-	-	-	-	7
Zambia	3	3	-	-	-	-	-	-	-
Algeria	2	2	-	-	-	-	-	-	-
Malaysia	12	11	-	-	-	-	-	-	1
South Sudan	29	-	-	-	-	-	-	-	29
Sudan	38	6	13	-	-	5	-	-	14
Burkina Faso	18	7	-	-	-	-	-	-	11
Gambia	2	2	-	-	-	-	-	-	-
Laos	1	-	-	-	-	-	-	-	1
Senegal	11	6	-	-	-	-	-	-	5
Sierra Leone	34	-	-	-	-	-	-	-	34
Thailand	19	8	8	-	-	-	-	-	3
TOTAL	442	156	66	0	1	7	0	13	196

*NVD: no virus detected

Conventional surveillance often relies on the recognition and reporting of obvious clinical cases by farmers, livestock workers or animal health service providers (Bates et al., 2003; Picado et al., 2011; Machira and Kitala, 2017). In areas where FMD is common, farmers may be able to easily identify the disease based on clinical presentation (Nyaguthii et al., 2019), and this has been shown to be comparable to the results of serological testing (Morgan et al., 2014).

Although passive surveillance is valuable, there are several limitations to this approach. For example, it is possible that farmers may not observe or correctly identify FMD, especially where clinical signs are mild (Knight-Jones et al., 2014), or in regions where FMD is uncommon. Furthermore, even when farmers identify FMD, they may not view it as serious enough to report to veterinary services, or may be deterred due to the repercussions of imposed control measures, as has also been observed for other diseases (Falzon et al., 2019). Moreover, where outbreaks are reported, extensive spread of the disease may have occurred between noticing the disease and actual reporting (Vosloo et al., 2002; Knight-Jones et al., 2016). As a consequence FMD is often under-reported, and it is difficult to determine the true incidence of the disease.

Passive surveillance activities may be supplemented by targeted case finding activities, but these may be infrequent due to the costs and labour involved (Hadorn and Stärk, 2008; Kasanga *et al.*, 2012). Alternative risk-based surveillance methods such as serological surveys can provide valuable information by identifying the presence of unreported infection (Caporale et al., 2012; Hoinville et al., 2013; Dhikusooka et al., 2016). Many studies have used the detection of NSP antibodies in serum samples for FMD surveillance (Kibore et al., 2013; Ehizibolo et al., 2014; Lyons et al., 2017; Souley Kouato et al., 2018), however due to the length of time that NSP antibodies may persist in the blood, these tests are unable to identify when exposure occurred (Elnekave et al., 2015), although stratifying animals into age groups and performing expensive longitudinal studies with frequent testing intervals may improve accuracy (Bertram et al., 2018; Farooq et al., 2018). Additionally NSP antibody tests are unable to identify the causal viral lineage, are time consuming, labour-intensive and expensive to perform, and they may be influenced by the use of non-NSP purified vaccines (Lee et al., 2006; Caporale et al., 2012).

Additionally, the capacity for undertaking outbreak investigation and collection of clinical specimens in resource-limited countries is often restricted (Kasanga et al., 2012; Namatovu et al., 2013). Even when samples are collected from suspect cases, the insufficient maintenance of a cold chain during sample transport, and/or poor quality sample laboratory storage due to failures in power supply, may lead to sample degradation and therefore potentially false negative test results (Vosloo et al., 2002; Namatovu et al., 2013; Zinsstag et al., 2016).

When outbreaks are reported and clinical samples collected, the recommended sample types submitted to laboratories for FMD diagnosis are epithelial tissue or fluid in vesicular lesions from acutely diseased animals, although blood and OP fluid are often received (King et al., 2006; OIE, 2018). Consequently, the majority of samples are taken from animals with recognisable clinical signs often at a single time point. This may result in sampling bias towards visibly clinically infected animals, in comparison to sub-clinically infected animals (such as vaccinated herds), although the role of subclinical infection in disease transmission is still to be elucidated (Sutmoller and Casas, 2002).

1.5.2 Reducing sampling bias

In order to reduce sampling bias, methods for sample collection using alternative sample types at points other than during the acute stage of disease have also been explored. For example, FMDV RNA has been detected in saliva and nasal swabs, and air samples before clinical signs became apparent (Alexandersen et al., 2003; Marquardt et al., 1995; Nelson et al., 2017; Stenfeldt et al., 2013). This may enhance early detection so that control measures may be put in place, if resources allow, before extensive spread of the disease may occur (Charleston et al., 2011; Nelson et al., 2017). The Chinese national surveillance program has also successfully identified FMDV in lymph node samples at slaughter (OIE, 2017b). Detection of FMDV has also been demonstrated in nasal fluid, saliva and OP fluid during late-stage infection (Stenfeldt et al., 2013; Parthiban et al., 2015), and in OP fluid in persistently infected (carrier) animals (Stenfeldt et al., 2013; Lohse et al., 2018). Surveillance of animals using this method may facilitate the clarification of freedom from disease in a herd or region, and may identify the likelihood of onward transmission (Caporale et al., 2012; Parthiban et al., 2015). Detection of virus at these late stages may identify infected animals that were previously overlooked due to mild clinical signs, or sub-clinical infection.

1.5.3 Detection of sub-clinical FMD infection

Several studies have identified the presence of FMDV RNA in sub-clinically infected animals, including cattle (Bertram et al., 2018; Hayer et al., 2018), Asian water buffalo (*Bubalus bubalis*) (Farooq et al., 2018) and African buffalo (*Syncerus caffer*) (Vosloo et al., 2007; Wekesa et al., 2015; Dhikusooka et al., 2016; Maree et al., 2016), although the proportion of animals affected is unknown. Additionally, the occurrence of sub-clinical FMDV infection in vaccinated animals has also been reported (Hafez et al., 1994; Hutber et al., 1999; Lyons et al., 2017; Hayer et al., 2018; Stenfeldt et al., 2018), and that newly infected sub-clinical animals may shed considerable amounts of infectious FMDV, despite no manifestation of clinical signs (Stenfeldt et al., 2015). Consequently, this information may affect control policies where vaccination is practiced, if FMDV can continue to circulate sub-clinically. Although the detection of NSP antibodies is a valuable tool in the identification of sub-clinical animals (Lyons et al., 2017; Farooq et al., 2018), previous infection of subject animals must be ruled out, which may involve costly longitudinal studies. Consequently, most studies have used the detection of FMDV in OP fluid to identify sub-clinically infected animals. This method however, must be performed by a trained individual, is labour intensive, and highly invasive for the animal (Kitching, 2002; Stenfeldt et al., 2013). Additionally, OP fluid samples are less likely to be collected from smaller animals such as sheep and goats, due to the invasive nature of the technique and the different sizes of instrumentation required.

The potential risk and significance for FMD epidemiology from sub-clinically infected animals is not fully understood. Further studies focussing on the detection of natural sub-clinical infection utilising novel surveillance strategies may reduce the bias observed with conventional surveillance methods and facilitate improved knowledge on viral prevalence, dynamics and the emergence of new lineages (Hutber et al., 1999; Sutmoller and Casas, 2002; Farooq et al., 2018; Stenfeldt et al., 2018).

1.6 Milk

Milk is a non-invasive sample type, collected from lactating cattle on a daily basis, and has the advantage that both FMD virus and antibodies can be detected (Blackwell and McKercher, 1982; Armstrong, 1997). Milk has been utilised for the detection of several pathogens and the antibodies raised against them, including *Brucella* spp. (Vanzini et al., 1998; Hamdy and Amin, 2002), bovine viral diarrhoea virus (BVDV) (Drew et al., 1999; Renshaw et al., 2000a; Hill et al., 2010), Schmallenberg virus (Daly et al., 2015), *Coxiella burnetti* (Kim et al., 2005), bovine respiratory syncytial virus (Elvander et al., 1995), and *Neospora caninum* (González-Warleta et al., 2011). Furthermore, the use of pooled milk samples from individual animals, or collecting samples of milk from a bulk tank or milk line enables a cost-effective surveillance approach which has been validated for the routine surveillance of diseases such as brucellosis (DEFRA, 2015a) and mastitis caused by *Mycoplasma* spp. (APHIS, 2008).

An example of using pooled milk in a national surveillance system is for brucellosis in the United Kingdom (U.K.). The Brucellosis (England) Order 2000 (DEFRA, 2015a) aims to maintain the 'Officially Brucellosis Free' status by ensuring animals are free from infection. This programme involves the submission of quarterly bulk milk samples from commercial dairy farms to the Animal and Plant Health Agency (APHA) for testing by the Brucellosis Bulk Milk ELISA (DEFRA, 2015b; Musallam et al., 2017). This method is non-invasive, involving milk already collected and destined for consumption, and therefore is more cost-effective than the serological sampling of individual animals (Rolfe and Sykes, 1987).

Additionally, many studies have investigated the surveillance of bovine viral diarrhoea virus using pooled milk samples (Niskanen et al., 1991; Paton et al., 1998; Kuijk et al., 2008, among others), determining this method to be useful for cost-effective surveillance. Consequently, as a part of the BVDFree Scheme in the U.K. (BVDFree, 2019), bulk milk samples are collected from dairy farms, and sent to various laboratories for either antibody or virus detection by ELISA and RT-PCR respectively. This scheme identifies infected herds so that control measures may be implemented, with the aim of eliminating BVDV from all cattle by 2022.

It is hypothesised that similar schemes could be designed for FMD surveillance in endemic regions or during outbreak scenarios in FMD free countries. However, unlike infection with BVDV, in which animals may become persistently infected and shed large amounts of virus throughout their lifetime (Brock et al., 1998), FMD infection is generally not maintained within an individual or herd for long periods, other than in OP fluid (Stenfeldt et al., 2013), which requires invasive sampling. Consequently, the window of detection for cost-effective FMD surveillance is much smaller than that of BVDV. The potential of milk as a suitable sample type for FMD surveillance is discussed in more detail below.

1.6.1 Detection of FMDV in milk

Experimental inoculation of the mammary gland with FMDV has shown that it is an organ that is highly susceptible to viral replication (Burrows et al., 1971), and that virus is readily excreted in the milk of infected animals (Lebailly, 1920; Terbruggen, 1932; Burrows, 1968). After experimental infection of dairy cattle with FMDV, live virus has been detected in milk by virus isolation and plaque assay before the appearance of clinical signs (Burrows, 1968; Hedger and Dawson, 1970; Blackwell and McKercher, 1982). Live FMDV was also detected up to 51 days post-infection (Burrows et al., 1971), although this experiment involved inoculation of virus directly into the udder, which is unlikely to be a natural route of infection. Additionally, experiments have inoculated FMDV infected milk into various animals such as suckling mice, guinea pigs and calves (Sellers, 1969; Felkai et al., 1970) to determine virus infectivity and the onward risk of transmission with similar limitations from the unnatural inoculation routes. Nevertheless, these experiments demonstrate the possible extended excretion periods of virus into milk, when compared to other sample types such as serum and vesicular epithelium, and highlight the potential risk of infected milk for virus transmission.

1.6.2 Detection of FMDV RNA in milk

With a transition towards rapid, automated and high-throughput diagnostic methods, it has also been demonstrated that FMDV RNA can be detected in the milk of infected animals by real-time RT-PCR (Reid et al., 2006; Ahmed et al., 2017), multiplex PCR and RT-LAMP (Ranjan et al., 2016). A study by Reid et al. (2006) showed that after lactating cattle were kept in direct contact with experimentally infected lactating cattle to simulate a natural route of infection, virus was detected in milk by rRT-PCR for a longer time period than by conventional virus isolation methods. In some of the animals, FMDV RNA was detected before the appearance of clinical signs, and up to 23 days post infection in milk from the inoculated cattle. Additionally, in a study by Ahmed et al., (2017) FMDV RNA could be detected by rRT-PCR in the milk of apparently healthy NSP positive vaccinated Asian water buffaloes (*Bubalus bubalis*), suggesting the presence of sub-clinical infection in this species.

Serotype identification of FMDV in milk samples has also been demonstrated by molecular methods (Saeed et al., 2011; Ahmed et al., 2017). Furthermore, Saeed et al. (2011) reported successful FMDV sequence analysis of milk collected from acutely infected animals, and studies on different viruses support the potential of obtaining sequence data from milk samples, for example vaccinia virus in milk from dairy cattle (Abrahão et al., 2009) and human immunodeficiency virus from human breast milk (Salazar-Gonzalez et al., 2011).

1.6.3 Detection of FMD antibodies in milk

It has also been demonstrated that antibodies to FMDV can be detected in cattle milk, from 7 days post infection and up to 12 months post vaccination (Stone and DeLay, 1960). Antibodies to FMDV in milk samples have been detected using a liquid-phase blocking ELISA (LPBE) and a specific isotype assay (SIA) for bovine Immunoglobulin G, (Armstrong, 1997; Armstrong et al., 2000), and the authors hypothesised that both of these tests could be applied in surveillance schemes to identify exposed cattle/herds. A significant correlation between antibody levels in serum and milk has also been observed (Armstrong and Mathew, 2001; Fayed et al., 2013) and it has been

hypothesised that milk collected from infected cattle may contain higher levels of virus-specific antibody than serum, as serum antibody is concentrated into mammary secretions (Stone and DeLay, 1960). However in a study using milk from Asian buffaloes, Yadav et al. (2007) observed lower levels of FMDV-specific antibodies than in serum. Nonetheless, milk could be a useful alternative sample type to blood for surveillance or post-vaccination monitoring (Fayed et al., 2013), to complement current systems.

1.6.4 The impact of FMDV infection on milk yield

For milk to comprise a suitable sample type for FMD surveillance, it is important to have confidence that the ability to collect this sample is not affected by disease presence. Many studies document a reduction in milk yield during FMDV infection (see Table 1.2)(James and Rushton, 2002; Senturk and Yalcin, 2005; Knight-Jones and Rushton, 2013; Jemberu et al., 2014; Casey-Bryars et al., 2018). Interestingly, local breeds were estimated to have significantly smaller losses than Holstein dairy cattle (Senturk and Yalcin, 2005). These studies report highly variable losses although are all estimates based on farm surveys or expert opinion with relatively few empirical studies quantifying the reduction in milk output (see Table 1.2). One such example is from a longitudinal study in Pakistan carried out by Ferrari et al., (2014) that demonstrated a significant reduction in milk yield from individual cattle and buffaloes in the 60 days following the onset of acute clinical FMD. Additionally, Ansari-Lari et al., (2017) demonstrated a significant decline in individual daily milk production after an FMD outbreak compared to before the outbreak, although the drop was small (up to 8% over a 42 day outbreak period). Furthermore, Lyons et al., (2015) aimed to quantify objectively the impact of FMD in milk yields. During an outbreak of FMDV SAT 2 on a large scale dairy farm housing mainly European breed cattle in Kenya, they observed that although there was up to an approximate 35% decrease in milk production at the herd level, no statistical evidence was found to indicate a significant decrease in milk yield between FMD clinical animals and non-clinical cases (Lyons et al., 2015).

Interestingly, reports to date on the effect of FMDV infection on milk yield based on empirical data do not specify a complete cessation of milk production at any time. This

demonstrates the potential availability of milk as a diagnostic sample type during infection, as it is expected that it would still be possible to collect milk from individual lactating cows before, during and after FMD infection. However, factors such as the development of clinical mastitis as a result of vesicular lesions on the teats (Kitching, 2002; Sharma, 2010; Lyons et al., 2015), may mean that milk from this animal is not contributed for herd level milk sampling.

Table 1.2 Summary of studies reporting the impact of FMD on milk yield in cattle. Adapted from Lyons etal. (2015)

Country	Study period	Farming system	Breed	Type of study	Estimated milk yield loss	Reference
UK	N/A	UK based dairy farms	Not specified	Expert opinion	25% in first four years nationally after incursion into FMD free UK; 12.5% thereafter if becomes endemic	Power and Harris (1973)
Pakistan	1976	Large-scale	Sahiwal	Longitudinal	74.4 litres of milk lost per affected lactation	Kazimi and Shah (1980)
Bangladesh	1988-1991	Not specified	Not specified	Post-outbreak farm surveys	66% reduction in average daily yield	Chowdhury *et al.* (1993)
India	1991	Smallholder	Indigenous and cross-breed	Post-outbreak farm surveys	14–19% reduction in the annual yield of an affected animal	Saxena (1994)
Turkey	N/A	Turkish dairy farms	Holstein-Friesan	Expert opinion	22% and 10% milk yield loss in current lactation for Holstein Friesian and local breeds, respectively	Senturk and Yalcin (2005)
South Sudan	2005	Agropastoralists	Indiginous	Post-outbreak interviews (Participatory epidemiology methodology)	62% reduction while sick. Average 14 day illness. Mean daily loss 1.6 litres per cow compared to normal 2.6 litres	Barasa *et al.* (2008)
Ethiopia	2008	Large-scale	Fogera	Longitudinal	50% of pre-outbreak level	Mazengia *et al.* (2010)
Ethiopia	2008	Pastoral and agro-pstoral	Borana	Post-outbreak farm surveys	Acute phase: 1.37 litres/cow/day for average 25.5 days (73.3% reduction while sick; 7.7% reduction per lactation). Chronic phase: 0.67 litres/cow/day for 3.8 months (78% reduction per lactation)	Bayissa *et al.* (2011)
Kenya	Not specified	Pastoralists	Indigenous	Farmer surveys (Participatory epidemiology methodology)	53% reduction in a herd during outbreak period	Onono, Wieland and Rushton (2013)

Country	Study period	Farming system	Breed	Type of study	Estimated milk yield loss	Reference
Pakistan	Not specified	Smallholder	Not specified	Longitudinal	51.8% of potential during outbreak	Ferrari *et al.* (2014)
Ethiopia	2012-2013	Pastoral and smallholder	Indigenous	Post-outbreak farm surveys	75% of pre-outbreak level (average loss of 1.8 litres/cow/day)	Jemberu *et al.* (2014)
Kenya	2012	Large-scale	European-breed cattle	Longitudinal	Animals in parity ≥4, between 0 and 50 DIM at the start of the outbreak, produced on average 688.7 kg (95%CI 395.5, 981.8) less milk than predicted for their remaining lactation. This represents an average 15% reduction in the 305 day production.	Lyons *et al.* (2015)
India	2013-2014	Organised dairy cattle farms	Crossbred	Milk production records - longitudinal	Milk production reduced by 85%, 67%, 45 % and 81 % on four dairy farms.	Ranjan, Biswal, A. K. Sharma, *et al.* (2016)
Pakistan	Not specified	Not specified	Cattle and Asian buffalo	Post-outbreak farm surveys	Total loss of 225 litres (51% of the predicted value) per lactating buffalo, and 195 litres (31% of the predicted value) per lactating cattle.	Ali *et al.* (2017)
Iran	2014	Industrial dairy herd	Holstein	Post-outbreak farm surveys	Total reduction of 8.0 (lactation one cows) and 4.7% (lactation ≥2 cows) in mean daily milk production per cow after the outbreak when compared with before (over a 42 days outbreak period).	Ansari-Lari *et al.* (2017)
Tanzania	2011–2014	Pastoral, agropastoral and rural small-holders	Not specified	Post-outbreak farm surveys	FMD was associated with considerably lower herd milk yield (mean percentage decrease 67%), with 90% of respondents reporting reduced cowmilk production during outbreaks.	Casey-Bryars *et al.* (2018)

1.6.5 FMDV detection in pooled or bulk tank milk

The potential for detection of FMDV in milk samples from individual animals has been described above. Theoretically, sampling of milk at the herd level or even further up the milk production chain could also offer a cost-effective framework for FMD surveillance. Currently, the impact of pooling on the detection sensitivity of FMDV in milk has not been sufficiently studied. Using milk samples from experimentally 'in-contact' infected cattle diluted in uninfected whole milk, Reid et al., (2006) demonstrated that FMDV RNA could be detected by rRT-PCR down to a dilution of 10^{-4}, and by virus isolation down to a dilution of 10^{-3}. Consequently, they hypothesised that the FMDV rRT-PCR assay could detect the presence of a single infected animal in a sample from the bulk milk of a herd of up to 10,000 animals. Utilising these data, simulation modelling studies by Thurmond and Perez, (2006), and Garner et al., (2016) aimed to estimate when FMD virus could be detected by rRT-PCR in bulk milk during an outbreak, and found that this approach could be useful for the detection of pre-clinical infection, before the appearance of clinical signs in the herd. As a result, they suggested that pooled milk could be a useful tool in enhancing a surveillance system for FMD, and that this approach should be considered for regional FMD surveillance.

However, to estimate prevalence of FMDV infection from bulk tank milk, pooling and sampling schemes should be carefully assessed, taking into account the analytical sensitivity of the chosen detection assay (Christensen and Gardner, 2000; Reichel et al., 2016). Ahmed (2015) investigated the effect of pool size and found that larger pool sizes maximised pooling efficiency at low disease prevalence, whereas smaller pool sizes maximised efficiency at a higher prevalence. It is likely then that a bulk tank milk testing system could indeed detect small quantities of FMD virus, and identify the presence of even one newly infected animal, which may provide a useful surveillance tool for rapidly detecting infected herds. Additionally, farm/farmer selling trends which are difficult to quantify and therefore may not be incorporated into models, should be taken into consideration as these may have a large impact on the utility of pooled milk for FMD surveillance.

1.7 Scientific aims

Milk has the potential to be a suitable alternative sample type to those currently used for FMD diagnosis. The benefits of this sample type are clear:

- Simple to collect – it may already be collected as part of routine surveillance of other pathogens
- Non-invasive compared to other sample types such as vesicular epithelium or blood
- FMD live virus, FMDV RNA and FMDV antibody can be detected
- FMDV RNA may be detected by rRT-PCR for a longer window than other sample types i.e. before, during and after the appearance of clinical signs
- Potential for the identification of sub-clinical infected animals and herds
- rRT-PCR assay can detect FMDV in heavily diluted milk demonstrating the potential for bulk tank milk testing.

Despite the clear advantages of milk as an alternative sample type, and although FMDV detection in milk samples has been well described during *in-vivo* experiments (Burrows et al., 1971; Blackwell and McKercher, 1982; Reid et al., 2006), only a small number of studies have described the detection of FMDV RNA in milk from naturally-infected animals. These include FMDV detection in milk from cattle and buffaloes in Pakistan (Saeed et al., 2011; Ahmed et al., 2017) and in cattle in India (Ranjan et al., 2016). Furthermore, the limited milk samples used in these studies were collected either as an additional sample type to validate molecular assays, or to investigate the possible role of milk in FMDV transmission.

Given the benefits described above, further investigation into the potential of milk as an alternative sample type for routine FMDV detection and surveillance is warranted, especially as this approach is already successfully utilised for a number of other pathogens. Sampling of milk both at an animal and herd level could offer a more representative sampling framework compared to established surveillance methods, reducing sample selection bias, increasing surveillance sensitivity, and may facilitate a more thorough understanding of herd/district level epidemiology.

It is hypothesised that sampling of milk at the herd level, for example by taking aliquots of milk from bulk tanks, may be useful for targeted/risk-based surveillance to:

- improve knowledge on the epidemiology of FMD in endemic areas, including determination of circulating serotypes, and identification of sub-clinical infections
- rapidly identify infected herds in response to an outbreak in a disease-free country
- screen infected premises after an outbreak to ensure disease freedom

Therefore the aim of this thesis was to expand on previous studies to determine whether milk may be utilised for FMDV detection and surveillance. Consequently, this thesis is organised to answer five specific research objectives (see figure 1.9):

(i) Optimise a high-throughput nucleic acid extraction and one-step real-time RT-PCR method to detect foot-and-mouth disease virus (FMDV) in milk samples.

(ii) Determine the utility of milk samples compared with sample types currently used, collected from individual experimentally and naturally infected animals.

(iii) Evaluate the stability of FMDV in milk samples during transportation.

(iv) Determine the effects of pooling milk on the sensitivity and specificity on the FMDV detection system.

(v) Assess the potential of FMDV testing of pooled milk in different farming systems as an alternative surveillance approach, by comparison with established surveillance methods.

Chapter 2:
Optimisation and evaluation of a high-throughput screening method for the detection of foot-and-mouth disease virus in milk samples
(i),(ii),(iv)

Chapter 3:
Opportunities for enhanced surveillance of foot-and-mouth disease in endemic settings using milk samples from individual cattle
(ii)

Chapter 4:
Moving from individual to pooled milk: considerations for the treatment of samples collected from herds in endemic countries
(iii),(iv)

Chapter 5:
Utilising milk from pooling facilities as a novel approach for foot-and-mouth disease surveillance
(v)

Chapter 6:
Pooled milk for foot-and-mouth disease surveillance on large-scale dairy farms in endemic settings
(v)

Chapter 7:
Discussion and future research

Figure 1.9 Overview of thesis. Numerals in parenthesis indicate the research objectives focussed on in each chapter.

CHAPTER 2

Optimisation and evaluation of a high-throughput screening method for the detection of foot-and-mouth disease virus in milk samples

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Detection of foot-and-mouth disease virus in milk samples by real-time reverse transcription polymerase chain reaction: Optimisation and evaluation of a high-throughput screening method with potential for disease surveillance

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2.1 Summary

This study aimed to assess the performance of an optimised nucleic acid extraction protocol utilising robotic equipment in combination with a one-step real-time RT-PCR method to detect foot-and-mouth disease virus (FMDV) in milk samples, in order to assess the utility of milk as a non-invasive sample type for surveillance. Four milking Jersey cows were infected via direct contact with two non-milking Jersey cows that had been previously inoculated with FMDV (isolate O/UKG/34/2001). Milk and blood were collected throughout the course of infection to compare two high-throughput real-time reverse transcription polymerase chain reaction (rRT-PCR) protocols with different RT-PCR chemistries. Using both methods, FMDV was detected in milk by rRT-PCR one to two days before the presentation of characteristic foot lesions, similar to detection by virus isolation. Furthermore, rRT-PCR detection from milk was extended, up to 28 days post contact (dpc), compared to detection by virus isolation (up to 14 dpc). Additionally, the detection of FMDV in milk by rRT-PCR was possible for 18 days longer than detection by the same method in serum samples. FMDV was also detected with both rRT-PCR methods in milk samples collected during the UK 2007 outbreak. Dilution studies were undertaken using milk from the field and experimentally-infected animals, where for one sample it was possible to detect FMDV at a dilution of 10^{-7}. Based on the peak C_T values detected in this study, these findings indicated that it was possible to identify one acutely-infected milking cow in a typical-sized dairy herd (100-1000 individuals) using milk from bulk tanks or milk tankers. These results motivated further studies using milk in FMD-endemic countries for FMD surveillance.

2.2 Introduction

Rapid and accurate detection is central to facilitate the control of FMD. Real-time reverse transcription polymerase chain reaction (rRT-PCR) assays have been developed with high diagnostic and analytical sensitivity (Shaw et al., 2004), and since they detect viral RNA (or even degraded genome) instead of intact viral antigens and/or live virus, these assays can be used on a number of sample types (Reid et al., 1998; Reid et al., 2003).

Milk is a non-invasive sample type that does not require qualified veterinary practitioners for collection, unlike traditional sample types such as vesicular lesion tissue or fluid, oesophageal-pharyngeal fluid, and blood. Previous experiments have shown that the mammary gland is highly susceptible to FMDV replication, and that FMDV can be detected in milk before the appearance of clinical signs (Burrows et al., 1971; Blackwell and McKercher, 1982; Reid et al., 2006). Milk therefore represents a potentially valuable sample source for FMDV detection and surveillance during, and in recovery from a disease outbreak.

Previous studies have investigated FMDV detection by rRT-PCR in milk samples from experimentally infected Holstein-Friesian cattle (Reid et al., 2006) using two-step amplification protocols. This chapter aims to build on this previous work, to assess the performance of a more recently developed nucleic acid extraction protocol utilising rapid, higher throughput robotic equipment and newer one-step real-time RT-PCR kits to detect FMDV in milk. Two protocols were compared employing the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems®) for RNA extraction, in combination with either the TaqMan® Fast Virus 1-Step kit (Applied Biosystems®) (Method A), or the Superscript III Platinum® One-Step qRT-PCR Kit (Invitrogen™) (Method B). Although these methods are currently utilised in FMD diagnostic laboratories for the traditional samples mentioned above, they have not been fully validated for use on milk samples. Unlike the other sample types, milk contains high concentrations of calcium, proteinases and fat globules that have been shown to inhibit amplification efficiency (Rossen et al., 1992; Bickley et al., 1996). Although this was not observed in the previous study by Reid et al. (2006), they used milk from Holstein-Friesian cattle, which has a lower fat concentration than milk from Jersey cattle, for example

(Palladino et al., 2010). Therefore, in order to fully challenge the RNA extraction conditions of the methods tested in this chapter, Jersey cows, which produce milk with a high fat content, were used. Additionally, the effect of FMD on milk yield in Jersey cows was also assessed.

It is anticipated that the results from this study can be used to support the development of an FMD surveillance plan utilising pooled milk in endemic settings, or from bulk tanks as part of preparedness for combating a possible FMD outbreak in disease-free settings.

2.3 Materials and Methods

2.3.1 Experimental samples

In-vivo studies were carried out in the high containment unit at The Pirbright Institute, UK and all procedures were approved by the Home Office (Project Licence number:70/718) and complied with the Animals (Scientific Procedures) Act 1986, EU Directive 2010/63/EU. Four naïve Jersey dairy cows (aged between 2 years, 9 months, and 8 years, 1 month), were infected via direct contact (day 0) with two non-milking Jersey cows that had been inoculated by intra-dermolingual injection with 10^5 $TCID_{50}$ FMDV O/ME-SA/PanAsia, O/UKG/34/2001 (0.25 mL per inoculation site [n=2]) two days previously, and that were displaying clinical signs. Animals were observed for clinical signs, and sampled every day for blood and milk on days -5 to 7, and 10, 12, 14, 19, 21, 26 and 28 days post contact (dpc). Blood was collected in plain vacutainers and serum used in the testing. Milk was collected by machine twice a day until 7 dpc, and once a day thereafter on the days stated, and daily milk yields recorded by weight. Skimmed milk was separated from the cream and cell fraction by centrifuging an aliquot of each whole milk sample at 3000xg (Hettich Rotanta 460R) for ten minutes.

2.3.2 Field samples

Twelve milk samples collected during the FMDV outbreak in the UK in 2007 (caused by a derivative of FMDV O_1 BFS 1860) were used to compare diagnostic screening methods. These samples were from individual cows displaying clinical signs held at one of the infected premises (IP) 2 (Cottam et al., 2008; Ryan et al., 2008).

2.3.3 Cell culture isolates

FMDV cell culture isolates (isolated once in primary bovine thyroid [BTY] cells) were obtained from the FMDV repository held at the World Organisation for Animal Health (OIE) Reference Laboratory and United Nations Food and Agriculture Organization (FAO) World Reference Laboratory for foot-and-mouth disease (WRLFMD), Pirbright, UK. Positive controls for rRT-PCR assays were prepared by spiking unpasteurised whole Jersey milk with a 10^{-2} dilution of cell culture isolate O/SAU/1/2016. Analytical sensitivity of the diagnostic screening methods was assessed using a ten-fold dilution series (10^{-1} to 10^{-8}) of cell culture isolate A/KEN/6/2012 in whole Jersey milk.

2.3.4 Virus Isolation

Virus isolation was carried out on primary bovine thyroid (BTY) cell cultures (Snowdon, 1966), on all experimental samples on the day of collection. Titrations were later performed on milk samples using BTY cell cultures after storage at -80^{o}C, and the viral titre was calculated using the Spearman-Kärber method, as described by the FAO (Karber, 1931; OIE, 2018) and expressed in units of $TCID_{50}$/mL.

2.3.5 Diagnostic screening methods

Diagnostic screening methods for the detection of FMDV genome in milk samples are defined as Method A and Method B for the purpose of this study, and are described in Table 2.1.

Table 2.1 Comparison of the two high-throughput foot-and-mouth disease virus detection methods.

	A	B
Extraction kit	MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems®)	MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems®)
Internal Control	VetMAX™ Xeno™ Internal Positive Control RNA (Applied Biosystems®)	VetMAX™ Xeno™ Internal Positive Control RNA (Applied Biosystems®)
Sample input	200 µL	200 µL
rRT-PCR kit	'TaqMan® Fast' Virus 1-Step Master Mix (Applied Biosystems®)	'Superscript' III Platinum® One-Step qRT-PCR Kit (Invitrogen™)
Internal control assay	VetMAX™ Xeno™ Internal Positive Control LIZ™ Assay (Applied Biosystems®)	VetMAX™ Xeno™ Internal Positive Control LIZ™ Assay (Applied Biosystems®)
Primers and Probes	Targeting 3D polymerase (Callahan et al., 2002)	Targeting 3D polymerase (Callahan et al., 2002)
RNA template input	2.5 µL	5 µL

2.3.6 RNA extraction

RNA extractions for both methods were carried out using the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems®) on a MagMAX™ Express 96 Extraction Robot (Applied Biosystems®) with a sample input of 200 µL, and elution volume of 90 µL. One µL per reaction of VetMAX™ Xeno™ Internal Positive Control RNA (10,000 copies/µL) (Applied Biosystems®) was also added to the lysis buffer prior to extraction.

2.3.7 rRT-PCR

Two commercially available rRT-PCR kits were evaluated as listed in Table 2.1. In Method A, the TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems®) was used with the following thermal cycling conditions: 50°C for 5 min, 95°C for 20 sec, then 45 cycles of 95°C for 3 sec and 60°C for 30 sec. For this method 2.5 µL of RNA template were added to the rRT-PCR reaction mix containing 6.25 µL of 1-step mastermix (4x, supplied with the kit), 0.25 µL each of forward and reverse primer (20 µM), 0.25 µL probe (10 µM), and 14.5 µL of nuclease free water. In Method B, the Superscript III Platinum® One-Step qRT-PCR Kit (Invitrogen™) was used with the

reagents, parameters and thermal cycling conditions previously reported (Shaw et al., 2007), with an RNA template volume of 5 µL. Primers and probes targeting the conserved 3D region of the FMDV genome (Callahan et al., 2002) were used for both methods. This assay has been previously shown to reliably detect viral RNA representing all seven FMDV serotypes (King et al., 2006) and is a widely adopted diagnostic assay recommended by the OIE for use in FMD Reference Laboratories. One µL VetMAX™ Xeno™ Internal Positive Control LIZ™ Assay (Applied Biosystems®) per reaction was also included in the reaction mix. The Applied Biosystems® 7500 Real-time PCR System was used on the 'fast' setting for Method A and the 'standard' setting for Method B. Evaluation of the RNA extraction and rRT-PCR methods were performed using experimental and field milk samples. Samples were considered positive for all C_T values observed until the end of the assay: ≤45 for Method A and ≤50 for Method B.

2.3.8 Statistics

In order to measure the agreement between the two methods using experimental whole milk samples, Cohen's Kappa statistic (κ) and the proportion of observed agreement (A_{obs}) were performed in R version 3.5.3 (R Core Team, 2019) using the package 'fmsb' (Nakazawa, 2017), and interpreted as described by Landis and Koch (Landis and Koch, 1977), and linear regression was used to compare C_T values. A paired t-test was used to compare C_T values from both methods using field samples. Unpaired t-tests were used to compare average milk yields before (-6-0 dpc) and during infection (1-6 dpc), both performed in Prism version 7 (GraphPad Software, Inc.).

2.4 Results

2.4.1 Comparison of detection methods with field samples

Twelve milk samples positive for FMDV collected from individual cows during the UK 2007 FMD outbreak were tested using both methods (A and B). Comparisons between the methods demonstrated lower C_T values in all samples when using Method B (Table 2.2) ($p = <0.001$), with a mean C_T difference of 5.00 between the two methods. Positive rRT-PCR results were observed in 12/12 (100%) for both methods.

Table 2.2 C_T values of individual milk samples collected from individual cows obtained from infected premises (IP) 2, from the 2007 UK outbreak of foot-and-mouth disease (FMD) for both methods. (Verification of clinical signs from these animals and formal confirmation of the FMD outbreak was completed by the Pirbright Institute (Ryan et al., 2008)).

Sample ID	Age of oldest lesion	Method A	Method B
c27	Not dated	21.19 (±0.45)	16.50 (±0.28)
105	2 days	21.59 (±0.22)	17.18 (±0.20)
036	5 days	26.18 (±0.17)	22.03 (±0.28)
027	6 days	27.07 (±0.15)	21.46 (±0.15)
369	6 days	24.98 (±0.17)	19.67 (±0.15)
341	6 days	27.15 (±0.14)	21.81 (±0.12)
069	4 days	25.26 (±0.11)	20.15 (±0.20)
030	5 days	27.79 (±0.25)	21.78 (±0.43)
161	2 days	29.58 (±0.08)	24.38 (±0.19)
092	5 days	32.27 (±0.19)	27.94 (±0.30)
241	3 days	22.04 (±0.39)	16.81 (±0.29)
093	5 days	24.64 (±0.27)	20.09 (±0.74)

Data shown are mean C_T values of rRT-PCR performed for Methods A and B, with standard deviations in parentheses. C_T values are the mean of three replicates from independent extractions.

2.4.2 Comparison of detection methods by limit of detection

The limit of detection of both methods was compared using the ten-fold dilution series of FMD A/KEN/6/2012 spiked into whole Jersey milk (10^{-1} to 10^{-8}) (Figure 2.1). Without normalising for different sample input volumes, Method B demonstrated a one $\log_{10}$ increase in the limit of detection when compared with Method A when all wells were positive, and a range in the difference in average C_T value of between 5.33 and 6.30, for Methods A and B. For each dilution, the maximum standard deviation between three technical replicates was 3.55 (Method B, 10^{-7}).

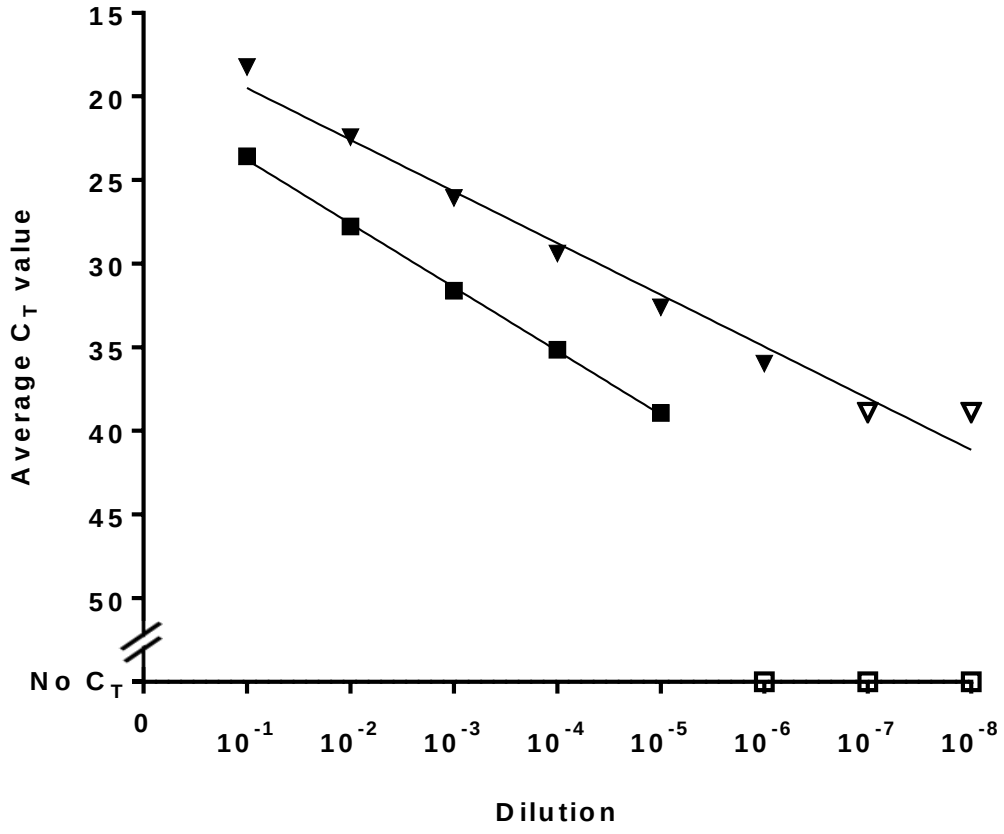


Figure 2.1 Comparison of the limit of detection for Methods A (used the TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems®)) and B (used the Superscript III Platinum® One-Step qRT-PCR Kit (Invitrogen™)). C_T values are the average of three replicates, and bars represent standard deviation. ■ : Method A, ▼: Method B. Open symbols indicate where no amplification was observed in at least one of the three replicates.

2.4.3 Experimental samples

The dairy cows (identified as animal numbers 108, 825, 867 and 951) exhibited clinical signs within 3-4 days after exposure to the inoculated cattle. Cows 108 and 825 developed mastitis and were euthanised at 3 dpc and 14 dpc, respectively. Both 867 and 951 survived to 28 dpc when the experiment was terminated.

Experimental samples were tested with both methods, after a freeze thaw and storage at -80°C for five years. Based on the testing of 67 whole milk samples, there was agreement (in at least one replicate) between positive and negative results in 61/67 (91.0%) samples across both methods (Figure 2.2).

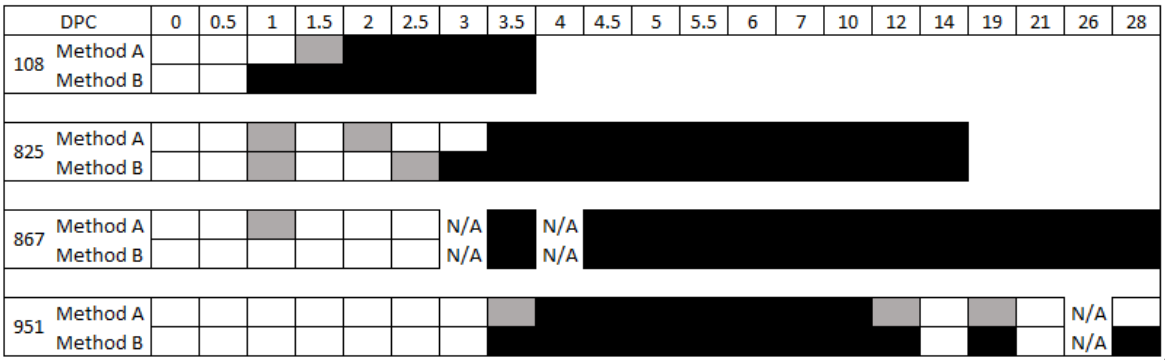


Figure 2.2 Comparison of both methods tested with whole milk samples from four experimentally infected cows. Each square represents the average C_T value of the whole milk sample at each day post contact (DPC). White squares represent a 'No C_T' value – no detection. Black squares represent any C_T value ≤45 (Method A) or ≤50 (Method B) in all replicate wells – FMDV positive. Grey squares represent instances where a 'No C_T' value was observed in one or two wells, but a positive result was observed in the other replicates. N/A represents where there was not sufficient sample available for testing.

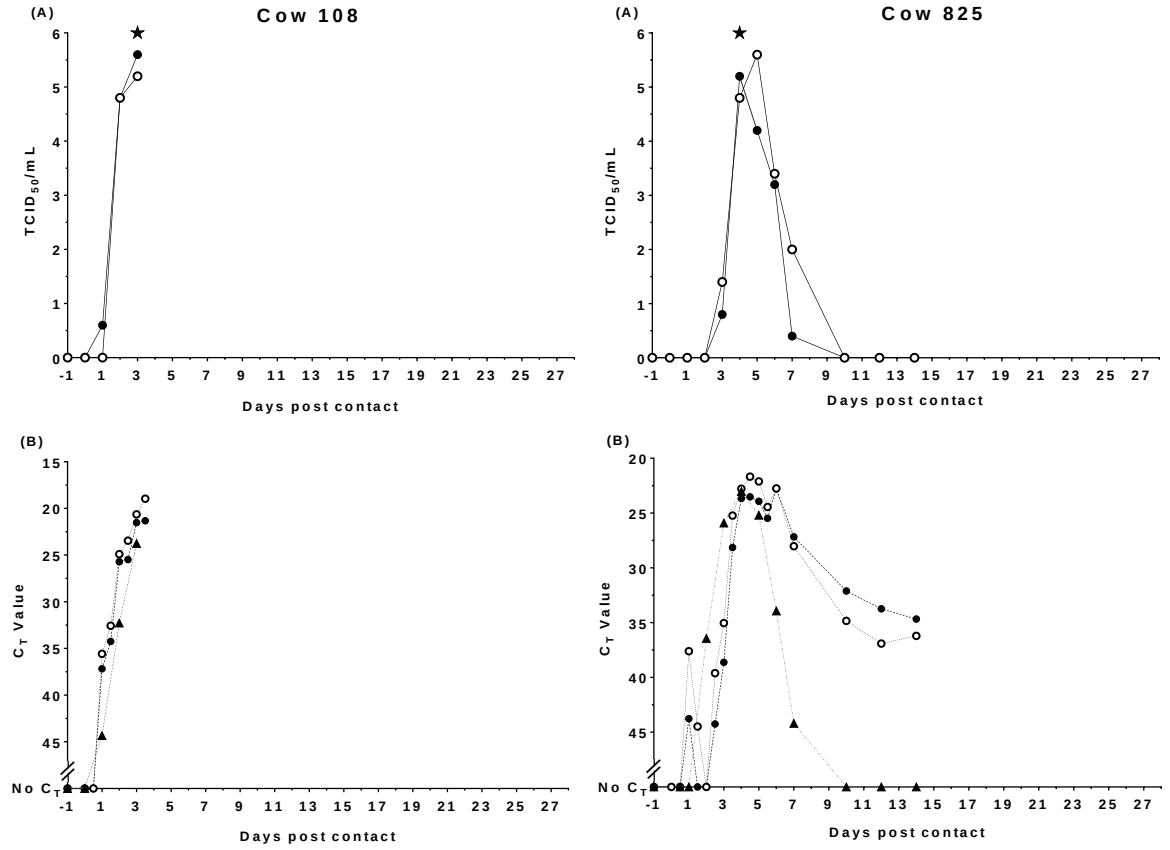
When comparing the two methods, almost perfect agreement was observed between the number of positive/negative samples identified ($\kappa = 0.811$; $p = <0.001$; $A_{obs} = 0.910$) (Table 2.3). Additionally, for the milk samples that were positive using both methods, the average C_Ts generated were lower when using Method B ($R^2 = 0.704$, $p = 0.001$) (see Appendix I, Figure 8.1). C_T values of the internal controls in all whole milk samples (n=67) were considered positive by both methods (Method A: mean: 35.37±0.83, Method B: mean: 38.23±2.42). Results from Method B were therefore used to determine the window of virus detection in dairy cows.

In most instances at the onset of infection, FMDV detection in milk by rRT-PCR coincided with detection by virus isolation, 1-2 days before the appearance of characteristic foot lesions, and concurrent with the development of nasal discharge in animals 867 and 951. FMDV detection by rRT-PCR in whole milk was observed for animals 108 and 825 until they were euthanised at 3 dpc and 14 dpc respectively (Figure 2.3). In addition to early detection, FMDV detection in both milk fractions (whole and skimmed) by rRT-PCR was prolonged, and was extended in whole milk (detected up to dpc 28 for animals 867 and 951), in comparison to virus isolation (detected up to dpc 7 for all three remaining cows). At the onset of infection, rRT-PCR detection of FMDV in serum coincided with FMDV detection in milk, 1 day prior (animals 867, 825 and 951) and the same day (108). In contrast, rRT-PCR FMDV detection in serum ended at 7dpc and 10 dpc, compared to at 28dpc in milk for animals 951 and 867, respectively.

Table 2.3 Comparison of Method A and Method B using experimental whole milk samples.

		Method B		
		Positive*	Negative	Total
Method A	Positive*	38	2	40
	Negative	4	23	27
	Total	42	25	67
		$\kappa = 0.811$; $p = <0.001$; $A_{obs} = 0.910$		

*Positive results are those with at least one well giving a C_T of ≤45 (Method A)/≤50 (Method B).


(A)
Cow 108
TCID50/mL
Days post contact
(B)
CT Value
No CT
Days post contact
(A)
Cow 825
TCID50/mL
Days post contact
(B)
CT Value
No CT
Days post contact

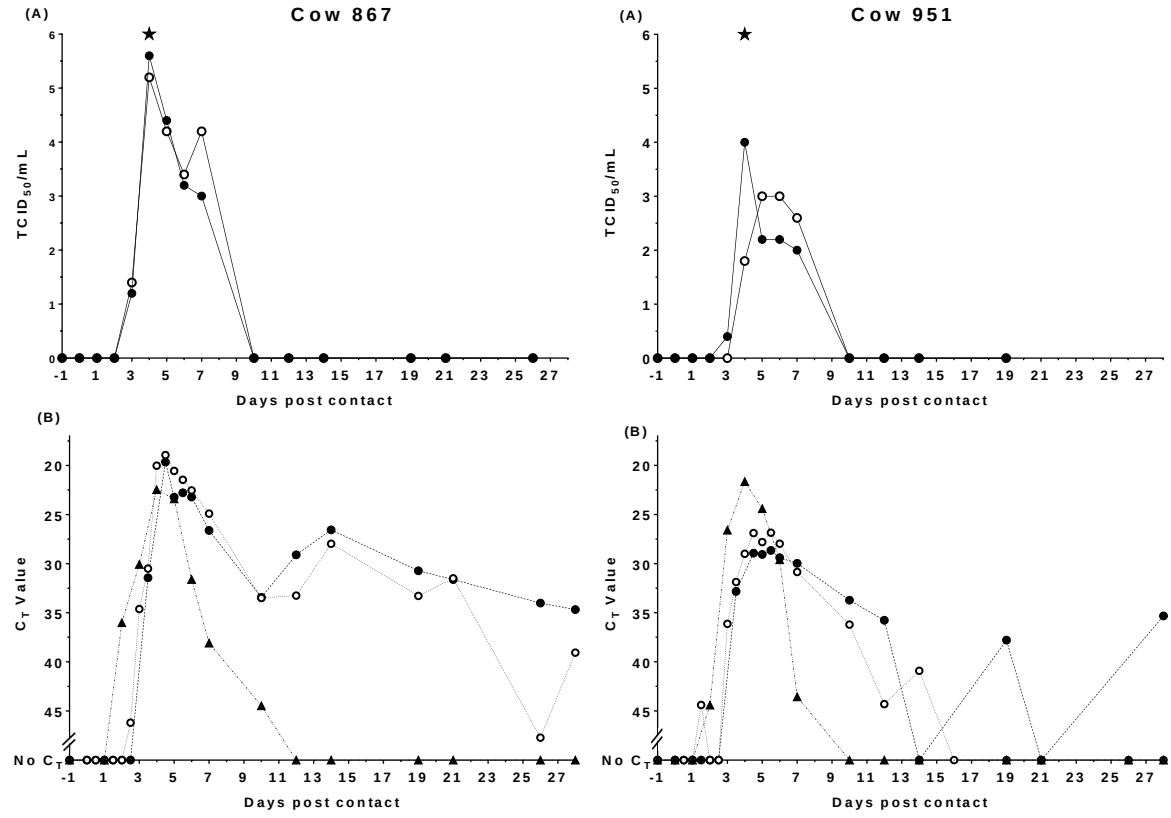

(A)
Cow 867
TCID50/mL
Days post contact
(B)
CT Value
No CT
Days post contact
(A)
Cow 951
TCID50/mL
Days post contact
(B)
CT Value
No CT
Days post contact

Figure 2.3 FMDV detection in samples collected at regular intervals from all cows. Virus titrations in BTY cells (A) and rRT-PCR using Method B (B) for skimmed and whole milk fractions and serum (B only). Average C_T is derived from the mean of 2 replicates. The development of lesions in at least one foot indicates the onset of clinical signs. ★: Onset of clinical signs, ●: whole milk, ○: skimmed milk, ▲: serum.

2.4.4 Impact of FMDV infection on milk yields

Milk yields were recorded by weight on -5 to 6 dpc. The average daily milk yield before cows were infected by direct contact (-6 to 0 dpc) was 22.14±0.51kg, 20.29±0.45kg, 18.17±0.86kg and 18.36±0.43kg for animals 108, 825, 867 and 951, respectively, these values were used as a baseline to calculate the change in milk yield after infection. The average daily milk yield after infection between days 1-6 dpc, was 23.00±0.58kg, 22.44±0.82kg, 16.58±1.96kg and 15.08±1.59kg, with an average change of +3.88%, +12.15%, -8.73% and -17.85% for animals 108, 825, 867 and 951, respectively. No significant difference was observed between average yields before and after infection [$p = 0.356$ (108), $p = 0.450$ (867), $p = 0.056$ (951)], apart from for animal 825 [$p = 0.032$ (825)], which demonstrated an increase in average milk yield after infection. The maximum reduction in milk yield recorded on any one day was 50.47% for cow 867, on 6 dpc. The mean difference in milk yield between -6 to 0 dpc and 1 to 6 dpc was greatest for cow 951 (-17.85%, range:-48.26% to +3.49%).

2.4.5 Limit of detection

To estimate the dilution at which FMDV may still be detected from a pooled milk sample, the limit of detection was determined using the more sensitive Method B, using one milk sample from the animal experiment (867, 4.5 dpc, mean C_T value: 19.65) and one milk sample from the 2007 outbreak (animal number c27, mean C_T value: 16.50 [Table 2.2]). Ten-fold serial dilutions were conducted in clean Jersey milk (Figure 2.4). Limits of detection were 10^{-7} for sample c27 and 10^{-5} for sample 867 (4.5 dpc) with mean C_T values of 40.61 and 38.70, respectively.

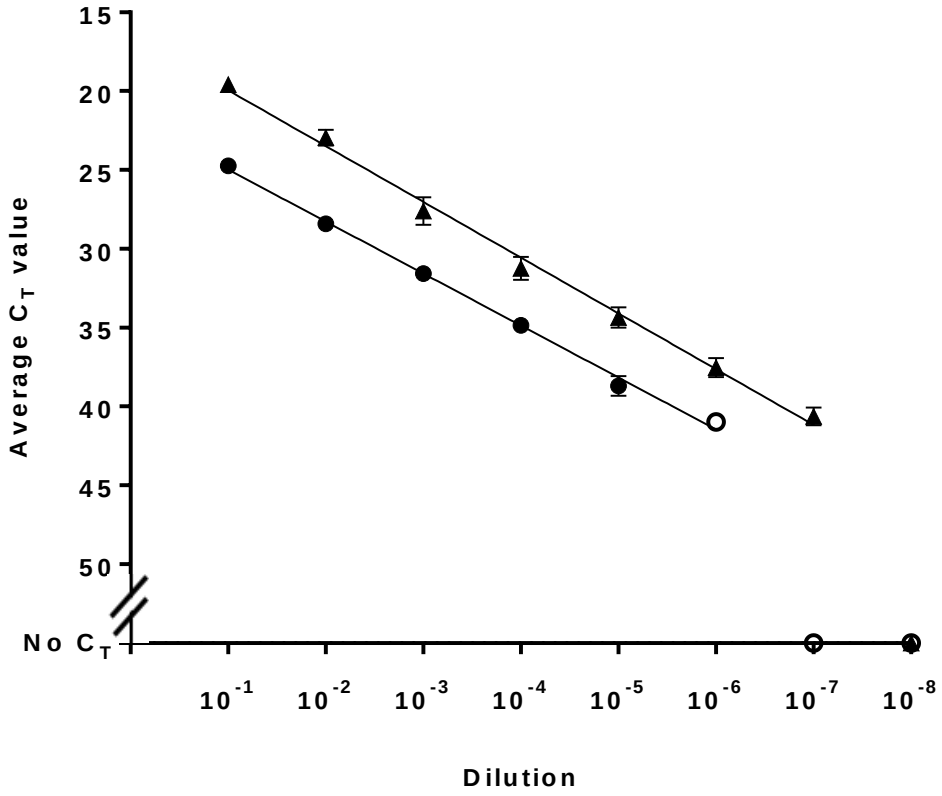


Figure 2.4 Detection of FMDV by rRT-PCR using Method B on ten-fold dilutions in Jersey whole milk of two milk samples: animal 867 (4.5 days post contact infection) and 200017, a field sample from the UK 2007 outbreak (Table 2.2). C_T values are the average of three replicates with standard deviation error bars. ●: 867 (4.5 dpc), ▲: 200017. Open symbols indicate where no amplification was observed in at least one of the three replicates.

2.5 Discussion

Two RNA extraction and rRT-PCR combinations (Methods A and B) were evaluated utilising experimental milk and serum samples, and opportunistic milk samples collected in the field during the UK 2007 outbreak (Ryan et al., 2008). These two methods employ different RT-PCR kits (with different thermocycling conditions) and have been optimised for different RNA template volumes (2.5 µL and 5 µL for Methods A and B, respectively). These specific methods were selected for comparison since they were already used in two of the laboratories that participated in this study. Comparison of these RT-PCR kits using milk samples collected from the UK 2007 outbreak generated lower C_T values for all samples with Method B (the MagMax™ Pathogen

RNA/DNA kit in combination with the SuperScript™ III Platinum™ One-Step qRT-PCR Kit). It is possible that increasing the RNA template volume for Method A to 5 µL would reduce the number of PCR cycles required to generate signal in the assay. However, the C_T differences (i.e., >4) observed in these comparative experiments were greater than would be expected from a two-fold dilution in the volume starting template. Samples from experimentally infected cows were tested by both methods, where more samples were identified as positive using Method B, than Method A, and a higher limit of detection was also observed for Method B using the spiked milk dilution series. Based on these results, Method B was used to determine the window of virus detection during FMDV infection and was carried forward as the method of choice for FMDV genome detection in milk samples, for chapters 3 – 6 of this thesis. It was demonstrated that FMDV could be detected in whole milk by rRT-PCR coincident with, and up to 24 days after the onset of early clinical signs of FMD (28 dpc). This was longer than when tested by virus isolation, and for a longer period than with traditional surveillance samples such as serum, from which FMDV was detected only up to six days after the onset of clinical signs. Reid et al. (2006) were only able to detect FMDV RNA in milk up to 23 days post infection, but identified the presence of low copy numbers of FMDV RNA in the mammary lateral lymph node on post-mortem analysis at day 28 post infection. However, for this study, Jersey cattle were used, instead of the Holstein-Friesian cattle that were utilised by Reid et al. (2006), and therefore it is unknown if this extended detection is due to the higher fat content of the milk from this breed, as FMDV has been shown to be particularly concentrated in the cream component (Reid et al., 2006). Additionally, it is likely that it could be due to the higher limit of detection of the newer detection methods, although as there was no absolute quantification of the virus stocks in my study, analytical sensitivity cannot be directly compared. Ranjan et al. (2016) demonstrated the presence of FMDV in milk samples up to 37 days post clinical manifestation by multiplex (m) PCR and reverse transcription loop-mediated isothermal amplification (RT-LAMP). In this study, animals 867 and 951 were terminated at 28 days post contact, and therefore it is unknown how much longer FMDV RNA might have been detected in these animals. Previous studies have reported FMDV detection up to 51 days post inoculation (Burrows et al., 1971), however this involved the inoculation of FMDV directly into the mammary gland which is not a method of transmission in field situations.

During the study, animal 108 displayed lesions on the teats, and animals 108 and 825 developed clinical mastitis (108 and 825). Vesicular lesions on the teats are common in lactating cows with FMD, with infection of the ruptured lesions predisposing animals to the development of secondary mastitis (Kitching, 2002), and field studies have supported this association between FMD and clinical mastitis (Sharma, 2010; Lyons et al., 2015). FMDV infection has been shown to cause a reduction in milk yield (Knight-Jones and Rushton, 2013), where secondary mastitis may play a part. However, in our study, when average milk yields were compared before (-6 to 0 dpc) and after (1 – 6 dpc) infection, no significant decrease was observed, even in cow 108 with secondary mastitis, although the maximum decrease observed on any one day was 50.47% for animal 867. This is comparable to previous experimental studies that demonstrated a maximum reduction of 62.1% on 10 dpc (Reid et al., 2006), and during an outbreak of FMDV in Iran, a total reduction of 8.0% and 4.7% in mean milk production for first and second lactation cows, respectively (Ansari-Lari et al., 2017). These published studies and our study support data reported by Lyons et al. (2015) who observed that although there was a decrease in milk production at the herd level, clinical FMD was shown to be a poor predictor of milk yield, and that no statistical evidence was found to indicate a significant decrease in milk yield between FMD clinical animals and non-clinical cases when lactation curves were modelled.

This study has demonstrated that milk from individual animals could be utilised as a less invasive sample type with simple collection procedures. Pooling these milk samples, or collecting milk from bulk storage tanks would allow for a testing method where there would be no requirement to test all samples individually, thus reducing the cost of testing. Bulk tank milk is used as a sample for a number of other diseases, including bovine viral diarrhoea virus (BVDV) (Renshaw et al., 2000a; Hill et al., 2010) and *Coxiella burnetii* (Bauer et al., 2015). In our study, the limit of detection was determined using the better performing Method B, to establish how far a positive milk sample could be diluted in whole Jersey milk and still be detected, simulating the detection of one infected animal from a herd. As expected, the ability to detect FMDV at high dilutions was related to the viral load of FMDV in the individual positive milk, and for one sample, FMDV was detected at a dilution of up to 10^{-7}. Based on the peak C_T values detected in this study, these observations of the limit of detection indicate

that it should be possible to identify one acutely-infected milking cow in a typical sized dairy herd (100-1000 individual) using bulk milk sampling. However, further research on the impact of pooling on detection sensitivity is recommended, and is performed in chapters 5 and 6. If virus can be detected in bulk tank milk, this may provide a useful surveillance tool for rapidly detecting infected herds, whilst involving minimal stress to the animal for sample collection. Additionally, the likelihood of detecting FMDV infected animals may be increased due to the extended period of FMDV detection in milk compared to serum.

Before investigating pooling, research is required to demonstrate that FMDV can be detected in milk samples collected from individual cattle in endemic settings, and therefore this is investigated in Chapter 3. This may be particularly important where multiple serotypes are circulating, and vaccination may be practiced, as it unknown what effects these factors may have on the detection of FMDV in milk from animals in these regions. Data obtained in these experiments may then inform and facilitate the design and implementation of surveillance testing plans for FMD. This could be in readiness for a potential outbreak, for example by testing bulk milk samples to rapidly identify infected herds in response to an outbreak in a disease-free country. Additionally, alternative surveillance approaches using pooled milk from large-scale dairy farms or pooling facilities may be useful for FMD epidemiological studies in endemic regions, to identify disease presence and the circulating serotypes.

CHAPTER 3

Opportunities for enhanced surveillance of foot-and-mouth disease in endemic settings using milk samples from individual cattle

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SHORT COMMUNICATION

WILEY

Opportunities for enhanced surveillance of foot-and-mouth disease in endemic settings using milk samples

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Milk samples were shipped to The Pirbright Institute and tested by Bryony Armson (BA). BA also carried out the interpretation of results, data analysis and writing. Jemma Wadsworth, Nick Knowles and Kasia Bankowska provided technical assistance and experience when performing VP1 sequencing, phylogenetic analyses, and analysing the results. Thanks go to Valérie Mioulet and colleagues in the WRLFMD for the supply of original lesion material and cell-culture isolates.

3.1 Summary

The aim of this study was to examine the application of milk from individual cattle as an alternative sample type for FMDV detection and typing, and to evaluate milk as a novel approach for targeted surveillance of FMD in East Africa. FMDV RNA was detected in 73/190 (38%) individual milk samples collected from naturally infected cattle in northern Tanzania. Further, typing information by lineage-specific rRT-PCR assays was obtained for 58% of positive samples, and correlated with the virus types identified from traditional sample types collected during outbreak investigations in the study area. The VP1-coding sequence data obtained from milk samples matched the sequence data generated from paired epithelial samples collected from the same animal. This study demonstrates that milk represents a potentially valuable sample type for FMDV surveillance and might be used to overcome some of the existing biases of traditional surveillance methods. However, it is recommended that care is taken during sample collection and testing to minimise the likelihood of cross-contamination. Such approaches could strengthen FMDV surveillance capabilities in East Africa, both at the individual animal and herd level.

3.2 Introduction

Accurate and rapid identification of the FMDV serotype responsible for a particular outbreak is of the utmost importance for informing appropriate control strategies. Current methods of detection and characterisation of FMDV include virological [e.g. virus isolation (Snowdon, 1966)], molecular [e.g. reverse transcription polymerase chain reaction (rRT-PCR)(Reid et al., 1998)] and serological tests [e.g. virus neutralisation test (OIE, 2018)]. Various pan-serotypic rRT-PCR assays (which detect all serotypes but which do not differentiate between them) have been described (Callahan et al., 2002; Reid et al., 2002; King et al., 2006; Shaw et al., 2007). For serotype identification, the antigen detection ELISA (enzyme-linked immunosorbent assay) (Ferris and Dawson, 1988) is traditionally employed. However, the analytical sensitivity is often limited, and the test is only suitable for epithelium samples and cell culture material.

Various authors have demonstrated the utility of typing of FMDV by conventional RT-PCR assays (Callens and De Clercq, 1997; Giridharan et al., 2005; Bao et al., 2008), and more recently, rRT-PCR assays for the detection of strains specific to a particular region, for example the Middle East (Reid et al., 2014; Knowles et al., 2015), West Eurasia (Jamal and Belsham, 2015; Saduakassova et al., 2017), and East Africa (Bachanek-Bankowska et al., 2016). To enable the characterisation of circulating FMDV strains, nucleotide sequence analysis is also commonly used (Baxt et al., 1984; Wittwer and City, 1989; Jamal and Belsham, 2013). VP1 sequencing (Sanger et al., 1977; Knowles et al., 2016) has value in deducing evolutionary dynamics, genetic and epidemiological relationships, and in the tracing of outbreaks and monitoring of the transboundary movements of the disease (Marquardt and Adam, 1990; Knowles and Samuel, 2003; Logan et al., 2014).

Currently, the most common sample type submitted to laboratories for FMD diagnosis is epithelial tissue or sometimes the fluid found within a vesicular lesion. These are labour intensive to collect (King et al., 2006) and consequently, reporting of disease is inherently biased towards clinical animals, and samples are often not collected due to the effort involved. Therefore viruses circulating sub-clinically may not be represented and the true prevalence of the disease is under-recognised (Knight-Jones et al., 2016).

Milk is a simple-to-collect, non-invasive sample type that has already been utilised for the surveillance of a number of diseases (Sekiya et al., 2013; Bauer et al., 2015; Nielsen et al., 2015). Although FMDV detection in milk samples has been well described during *in-vivo* experiments as shown in Chapter 2 and by others (Burrows et al., 1971; Blackwell and McKercher, 1982; Reid et al., 2006), only a small number of studies have demonstrated that FMDV RNA can be detected in milk from naturally-infected animals. These include FMDV detection in milk during the 2007 FMD outbreak in the United Kingdom (Armson et al., 2018, Chapter 2), in cattle and buffaloes in Pakistan (Saeed et al., 2011; Ahmed et al., 2017) and in cattle in India (Ranjan et al., 2016). The limited milk samples used in these studies were collected either as an additional sample type to validate molecular assays, or to investigate the possible role of milk in FMDV transmission. Nonetheless these studies provide useful evidence that FMDV RNA can be detected in milk from naturally infected animals and typed by rRT-PCR. Consequently, further investigation into the potential of milk as an alternative sample type for routine FMDV detection and surveillance is warranted, particularly in areas where surveillance infrastructure is limited.

For example, Tanzania has the third largest cattle population in Africa, and a report prepared in 2012 estimated milk production at 1.6 billion litres per annum (Kurwijila et al., 2012). However, FMD is of a high concern in Tanzania, with adverse impacts on livestock production, trade, and farmer livelihoods (Kivaria, 2003; Casey-Bryars et al., 2018). Although the recent introduction of the Progressive Control Pathway for FMD control (PCP-FMD) in eastern Africa has driven improved knowledge of the distribution of FMD, the epidemiology is still inadequately understood. Additionally, control of the disease remains challenging for many reasons, including insufficient surveillance and diagnostic capacity, a lack of comprehensive animal movement records, and inconsistent, costly vaccination programmes. The presence of at least 4 serotypes (O, A, SAT 1 and SAT 2) with multiple topotypes further complicates the control of the disease (Food and Agriculture Organization (FAO), 2011; Kasanga et al., 2012; Namatovu et al., 2013; Casey-Bryars et al., 2018; Kerfua et al., 2018). Consequently, there is a requirement for improved surveillance of FMD, utilising simple, cost-effective tools.

Therefore, the aim of this chapter was to examine the use of milk from individual cattle for FMD surveillance in Tanzania where this approach had not been investigated previously. It is anticipated that results from this chapter may inform future studies focussing on the use of pooled milk samples for the simple, cost-effective herd-level surveillance of FMD.

3.3 Materials and Methods

3.3.1 Viruses and field samples

Milk samples (n=190) (see Appendix II, Table 8.1) were collected by hand from clinical and healthy cows during FMD outbreak investigations in northern Tanzania (Serengeti and Bunda Districts) between 2012 and 2015 (Casey-Bryars et al., 2018). For four of the FMD clinically affected cows (subsequently referred to as animals A – D) that supplied a milk sample, vesicular lesion material (epithelium or fluid) was also collected on the same day. This lesion material was submitted to the WRLFMD for confirmatory diagnostics, sequencing and phylogenetic analyses (WRLFMD, 2015). Cell culture isolates (isolated once in primary bovine thyroid [BTY] cells) TAN/39/2012 (serotype O), TAN/6/2013 (A), TAN/33/2014 (SAT 1) and TAN/19/2012 (SAT 2) from the EA region spiked in negative milk from a UK farm were used as positive controls for molecular assays. All samples had been stored at -80^{o}C (milk, epithelium and vesicular fluid) or -20^{o}C (isolates) before use.

3.3.2 RNA extraction and rRT-PCR

As samples in this chapter were tested before the molecular method optimisation occurred (see Chapter 2), the MagMAX™-96 Viral RNA Isolation Kit (Applied Biosystems®) was utilised, which is currently used for nucleic acid extraction from clinical samples in the WRLFMD. This kit uses the same chemistry as that described in method B, only the sample input volume is smaller (50 µL compared to 200 µL), resulting in an increase of approximately 1-2 C_Ts.

The pan-serotypic rRT-PCR assay described in Method B (Chapter 2) was carried out on an Applied Biosystems® 7500 Real-time PCR System, using the Superscript III Platinum® One-Step qRT-PCR Kit (Invitrogen™), with primers and probes targeting the conserved 3D region of the FMDV genome (Callahan et al., 2002; OIE, 2017a), and thermal cycling conditions as previously reported (Shaw et al., 2007). Positive samples were then tested using the O, A, SAT 1 and SAT 2 East Africa (EA) typing rRT-PCR assays, as previously described (Bachanek-Bankowska et al., 2016). For all rRT-PCR assays, positive samples were defined as those with a C_T value of ≤ 50.

3.3.3 VP1 nucleotide sequencing

Paired epithelial/fluid samples had been previously typed as SAT 1 (WRLFMD, 2015), therefore SAT 1 assay conditions were used. For amplification of the VP1 region of FMDV, a one-step RT-PCR described previously (Sanger et al., 1977; Knowles et al., 2016) was performed with the primers shown in table 3.1. Amplification products were visualised by electrophoresis on a 1.5% agarose-Tris-borate-EDTA gel containing 0.5ug/ml Gel Red, and compared with DNA size markers (GeneRuler 100 bp DNA Ladder Plus, Fermentas Inc, USA). Post-PCR purification was carried out using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, UK) according to the manufacturer's instructions, and the products eluted in 20 µL of elution buffer.

DNA sequencing of PCR products was carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies), reactions cleaned up by ethanol/EDTA precipitation, and loaded onto the ABI 3730 DNA Analyser. Primers used are listed in table 3.1. VP1 sequences were assembled using SeqMan Pro (Lasergene package, DNAstar Inc., Madison, WI, USA), and further sequence analysis performed using BioEdit v7.2.5 (Hall, 1999).

Phylogenetic analyses of the FMDV VP1 coding sequences of FMD virus isolates from milk and clinical samples were performed in MEGA7 (v0.26)(Kumar et al., 2016). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei,

1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) in the units of the number of base substitutions per site.

Table 3.1. One step RT-PCR (A) and DNA sequencing primers (B) for SAT 1. Sequences are described previously (Knowles et al., 2016)

(A) One step RT-PCR		**(B) DNA Sequencing**
Forward primers	Reverse primers	Sequencing primers
SAT1-1C559F	SAT-2B-208R	NK72
SAT1U-OS	SAT-2B-208R	SAT1U-OS
		SAT 1-1D200F
		SAT 1-1D394R

3.4 Results

3.4.1 Detection of FMDV serotypes within milk samples

An initial screen of all the milk samples was performed. FMDV RNA was detected in 73/190 (38%) milk samples (Figure 3.1A) and the FMDV type was identified in 42/73 (58%) FMDV positive milk samples (Figure 3.1B). SAT 1 was the most prevalent serotype detected (45%), followed by serotypes O (29%) and A (12%), with no evidence of SAT 2 in the milk samples tested (Figure 3.1B and Appendix II, Table 8.1). Typing of milk samples that were observed to have a C_T value of above 38 using the pan-serotypic rRT-PCR assay was not possible. In addition, a positive signal from more than one typing assay was identified in eighteen milk samples, including three samples each positive for multiple serotypes (O, A and SAT 1). In samples with a positive signal for two FMDV types, O and SAT 1 were the most common types detected, while types A and SAT 1 were identified in one sample only.

Published reports of clinical samples from the study region indicate circulation of all four serotypes during the study period (WRLFMD, 2015) (Figure 3.1C) and are mainly consistent with rRT-PCR typing results of the milk samples, apart from the absence of SAT 2 detection in the milk samples.

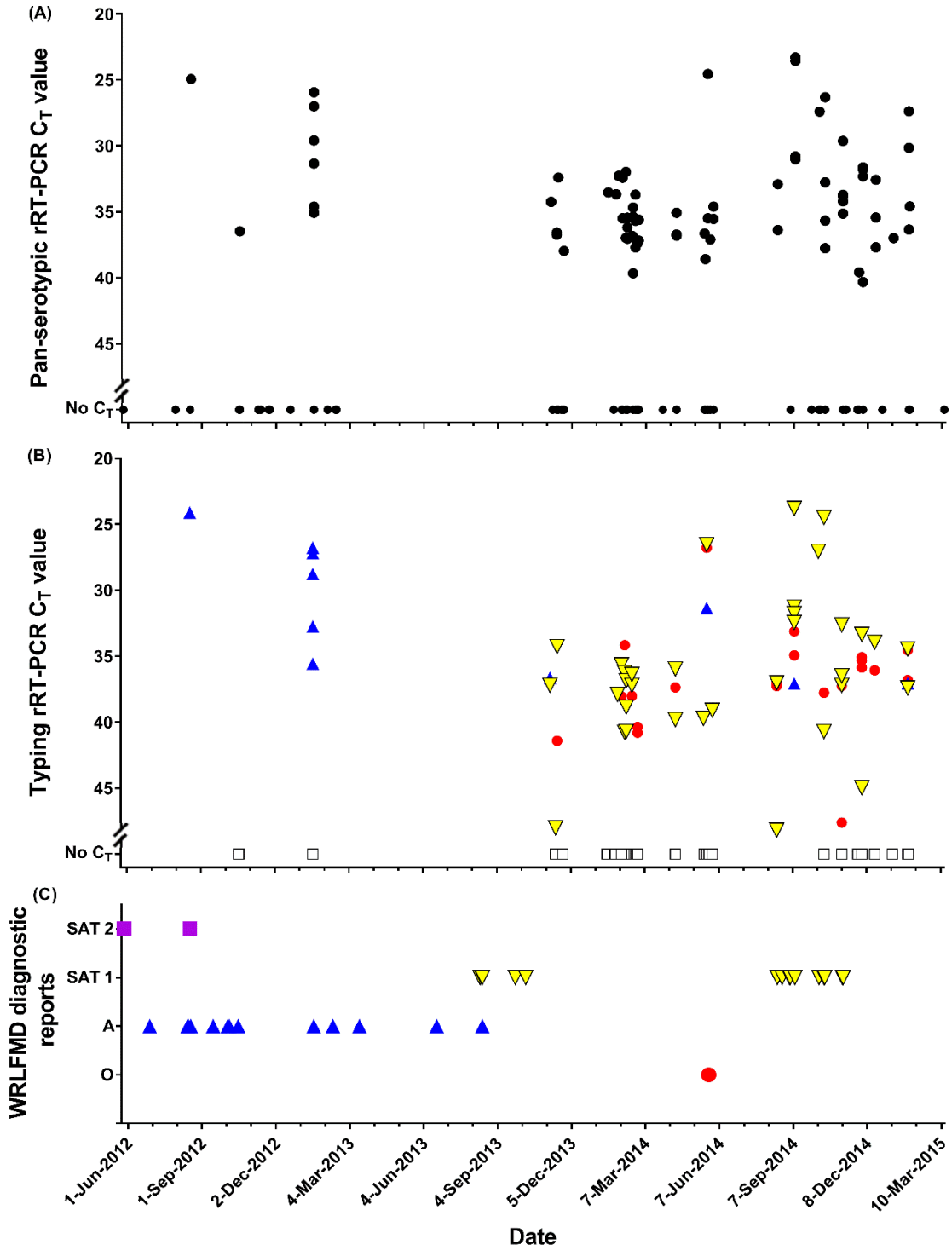


Figure 3.1 (A) C_T values from the pan-serotypic rRT-PCR assay (●) for milk samples collected from individual cows in northern Tanzania throughout the study period (n=190). (B) C_T values for each East African serotyping rRT-PCR assay for samples that tested positive ($C_T \leq 50$) in the pan-serotypic rRT-PCR assay. (C) Collection dates and the reported serotypes of clinical samples (vesicular epithelium/fluid) submitted to the World Reference Laboratory for Foot-and-mouth disease (WRLFMD). ▲: Serotype A. ▽: Serotype SAT 1. ●: Serotype O. ■: Serotype SAT 2. □: Sample that could not be typed.

3.4.2 Detection of FMDV RNA in milk samples, compared to lesion material

To determine if milk is a suitable alternative sample type to vesicular lesion material (epithelium/fluid) for FMDV detection and typing, both sample types collected from the same animal were tested and the results compared (Table 3.2). In the pan-serotypic assays, the C_T values of the lesion material samples were stronger (lower C_T values) than of the milk samples. Typing results were comparable for all pairs, with the exception of Animal A, where no signal was observed in any of the typing assays. In three animals (B, C and D), SAT 1 was detected in both milk and lesion material samples (Table 3.2). In animals C and D, the C_T values of the pan-serotypic and the SAT 1-specific assays were comparable, while in animals A and B the differences in the values were greater. In animal A, SAT 1 was detected in the vesicular lesion sample only. Two different FMDV types (O and SAT 1) were detected in both milk and vesicular fluid in animal B, but type O was not detected in the vesicular epithelium sample.

Table 3.2 FMDV detection in milk samples and epithelial samples.

Animal Reference	WRLFMD reference/ Milk sample	Sample Type	3D	O	A	SAT 1	SAT 2
A	TAN/20/2014	Vesicular epithelium	18.30	No C_T	No C_T	24.98	No C_T
	7736	Milk	33.31	No C_T	No C_T	No C_T	No C_T
B	TAN/22/2014	Vesicular epithelium	10.19	No C_T	No C_T	21.06	No C_T
	TAN/23/2014	Vesicular fluid	9.94	38.07	No C_T	19.76	No C_T
	7609	Milk	29.04	33.13	No C_T	33.42	No C_T
C	TAN/28/2014	Vesicular epithelium	16.00	No C_T	No C_T	20.33	No C_T
	TAN/29/2014	Vesicular fluid	9.05	No C_T	No C_T	10.3	No C_T
	7805	Milk	26.48	No C_T	No C_T	29.17	NP
D	TAN/34/2014	Vesicular epithelium	16.51	No C_T	No C_T	16.77	No C_T
	7815	Milk	25.08	No C_T	No C_T	25.69	NP

C_T values are the mean of duplicates for each rRT-PCR assay. NP – not performed.

3.4.3 VP1 nucleotide sequencing of milk samples, compared to lesion material

To determine if milk is a suitable alternative to the commonly used epithelial tissue and vesicular fluid sample types for characterisation of FMDV by VP1 sequencing, two sequences derived from the milk samples from northern Tanzania (7805 and 7815) were compared with previously reported sequences of paired epithelial/fluid samples from the same animal, held in the WRLFMD archive.

VP1 sequences obtained from milk samples 7805 (animal C; accession number MH791039) and 7815 (animal D; accession number MH791040) were found to be identical (animal D) or within one nucleotide difference (animal C) to reported sequences of paired vesicular samples from the same animals (animal C: accession number MF592687, animal D: accession number MF592691) (Figure 3.2). The nucleotide difference for animal C was a non-synonymous change at VP1 amino acid position 204.

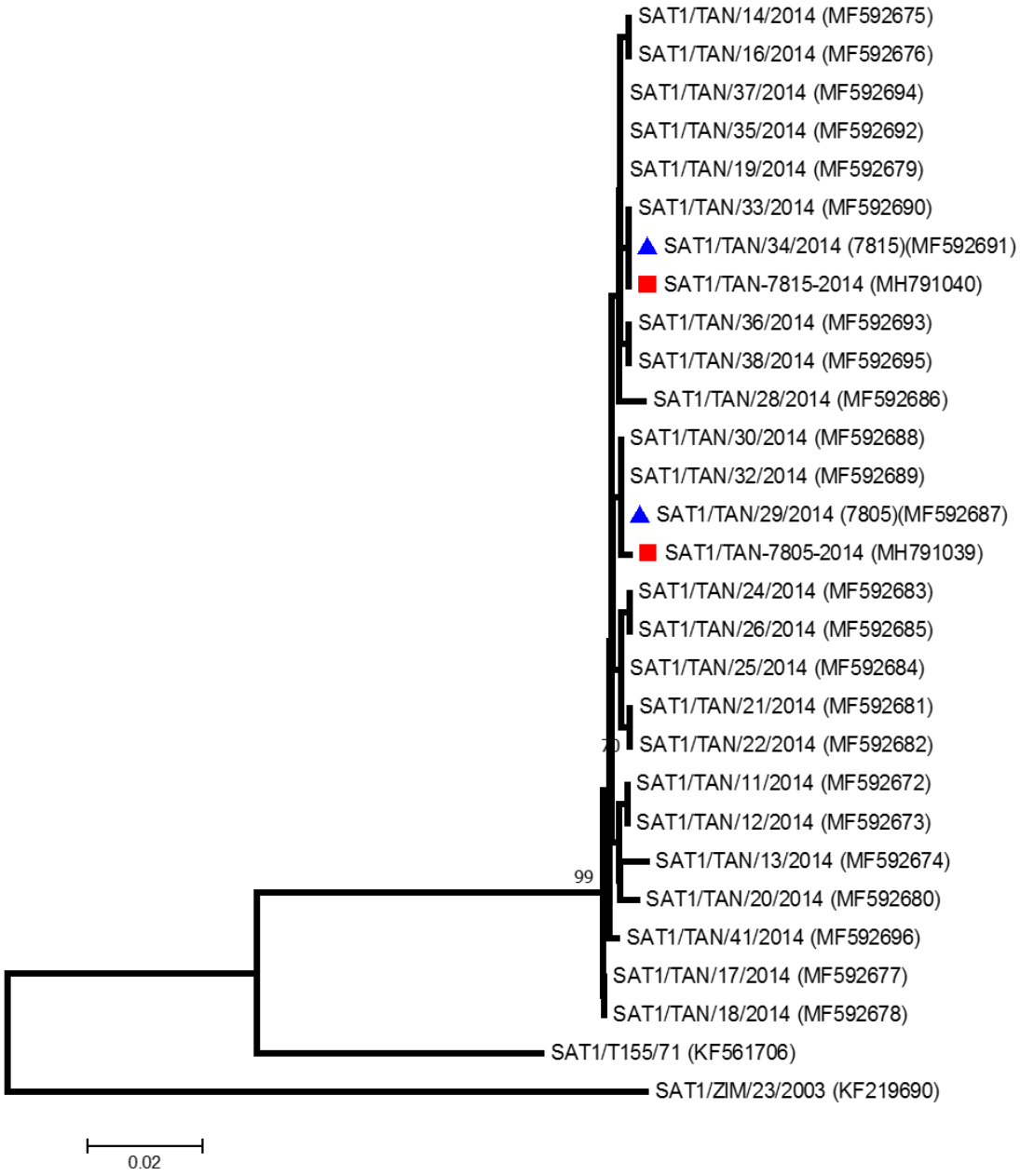


Figure 3.2 Phylogenetic analyses of FMDV VP1 coding sequences of FMD virus isolates (accession number in parentheses) collected from Tanzania in 2014. Blue triangles represent milk samples from cows 7805 and 7815, and red triangles represent clinical lesion material from the same animal. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA7(Kumar et al., 2016).

3.5 Discussion

The aim of this chapter was to examine the suitability of milk from individual cattle for FMD detection and typing so that it could be used for FMD surveillance in endemic settings such as East Africa. FMDV RNA was detected in 38% of the 190 milk samples tested, and of these, 58% of samples could be typed, with some milk samples positive for more than one serotype. It is possible that these animals were co-infected with multiple FMDV serotypes, as has been previously described in endemic areas (Woodbury et al., 1994; Ferris et al., 1995; Casey-Bryars et al., 2018). However, alternative explanations should also be considered, including the possibility that these results represent (*i*) contamination due to contact with materials infected with other FMDV types during sample collection in the field, transport or testing in the laboratory; or (*ii*) cross-reaction between the individual typing rRT-PCR assays, although no evidence of this has been observed during the validation of these tests (Bachanek-Bankowska et al., 2016). Samples that could not be typed were those with a low level of FMDV specific RNA, indicated by high C_T values (> 38) detected in the pan-serotypic assay. It is likely that these samples were beyond the analytical sensitivity of the typing assays, and methods to concentrate virus could be investigated to improve this in the future.

During the study period, all four serotypes were detected in clinical samples, as reported by WRLFMD (2015), which was mainly concurrent with the results of rRT-PCR testing of milk samples. However, there was an absence of serotype SAT 2 detection in the milk samples, likely due to milk samples not being collected from the specific locations where serotype SAT 2 was identified in clinical samples at the start of the study period. Additionally, on some dates, FMDV RNA was detected in a milk sample, but there were no confirmed diagnostic reports of this serotype in the region at this time. This could be due to poor farmer recognition of clinical signs, lack of disease reporting, or sample contamination (as discussed above). Alternatively, these results may indicate that FMDV can be detected in milk samples during the pre-clinical or convalescence phases of infection, as reported previously (Blackwell and McKercher, 1982; Reid et al., 2006; Armson et al., 2018 [see Chapter 2]), or even during subclinical infection.

In order to substantiate the use of milk as an alternative sample type for surveillance, typing assay results and VP1 sequences from both milk and the traditional diagnostic sample types (epithelium or vesicular fluid) from the same animal and collection date were compared. The stronger C_T values of the lesion material samples compared to those of the milk samples when tested by the pan-serotypic rRT-PCR confirm previous observations of higher virus concentrations in vesicular lesions (King et al., 2006; Stenfeldt et al., 2015). Typing results were comparable, with SAT 1 detected in all samples apart from the milk sample from animal A, possibly due to the reduced viral load observed in this animal. Additionally, the presence of type O in the milk and vesicular fluid from animal B, but not in the vesicular epithelium sample was interesting. As discussed above, contamination cannot be excluded as a reason for this result.

Additionally, two sequences derived from the milk samples from northern Tanzania were compared with previously reported sequences of paired epithelial/fluid samples from the same animal, held in the WRLFMD archive. Sequences from the same animal were found to be identical, or with one nucleotide difference, which may be explained by a mutation that could have occurred during viral replication, as sequences from the vesicular samples were obtained from virus isolated on primary bovine thyroid (BTY) cells. Upon comparison of the SAT 1-specific primers/probe with the VP1-coding sequence data obtained from milk and vesicular samples, it was evident that the difference in C_T values between the pan-serotypic and the SAT 1-type specific assay may occur due to nucleotide differences at the 3' end of the primer binding region of the typing assay. At least one nucleotide difference was identified within the SAT 1-specific typing assay binding region in sequences obtained from animals A and B, while no such differences were observed in sequence data obtained from animals C and D. As the VP1-coding sequence is the most variable genome region, mismatches between the primers and probes of the typing assays and the template are expected. Therefore, it is recommended to use typing assays alongside the more sensitive pan-serotypic assay as a screening tool (Bachanek-Bankowska et al., 2016). Only limited vesicular samples were available from the same animal for this study, therefore, generation of additional data when more samples are available will continue to further validate these preliminary results. Additionally, the detection of FMDV RNA in milk samples should

also be compared with that of other excretory samples such as nasal swabs, oral swabs, and OP fluid from the same animals at different stages of infection. Overall however, observations from paired samples indicate that, despite a weaker rRT-PCR signal, milk can be useful for the detection and typing of FMDV from individual animals.

This study demonstrates that milk could represent a valuable sample type as an alternative to the traditional diagnostic samples collected for FMD surveillance: vesicular epithelium or fluid. Milk from individual animals can be routinely collected and FMDV RNA can be detected and typed by rRT-PCR in milk samples in a region where FMD is endemic, albeit with weaker C_T values than from vesicular samples. Additionally, the identification of multiple FMD serotypes in an individual milk sample suggests the likely possibility of co-infection, however contamination should not be excluded. The study demonstrates that VP1 sequence data may be obtained from milk samples, enhancing the possibility of further, in-depth virus characterisation. Milk sampling as a targeted surveillance approach shows promise given the concordance between typing data from milk samples and confirmed reports from outbreak investigations. Due to the high analytical sensitivity of molecular tests used to detect FMDV, appropriate care needs to be taken to minimise the possibility for cross-contamination during sample collection, transport and testing in the laboratory. In conclusion, milk is a simple-to-collect, non-invasive sample type which might be utilised in targeted surveillance campaigns in FMD endemic regions. Follow-on studies are required to assess the application of pooled milk in combination with herd clinical status for improved FMDV surveillance.

CHAPTER 4

Moving from individual to pooled milk: considerations for the transport and storage of samples collected from herds in endemic countries

Acknowledgements: Bulk milk samples and MicroTabs, used for specificity and preservative testing respectively, were kindly provided by the Animal and Plant Health Agency (APHA), Surrey, UK.

4.1 Summary

The cost-effective, non-invasive collection of pooled milk samples for herd-level surveillance in endemic settings may help to improve the current understanding of FMD epidemiology. However, in many developing countries where FMD is endemic, a lack of infrastructure and resources can pose challenges in maintaining the cold chain for sample storage and transportation to testing laboratories. This chapter aimed to address some of the logistical challenges that might impact on the use of milk samples for surveillance purposes in endemic settings, including determination of the stability of FMDV RNA in milk samples in different scenarios, simulating conditions that might be experienced during storage and transport. Experiments performed demonstrated that FMDV detection was not significantly affected during long-term storage at -20^{o}C, after multiple freeze-thaw cycles (-20^{o}C/room temperature) or treatment with a common preservative (Bronopol). However, heat treatment at 56^{o}C as a method to inactivate virus is not recommended when rRT-PCR testing is to be performed, due to the large increase in C_T values that occurs, potentially resulting in a loss in diagnostic sensitivity. Separate experiments aimed to determine the occurrence of non-specific amplification, and the level of intra-assay contamination that may occur when testing milk samples. In these studies, only, 3/884 wells in the FMDV rRT-PCR assay were found to be weakly positive for FMDV RNA, resulting in a specificity of 99.66%. With these considerations in mind, experiments performed in this chapter further support the utility of pooled milk as an alternative sample for FMD surveillance, especially when samples may need to be shipped long-distances for FMDV testing.

4.2 Introduction

The potential of milk as an alternative sample type for FMDV detection has been described in Chapters 2 and 3 (Armson et al., 2018, 2019). Milk sampling is non-invasive and can be cost-effective as milk is routinely collected from dairy farms. Milk is also potentially less susceptible to selection bias than the routine sample type (vesicular material), as it does not rely on the observation of clinical signs. Of particular interest is the potential use of pooled milk for routine, low-cost, herd surveillance of FMD in endemic settings to determine disease prevalence and improve our understanding of FMD prevalence and epidemiology, so that appropriate control measures may be employed.

In many developing countries where FMD is endemic, a lack of infrastructure and resources can pose challenges in maintaining the cold chain for sample storage and transportation to reference laboratories (Fowler et al., 2014; Niedbalski, 2016). Consequently, temperature and/or pH changes may affect the preservation of clinical samples (Bachrach et al., 1957), which may be partially degraded upon arrival for testing, resulting in difficulties in fully characterising the field FMDV isolate (Shaw et al., 2004).

The same may be true for milk samples, although several publications have reported an increased survival of FMDV in milk samples subjected to high-temperature pasteurisation, pH variation, freeze-drying, and the preparation of dairy products (Felkai et al., 1970; Blackwell and Hyde, 1976; Tomasula and Konstance, 2004; Spickler and Roth, 2012). It is hypothesised that the high fat and protein content of milk may partially protect the virus from inactivation procedures (Spickler and Roth, 2012). These experiments mostly involved the use of non-molecular testing methods, and were mainly focussed on the stability of live virus under extreme inactivation conditions, to assess the risk of milk and milk products for onward transmission of disease. Consequently, little is currently known about how these conditions affect the stability of FMDV RNA genome, and the ability to detect it using the FMDV rRT-PCR assay described in Chapter 2 (Armson et al., 2018).

It is anticipated that variations in temperature and consequently a decrease in pH (due to an increased growth of contaminating bacteria [Christiansson, Bertilsson and Svensson, 1999]) may cause degradation of full length, high quality RNA in the sample (Wilson, 1997), although this may still be detectable by the rRT-PCR assay. Additionally, PCR inhibition may occur due to substances found in milk such as bacterial contamination, antibiotics, RNases, fats and proteins, although an efficient RNA extraction method should remove many of these factors (Wilson, 1997; Radström and Al-soud, 2001; Schrader et al., 2012). Consequently, if milk samples from developing countries may be utilised as an alternative sample for FMDV detection, more research is required on the effects that these small changes in temperature or pH, which may occur during transportation, may have on FMDV RNA genome stability and the ability to detect it using the rRT-PCR assay.

Milk samples collected from field settings in this project (see chapters 3, 5 and 6) show that FMDV RNA genome is detected by rRT-PCR consistently with high C_T values (>30 for pooled milk). Although limit of detection studies performed in Chapter 2 (Armson et al., 2018) demonstrate positive C_T values of up to 41, it is possible that results may be due to false amplification. False amplification may occur for three main reasons:

(i) Environmental contamination during sample collection, for example transferring equipment between farms without sufficient disinfection.
(ii) Intra-assay or inter-assay contamination of the plate wells during set-up, RNA extraction or rRT-PCR from the laboratory environment or positive controls.
(iii) Non-specific amplification of other template (e.g. from other organisms) present in milk.

During work performed for this thesis, environmental contamination was controlled as far as was reasonably practicable for example sample collectors wore disposable gloves and used disinfectant appropriately. It was therefore assumed to be negligible for the purpose of this study. 'False positives' may have occurred due to non-specific amplification, i.e. amplification of other nucleic acid other than that of the target gene found in milk samples, although it is anticipated to be negligible due to the high analytical specificity of the primers and probes used (targeting the 3D polymerase) (Callahan et al., 2002; Reid et al., 2009). Furthermore, the possibility for intra-assay

contamination during set-up and testing cannot be ignored, as this might lead to amplification in samples of known negative origin (Reid et al., 2009). However, in the study by Reid et al. (2009) a different RNA extraction protocol and sample matrices were used. Therefore, the specificity of the method used for FMDV detection in this project still requires assessing.

This chapter is divided into two main parts. First, experiments were performed to simulate a range of scenarios that may occur from the point of milk sample collection to final testing. These include investigating the effects of various factors on the stability of FMDV RNA and the ability of detection by the rRT-PCR. These factors include heat inactivation and the addition of a common preservative (Bronopol) prior to shipment, and variations in temperature during long term storage and transportation.
Second, sensitivity and specificity experiments were performed to test more robustly the FMDV rRT-PCR detection method used throughout this thesis. It was anticipated that results from these experiments would help to better inform the conditions employed for the storage, transport and testing of pooled milk samples from endemic regions.

4.3 General materials and methods

4.3.1 Clinical samples

FMDV viruses used in this chapter were obtained either from archival stocks of cell culture isolates (isolated once in primary bovine thyroid [BTY] cells) held in the WRLFMD repository (The Pirbright Institute, UK), or were archival samples previously collected from experimental studies as described in Chapter 2 (Armson et al., 2018). Negative unpasteurised milk from Jersey cattle used for preparing dilution series or as controls was purchased from a UK farm. Unpasteurised milk samples as a negative cohort used for specificity testing were kindly provided by the Animal and Plant Health Agency (APHA), collected from UK farms every quarter as part of the national Brucellosis surveillance programme (DEFRA, 2015a).

4.3.2 RNA extraction

All RNA extractions were carried out using the MagMAX™ 96 Viral RNA Isolation Kit (Applied Biosystems®) using a sample input of 50 µL on a MagMAX™ Express 96 Extraction Robot (Applied Biosystems®) according to manufacturer's instructions. Negative extraction controls consisted of unpasteurised whole milk added to lysis buffer.

4.3.3 Real-time reverse transcription PCR

All rRT-PCR assays were carried out on an ABI7500 rRT-PCR machine (Applied Biosystems®) using the reagents, parameters and thermal cycling conditions as previously described in Chapter 2 (Method B) (Callahan et al., 2002; Shaw et al., 2007; Armson et al., 2018). Replicates of samples with a C_T value of >50 were considered negative, and weren't included in statistical analyses.

4.3.4 Virus Isolation

Virus isolation of milk samples was carried out on primary bovine thyroid (BTY) cell cultures (Snowdon, 1966), with the following modification. After the 30 minute incubation of the test-sample cell-culture tubes to allow adsorption of virus into the cells, all tubes were washed 3-5 times with phosphate buffered saline (PBS, Severn Biotech Ltd.) before the addition of 2mL Eagle's maintenance medium (serum free, MEM with HEPES, phenol red & GlutaMax)(Gibco®, Life Technologies). Tubes were then returned to the incubator for 72 hours and observed each day for cytopathic effect (CPE). Titrations of the original virus spiked milk sample were performed and the viral titre was calculated using the Spearman-Kärber method, as described by the UN, Food and Agriculture Organization (FAO) and expressed in units of $TCID_{50}$/mL (Karber, 1931; OIE, 2017a).

4.4 The stability of FMDV RNA in milk samples

4.4.1 Treatment of milk samples prior to shipment

This section investigates the effect of preservative treatment, heat inactivation and storage of milk samples on the ability of the rRT-PCR assay described above to detect FMDV RNA genome. The latter two methods could be employed prior to shipment in order to increase the time before spoiling or to inactivate the virus, and consequently may reduce the cost of shipment to international reference laboratories by allowing shipment at a lower category level.

(A) Does treatment with a common preservative affect the ability to detect FMDV by rRT-PCR?

As part of the national brucellosis surveillance programme, bulk milk samples collected by large-scale farms and submitted to national laboratories are often treated with a preservative to increase their storage duration before spoiling. A common preservative used in the UK is Bronopol (2-bromo-2-nitro-1,3-propanediol) plus natamycin. If a similar scheme was employed for FMD surveillance in the UK, milk samples may contain this preservative.

Alternatively, this preservative could be added before shipment, so that samples may only need to be refrigerated during transportation. A previous experiment, using a different RNA extraction and rRT-PCR method to the one employed in this project, demonstrated that treatment with a similar Bronopol-based preservative had no significant effect on the detection of FMDV RNA in milk samples (Reid et al., 2006).

Method

One 'Broad Spectrum Microtabs II™' (Advanced Instruments) tablet, containing 8 mg Bronopol and 0.30 mg Natamycin, was added to 25 mL unpasteurised Jersey milk, to simulate the method used in samples collected in the UK for the surveillance of other diseases such as brucellosis and bovine viral diarrhoea. Two ten-fold serial dilutions (10^{-1} to 10^{-8}) of cell-culture isolate A/KEN/6/2012 were prepared, one in the preservative-treated milk, and one in untreated negative milk as a control. RNA

extraction and the rRT-PCR assay were performed on each dilution series in triplicate. A paired t-test was performed comparing the results of preservative treated versus untreated dilution series.

Results

Results showed that the limit of detection for the untreated sample was one $\log_{10}$ higher than for the treated sample, although this was near the limit of analytical sensitivity of the rRT-PCR assay (Figure 4.1). However, there was no significant difference between the mean C_T values of the treated and untreated dilution series (p=0.139).

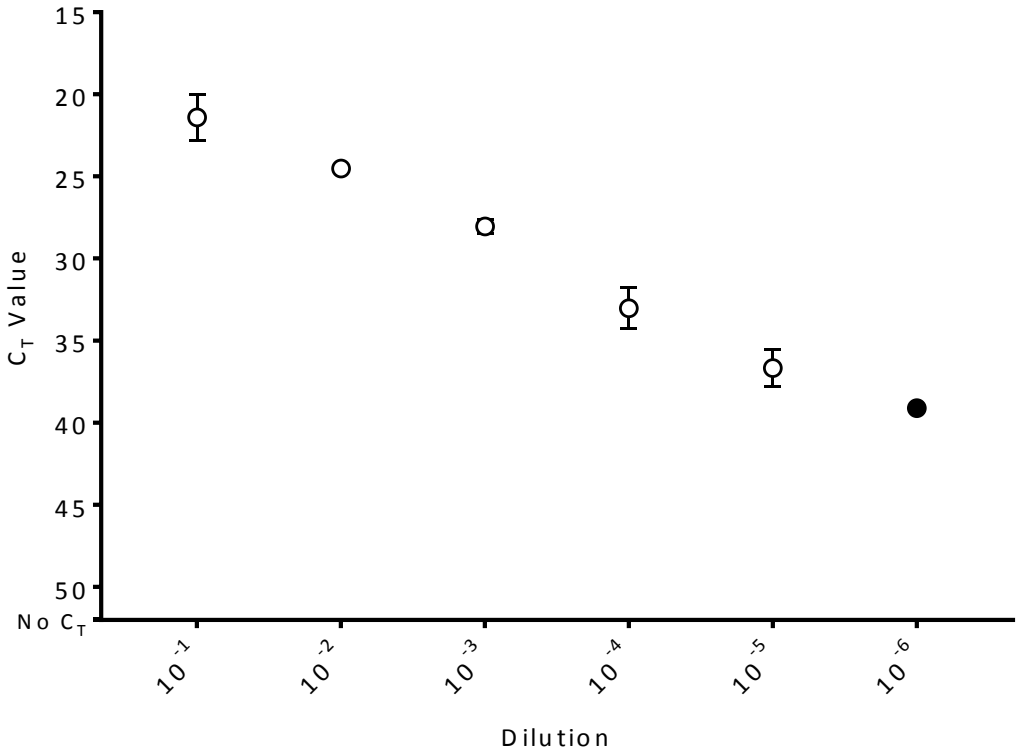


Figure 4.1 rRT-PCR results for the milk samples treated with preservative added (○) and untreated (△). Each point represents the mean of three replicates. Black symbols indicate where no amplification was observed for one or more replicate. Error bars indicate the standard deviation.

(B) What effect does heat-inactivation of samples have on the ability to detect FMDV RNA by rRT-PCR?

Strict procedures exist for the shipment of samples containing live FMD virus (IATA, 2019) and it is recommended that samples of unknown FMD status (i.e. could contain live virus) be shipped according to category A (The Pirbright Institute, 2019), which requires expensive packaging and shipping. Prior validated inactivation of any live virus present may therefore allow shipment of milk samples at a reduced cost, as samples may be sent at a lower category level. This allows more samples to be included in one shipment, and the requirements for packaging are fewer (IATA, 2019).

Heat inactivation is a cheap, simple inactivation protocol currently used for serum samples (The Pirbright Institute, 2019), involving heating samples in a water bath/heat block for two hours. It is anticipated that this method could be applied to milk samples, and could be performed by local low-resource laboratories before reduced-cost shipment to national/international reference laboratories for diagnostic testing. This section investigates the stability of FMDV RNA in milk and consequent detection by the rRT-PCR after heat-inactivation.

Method

A 10^{-1} dilution of cell culture isolate A/SAU/6/2015 was prepared in unpasteurised whole milk, and fifteen replicates of this dilution were aliquoted into 1.8 mL cryotubes. An additional four aliquots of milk were also prepared, as an FMD-negative control. Three tubes of the 10^{-1} dilution and an FMD negative control were placed in a water bath at 56^{o}C for different time periods (60 mins, 45 mins, 30 min or 15 mins). An additional 3 tubes and an FMD-negative control were placed at +4^{o}C for 60 minutes as non-treated controls.

After heat inactivation (or no treatment), virus isolation and RNA extraction-FMDV rRT-PCR were performed on all milk samples and FMD-negative controls. Additionally, virus titrations of the original virus spiked milk sample were performed on BTY cells (10^{-1} – 10^{-8}) to calculate the virus titre.

Results

The virus titre of the FMDV A/SAU/6/2015 sample when spiked in unpasteurised milk was 6.0 log $TCID_{50}$/mL.

No CPE was observed after heat inactivation of FMD infected milk samples for 30 minutes or more (Table 4.1). As observed by eye, a large difference in C_T value was observed when comparing no heat treatment with all heat inactivation times (C_T difference >10).

Table 4.1 Results of the pan-serotypic rRT-PCR assay and virus isolation after heat inactivation.

Heat inactivation at 56°C	C_T value (SD)	CPE observed in BTY cells
None	17.97 (0.08)	3/3
15 mins	28.47 (0.99)	3/3
30 mins	32.59 (0.42)	0/3
45 mins	34.28 (0.63)	0/3
60 mins	33.17 (0.44)	0/3

SD: standard deviation. CPE: cytopathic effect. BTY: bovine thyroid. C_T values are the mean of three biological and three technical replicates (n=9).

(C) Does long-term storage at -20°C affect the ability to detect FMDV by rRT-PCR?

International reference laboratories typically maintain those samples not preserved in glycerol for long-term storage in a -80°C freezer. However, low-resource laboratories may only have access to 'regular' freezer space that maintains a temperature of -20°C. Consequently, milk samples may need to be stored at this temperature for long periods until enough samples are collected for shipment.

Method

A ten-fold dilution series (10^{-1} – 10^{-8}) of cell culture isolate A/TAN/1/2013 was prepared in unpasteurised whole milk. Nine 500 µL aliquots of each dilution were prepared, and four aliquots of each dilution series stored at either -20°C or -80°C. At 3-month time intervals over a period of one year (i.e., at 0, 3, 6, 9 and 12 months), an aliquot stored at -20°C and -80°C was removed for testing. RNA was extracted in

triplicate from each dilution series and the pan-serotypic rRT-PCR performed on each replicate.

Paired t-tests were performed comparing the two storage temperatures at each time interval (3, 6, 9 and 12 months). Additionally, paired t-tests were carried out to compare the C_T values at Day 0, with -20^{o}C storage at each time interval.

Results

There was no significant difference in the C_T values obtained between storage at -80^{o}C and -20^{o}C at any of the time intervals, apart from at 6 months (p = 0.02) (Figure 4.2). However, the difference in average C_T values for the 6 month time interval was small (mean of difference = 1.22, 95% CI = 0.18 – 2.25). Additionally, when long term storage (12 months) is compared with testing on Day 0, no significant difference in the limit of detection was observed (p = 0.08).

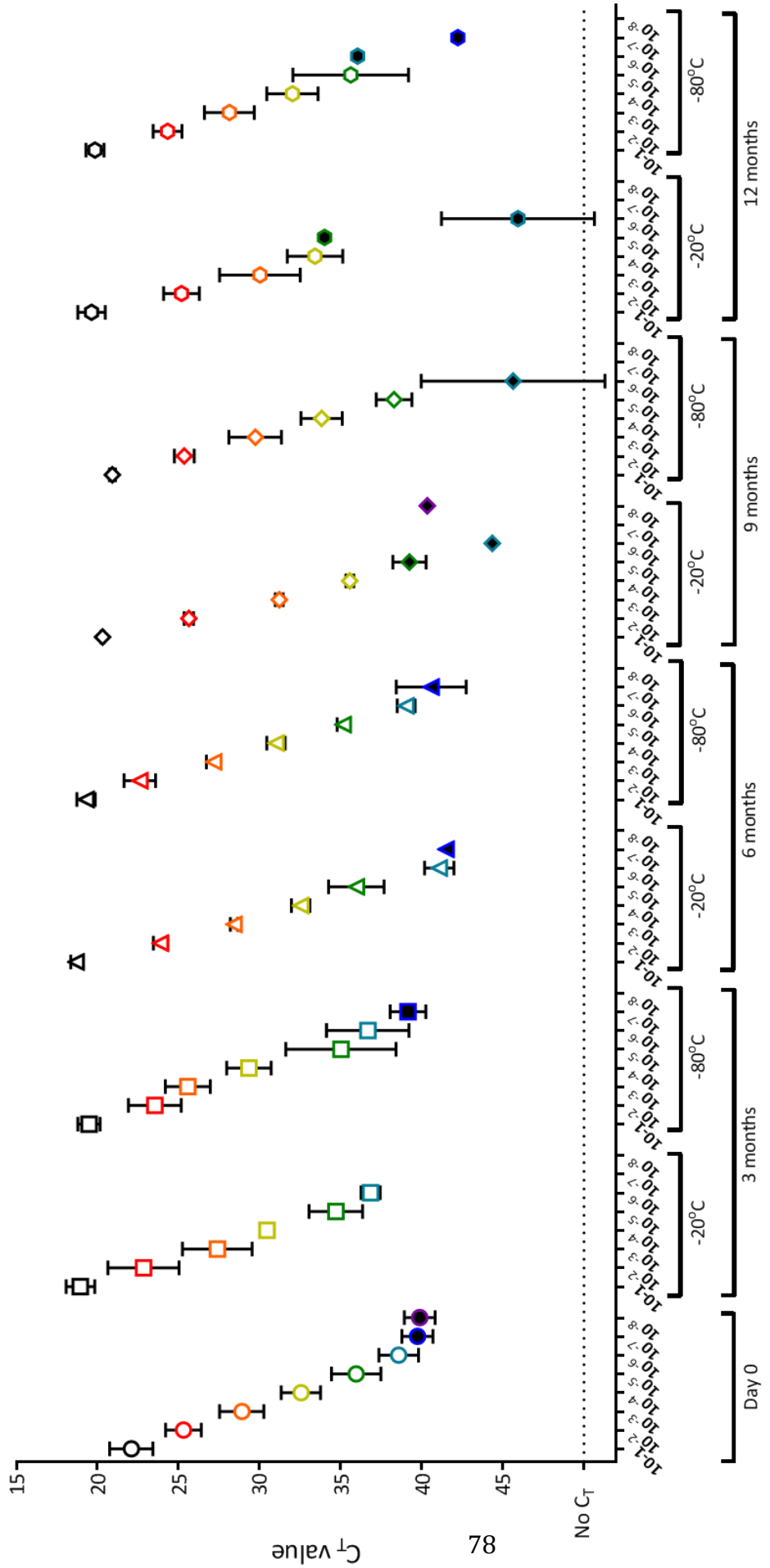


Figure 4.2 Limit of detection of the rRT-PCR of milk samples stored at -20°C and -80°C for 3 months (squares), 6 months (triangles), 9 months (diamonds) and 12 months (hexagons), and at Day 0 (circles). Each point represents the mean of three replicates. Black filled symbols represent where a No C_T result was obtained for one or more replicates. Error bars indicate the standard deviation.

4.4.2 Storage of milk samples during transportation

This section aims to determine the effect of temperature on rRT-PCR detection of FMDV changes that may occur during storage and transportation, in a number of experiments, described below.

(D) What is the effect of freeze-thawing samples on FMDV detection by rRT-PCR?

From sample collection through to testing, milk samples may be freeze-thawed multiple times, for example if further aliquoting of the sample is required, or during transportation, especially where delays may result in inadequate maintenance of the cold-chain.

Method

For experiments *(D)* and *(E)* two milk samples collected from the *in-vivo* experimental infection study (Chapter 2) were chosen with different levels of FMDV RNA present (based on original C_T value): Animal 867, 4 days post infection (DPI) (average C_T = 19.65) and Animal 867, 7 DPI (average C_T = 26.61), named 'high' and 'low' respectively. In order to ensure enough material was available, samples were diluted 1/20 in unpasteurised whole milk, and separated into different aliquots for each experiment *(D)* and *(E).*

For this experiment *(D)*, each sample ('high' and 'low') underwent five freeze-thaw cycles: samples were stored at -20^{o}C for at least 3 hours, and then defrosted for at least 30 minutes at room-temperature (RT) (approximately 21-23^{o}C). RNA was extracted in triplicate and the pan-serotypic rRT-PCR performed on each replicate at the start of the experiment, and also at each 'thaw' step (Figure 4.3). Additionally, the pH was measured at each 'thaw' step by adding a drop of each sample to universal pH indicator paper. This was to determine whether these temperature fluctuations also affected the pH of the milk sample, for example due to an increase in the growth of contaminating bacteria, as a decrease in pH may cause degradation of intact virus and nucleic acid, potentially leading to a 'false negative' result. One-way ANOVA tests were performed to compare C_T values obtained at each thaw stage for the 'high' and 'low' samples.

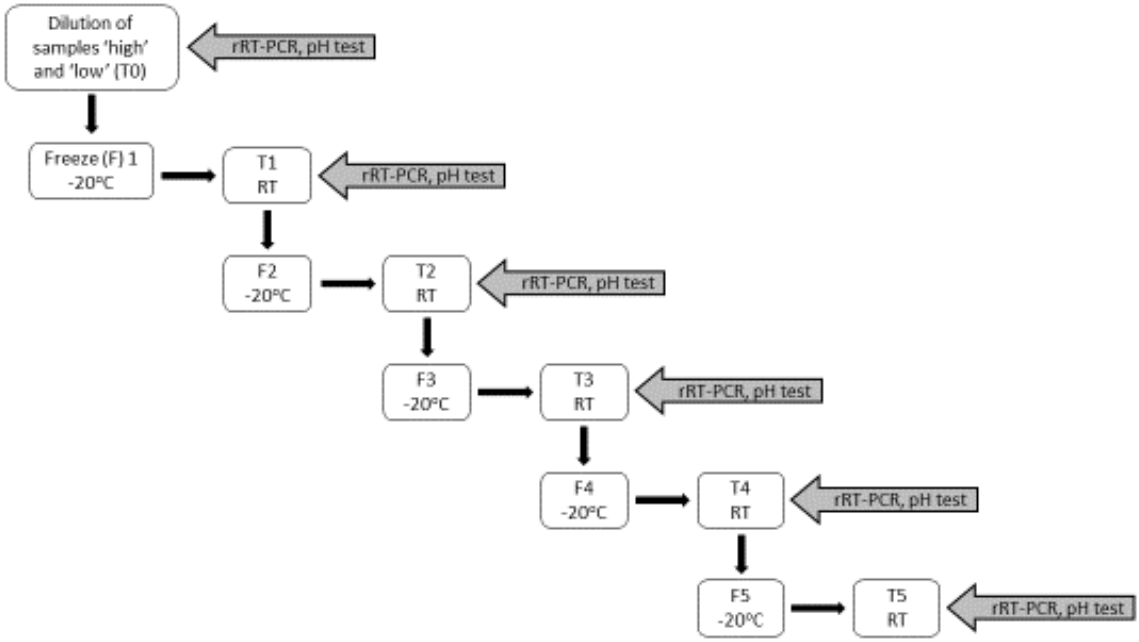


Figure 4.3 The freeze-thaw process carried out for each sample ('high' and 'low'), indicating the point at which rRT-PCR and pH testing was carried out.

Results

There was no significant difference between the mean C_T values between any of the thaw steps for the 'low' sample (p = 0.23) (Figure 4.4). A significant difference between thaw steps was observed for the 'high' sample (p = <0.01), however the difference between the lowest and highest C_T value for this sample was small (C_T=1.29). At each thaw step, the pH of each sample remained at 7.

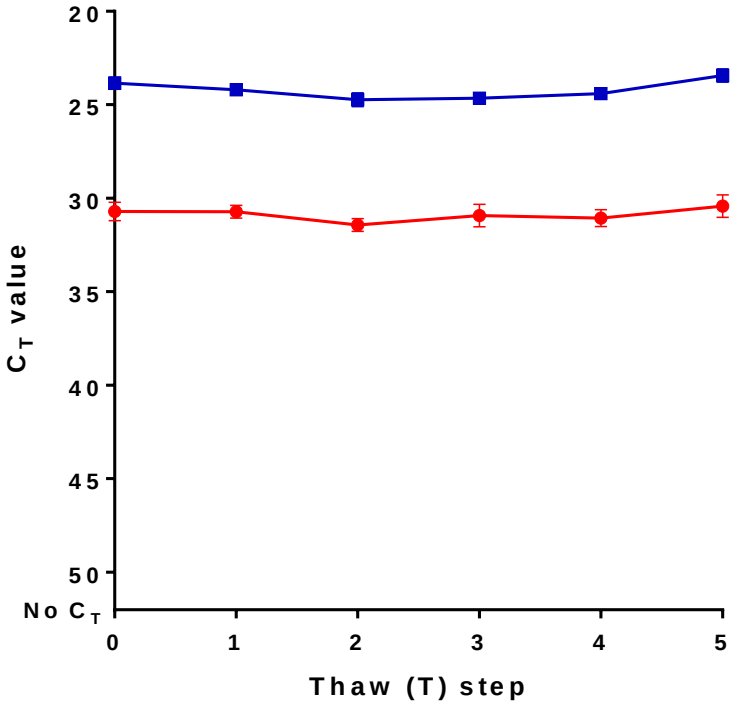


Figure 4.4 C_T values from the pan-serotypic rRT-PCR measured at each thaw (T) step for the 'high' sample (■) and 'low' (●) sample. Each point represents the mean of three replicates. Error bars indicate the standard deviation.

(E) What is the effect of short term storage at +4°C or room-temperature on FMDV detection by rRT-PCR?

If freeze-thawing samples does have a negative effect on FMDV detection, an alternative to shipping samples on dry ice to maintain samples in a frozen state would be to transport refrigerated samples. This may also reduce cost and the package size required.

Method

Thirteen aliquots of each sample ('high' and 'low') were prepared, and four aliquots of each were stored at +4°C, room temperature, and -20°C as a control (Table 4.2). Samples were stored for four days, and on each day, an aliquot was removed, RNA extracted in triplicate and the pan-serotypic rRT-PCR performed on all replicates. Additionally the pH was measured, as above.

Table 4.2 Storage temperatures and rRT-PCR testing intervals for samples 'high' (H) and 'low' (L).

Day 0	Day 1			Day 2			Day 3			Day 4		
Untreated	-20°C	+4°C	RT	-20°C	+4°C	RT	-20°C	+4°C	RT	-20°C	+4°C	RT
H	H	H	H	H	H	H	H	H	H	H	H	H
L	L	L	L	L	L	L	L	L	L	L	L	L

Results

Using a paired t-test, there was no significant difference between short term storage at -20°C (control) and +4°C for the 'low' sample (p = 0.57) or the 'high' sample (p = 0.08) over the 4 days (Figure 4.5). However, using a one way-ANOVA, there was a significant difference with these two temperatures compared with room temperature storage for the high sample (p = 0.001). Additionally, over the 5 days there was a significant difference in C_T value for the 'high' sample (p = 0.02), with an overall decrease in C_T value during storage at -20 °C and +4 °C, in contrast to an increase in C_T value for storage at room temperature. The pH remained at 7 for all samples/days, apart from for days 3 and 4 when both samples were stored at room temperature, where the pH lowered to 6.

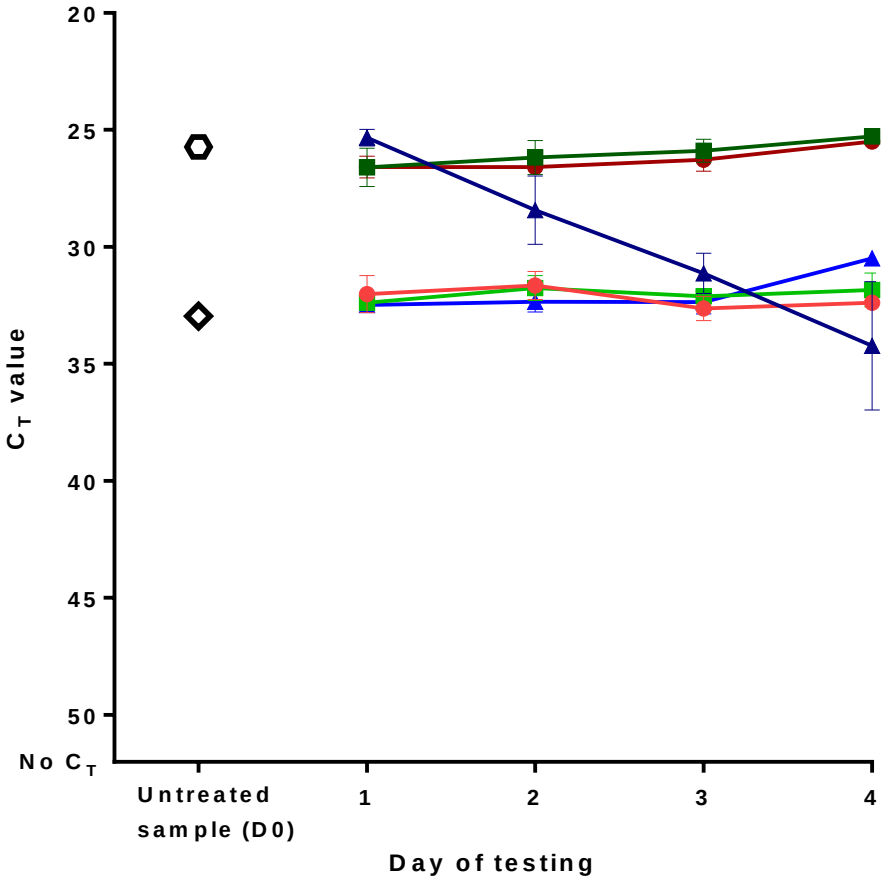


Figure 4.5 C_T values from the pan-serotypic rRT-PCR measured on each day after storage at -20°C (●), +4°C (■), and room temperature (RT) (▲) for the 'high' and 'low' samples. C_T values for the untreated high (⬡) and low (◇) milk samples prior to storage are also indicated. Each point represents the mean of three replicates, apart from 'high'-RT-D4, as no amplification was observed for one replicate. Error bars indicate the standard deviation.

4.5 Assessing the FMDV detection system using milk samples.

(F) Reproducibility of the limit of detection

Although limit of detection (LOD) experiments have previously been performed using the FMDV detection method employed in this project (see Chapter 2, Armson et al., 2018), these experiments have shown that at the LOD of the rRT-PCR assay amplification often only occurs in a proportion (e.g. one replicate out of two) of samples containing low amounts of viral RNA, as seen elsewhere in this chapter in experiments (A), (C) and (G-ii). This experiment aims to determine how consistently samples containing low amounts of viral RNA are detected at the LOD of the rRT-PCR.

Method

Ten-fold serial dilutions of FMDV cell culture isolate A/SAU/6/2015 were prepared in negative unpasteurised milk. The virus titre at a 10^{-1} dilution was 6.0 log $TCID_{50}$/mL (see experiment [B]). RNA was extracted from the dilutions 10^{-3} to 10^{-8} and 10 replicates of each dilution were tested using the pan-serotypic rRT-PCR.

Results

Amplification was observed for all ten replicates at a dilution of 10^{-6} (Figure 4.6). However, amplification was also observed for 4/10 (40%) of replicates at a dilution of 10^{-7}.

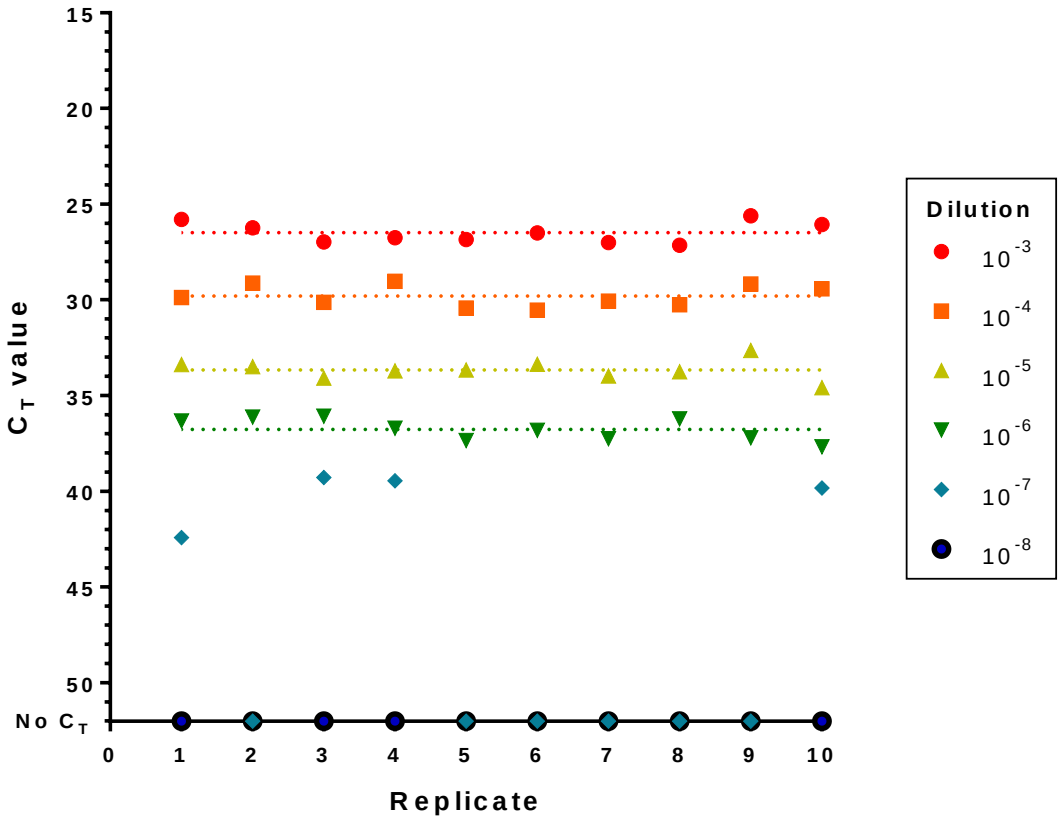


Figure 4.6 rRT-PCR (C_T) values of ten replicates of the ten-fold dilution series of cell culture isolate A/SAU/6/2015 spiked in unpasteurised milk. Dotted lines represent the mean of the ten replicates.

(G) Diagnostic specificity

In order to determine the diagnostic specificity of the FMDV detection system in milk, two experiments were performed:

(i) Checkerboard test to assess intra-run cross-contamination

(ii) FMDV rRT-PCR of milk samples from a known FMDV negative cohort

(G-i) Checkerboard test

Method

In these experiments (G-i and G-ii), cell culture isolate O/SAU/1/2016 was diluted to 10^{-2} in unpasteurised whole milk to prepare a FMDV positive control. Unpasteurised whole milk was used for the negative control.

Positive and negative control samples were added to the RNA extraction plate in a checkerboard layout as displayed in figure 4.7, and then an rRT-PCR performed in the same layout.

Results

No rRT-PCR signal indicative of contamination was observed in any of the negative wells. The mean C_T value of the positive wells was 24.24±0.71, with an inter-well coefficient of variation of 2.91%.

22.5	NEG	23.5	NEG	24.4	NEG	24.9	NEG	24.4	NEG	23.8	NEG
NEG	22.6	NEG	24.1	NEG	23.8	NEG	24.1	NEG	24.1	NEG	23.9
24.2	NEG	24.4	NEG	24.6	NEG	24.6	NEG	23.8	NEG	25.2	NEG
NEG	24.5	NEG	24.5	NEG	24.5	NEG	24.0	NEG	24.9	NEG	24.0
23.8	NEG	24.9	NEG	24.5	NEG	24.8	NEG	24.5	NEG	26.0	NEG
NEG	23.9	NEG	23.7	NEG	24.1	NEG	24.2	NEG	25.1	NEG	24.3
23.4	NEG	25.2	NEG	25.3	NEG	24.2	NEG	24.0	NEG	24.1	NEG
NEG	24.1	NEG	23.0	NEG	22.8	NEG	24.7	NEG	23.9	NEG	25.6

Figure 4.7 Checkerboard layout and C_T values after rRT-PCR of positive and negative spiked milk (white and grey wells, respectively). NEG: C_T value >50.

(G-ii) FMDV rRT-PCR of known negative samples

Method

Bulk milk samples (n = 442) collected from UK farms as part of the national brucellosis surveillance programme for England (DEFRA, 2015a) and submitted to the Animal and Plant Health Agency (APHA), Surrey, UK were used in this experiment. Broad Spectrum MicroTabs were added to each sample as a preservative, and they were refrigerated until tested. As the UK was free from FMD when these samples were collected, it was expected that all samples should be negative for FMDV on the rRT-PCR assay, allowing the specificity of the assay to be calculated.

RNA was extracted from the bulk milk samples, and then the rRT-PCR was performed on each sample in duplicate. Two positive and two negative control wells were included on each of the 11 rRT-PCR plates.

Results

Results of the rRT-PCR assays are shown in Table 4.3. Three wells (a maximum of one per plate) demonstrated false positive results with C_T values ranging between 39.11 and 41.09, leading to a specificity of the rRT-PCR assay of 99.66% (95% CI: 99.01% - 99.91%). Taking a 'positive' result as amplification in both duplicate wells the specificity of the rRT-PCR assay for the 442 milk samples tested was 100%. All controls were correct.

Table 4.3 rRT-PCR assay results of the 442 bulk milk samples tested.

rRT-PCR plate number	**Number of Bulk milk samples tested**	**Number of wells containing bulk milk samples**	**Number of wells positive* for FMDV (C_T value, % of total)**
1	43	86	0 (0%)
2	43	86	0 (0%)
3	43	86	0 (0%)
4	43	86	1 (41.09, 1.16%)
5	43	86	0 (0%)
6	43	86	0 (0%)
7	43	86	1 (39.39, 1.16%))
8	43	86	1 (39.11, 1.16%))
9	43	86	0 (0%)
10	43	86	0 (0%)
11	12	24	0 (0%)
Total	442	884	3 (0.34%)

*A positive result is defined as a C_T value ≤ 50

4.6 Discussion

Experiments performed in the first part of this chapter (section 4.4) aimed to simulate the storage, treatment and transport conditions of milk samples that may occur when they are collected from cattle in an endemic or outbreak setting.

After collection, milk samples may be stored locally either on the farm, or at a local laboratory. In order to increase the robustness of the samples during this temporary storage period, samples may be treated with a preservative, for example Bronopol, currently used in the UK when bulk milk samples are collected for the surveillance of a number of diseases (DEFRA, 2015a). Experiment *(A)* aimed to determine whether treatment with this common preservative has an inhibitory effect on the detection of FMDV, using the methods described in this thesis. Although there was no significant difference between the mean C_T values of the treated and untreated milk samples for the samples containing higher concentrations of FMDV RNA, there was a one $\log_{10}$ reduction in the limit of detection with treatment. Consequently, this method is only recommended where may be anticipated difficulties with maintenance of the cold chain, and where milk samples are not likely to contain low levels of FMDV RNA (e.g. pooled samples from large herds).

Together with treatment of milk samples with preservative, it was anticipated that prior inactivation of any live virus present may help to reduce the high cost of sample shipment by allowing milk samples to be sent at a lower category level. It has been demonstrated at TPI that FMDV infected serum samples that were heat treated for 2 hours at 56°C resulted in an inability to propagate the virus in tissue culture (The Pirbright Institute), although shorter time periods have also been reported to be effective (personal communication, Alison Morris). Prior to my study the ability to detect FMDV RNA following this treatment had not been investigated. However, it was anticipated that heat treatment for up to 2 hours may degrade FMDV RNA. Consequently, experiment (B) was designed to investigate whether the inactivation of live FMD virus was possible over shorter heat treatment times in milk samples, and following this, whether this affected FMDV RNA genome detection by rRT-PCR. Indeed, treatment of milk samples at 56^{o}C for over 30 minutes did inactivate any live virus present, however, it also significantly increased the resulting C_T value (>10). Although

this may be suitable for milk samples collected from individual cattle at the peak of FMD infection, this would not be appropriate for convalescing animals, or indeed pooled milk samples that typically demonstrate weaker C_T values. This experiment used a cell-culture isolate spiked milk sample, instead of milk from a naturally infected cow and it is thought that virus shed within cells of the milk from an infected cow may be further protected from inactivation procedures (Sellers, 1969; Tomasula et al., 2007). Therefore it is possible that the changes in C_T values/limit of detection may be reduced in this case, after heat inactivation or indeed after treatment with preservative. Consequently, further research should be performed to test heat inactivation of naturally infected milk samples, but also serum and other sample types, to determine whether the recommended 2 hour inactivation period could be shortened with the same inactivation effectiveness.

After collection in endemic countries, milk samples may need to be stored for a number of months in a -20^{o}C freezer, prior to testing or shipment to international reference laboratories. Experiment (C) demonstrated that, when compared to the 'gold standard' sample storage for international reference laboratories (-80^{o}C), there was no significant difference in FMDV RNA detection after 12 months of storage at -20^{o}C. This indicates that storage of milk samples in -20^{o}C freezers, which are more widely available in low resource laboratories, for up to 12 months would not affect the results of the rRT-PCR. However, for this experiment the temperature of the freezers remained in range (-90^{o}C to -50^{o}C for the -80^{o}C freezer, -30^{o}C to -5^{o}C for the -20^{o}C freezer), but in some endemic settings, power outages are a common occurrence, which may lead to freeze-thawing of the milk sample.

Consequently, experiment (D) aimed to simulate a scenario where multiple freeze-thaws of a milk sample may occur before testing. This may occur during storage, due to power outages as mentioned above, aliquoting of samples before transportation, or during a delayed/prolonged shipment. This experiment showed that even after five freeze-thaw steps, there was little change in C_T value (maximum change from starting value = 1.29 C_T). This indicates that unintentional freeze-thaws that may occur between sample collection and testing should not negatively affect the results generated by rRT-PCR.

In addition, experiment (E) was carried out to determine whether milk samples may be refrigerated during transportation to reduce shipping costs, instead of being maintained in a frozen state as currently recommended. Interestingly, C_T values of samples stored at +4°C were not significantly different from those stored at -20°C for four days for both the 'high' and 'low' samples. Storage at room temperature was also tested, and for the 'high' sample had a negative effect on the resulting C_T value after storage for 4 days. Interestingly however, no negative effect on the resulting C_T value for the 'low' sample was observed. The reason for this difference in the behaviour of the two samples is unknown. It is hypothesised that spoiling of the milk and lowering of the pH may have led to degradation of virus/nucleic acid, although it has been demonstrated that FMDV RNA detection is still possible from lateral flow device strips treated with citric acid (Romey et al., 2018). Consequently, it is possible that samples may be refrigerated during transportation, as long as this temperature can be maintained, as RNA detection from samples stored at higher temperatures is unpredictable. This may be used as a method to reduce shipment costs without affecting the viability of the RNA by permitting the use of simpler packaging types, decreasing the dimensions of packages, and removing the need to ship samples on dry ice.

Although these experiments demonstrate potential opportunities to reduce shipping costs, the use of a temperature monitor included in the shipment may be useful to record any fluctuations in the temperature of samples that may occur. This may then assist in informing the true status of test results, for example, whether there may be false negatives due to a prolonged increase in temperature during shipment. These experiments used limited isolates to test these methods and, although it is anticipated that differences in FMDV serotype/lineage would have little effect, this should be considered if a milk sampling surveillance system is to be implemented.

In the next part of this chapter (section 4.5), experiment (F) demonstrated the potential for false negative results when the levels of FMDV RNA genome in a pooled sample may be low, and at the limit of detection of the assay. Replicate rRT-PCR testing (e.g. duplicate/triplicate) is therefore recommended to increase the likelihood of FMDV detection.

Previous studies have investigated the diagnostic specificity of the FMDV rRT-PCR. For example, Reid et al. (2009) observed a 98.9% specificity for 3,004 samples collected from FMD-negative farms during field outbreaks in the United Kingdom in 2007. Additionally, as part of the collaborative work carried out for Chapter 2, the Foreign Animal Disease Diagnostic Laboratory (FADDL) observed a diagnostic specificity of 100% when testing 1,005 individual bulk milk tank samples in 5 US states (unpublished). However, both studies used different reagents/equipment/sample types to those used in this project. Therefore, in order to have confidence in the results from this project, it was important to determine the possibility of non-specific amplification, and the level of intra-assay contamination that may occur when testing milk samples by the chosen FMDV rRT-PCR assay.

For both experiments (G-i) and (G-ii), over 99% of samples known to be negative for FMDV were correctly identified as negative, with a 100% diagnostic specificity if a 'positive' result requires the presence of amplification in all replicate wells. It can therefore be assumed that amplification due to the presence of other template present in the milk cross-reacting with the FMDV rRT-PCR assay, is negligible, as observed previously. However, there were three samples where amplification did occur, indicating that some intra-assay contamination may occur during set-up or testing. C_T values of these false positives were those that may be expected from truly FMDV positive pooled milk samples. It is possible that this level of intra-assay contamination and the associated C_T values may be due to the strong positive controls used in this experiment (approximately $C_T = 20$), and using controls with higher C_T values may reduce this level of contamination. It is therefore necessary that great care is taken to minimise the risk of contamination when performing these assays, as it is difficult to determine whether a positive result is the result of false amplification, especially when samples containing low concentrations of FMDV RNA may not demonstrate amplification in all replicates tested. Additionally, this experiment was limited by the cost of reagents and the availability of samples. To more accurately determine the specificity of the rRT-PCR assay, it is recommended that a greater number of negative milk samples are tested, including those from a range of cattle breeds from different geographical regions. Indeed it should be considered that although the FMDV pan-

serotypic assay has been shown to be highly specific, organisms that may be present in the milk of animals not from the UK have the potential to cause false positive results.

4.7 Conclusion

Experiments performed in this chapter have tackled some of the logistical issues that might impact the use of pooled milk collected from outbreak scenarios or endemic settings, and shipment to national/international reference laboratories for FMDV detection by rRT-PCR.

Experiments in this chapter have demonstrated that the integrity of FMDV RNA present in milk samples does not seem to be significantly affected by changes such as chemical treatment with Bronopol, during freeze-thaw, and during refrigerated storage. It is likely, as alluded to previously (Blackwell and Hyde, 1976; Tomasula and Konstance, 2004; Spickler and Roth, 2012), that the high fat and protein content of whole milk may offer protection against these changes, making milk a particularly suitable sample type for FMDV detection.

Additionally, although the high analytical sensitivity of the FMDV rRT-PCR assay is encouraging, care should be taken when interpreting the test results of pooled milk samples from cattle in the field. In order to maximise the likelihood of FMDV detection in pooled milk samples containing a low FMDV RNA concentration, it is recommended that multiple replicates of samples are tested. In addition, great care should also be taken to minimise the likelihood of intra-assay contamination during testing using appropriate methods. These include the use of individual laboratory space and personal protective equipment for each stage of the testing process to minimise contamination of 'clean' reagents, laboratory/workspace disinfection and regularly changing gloves (Wilson, 1997; CDC, 1999; Schrader et al., 2012).

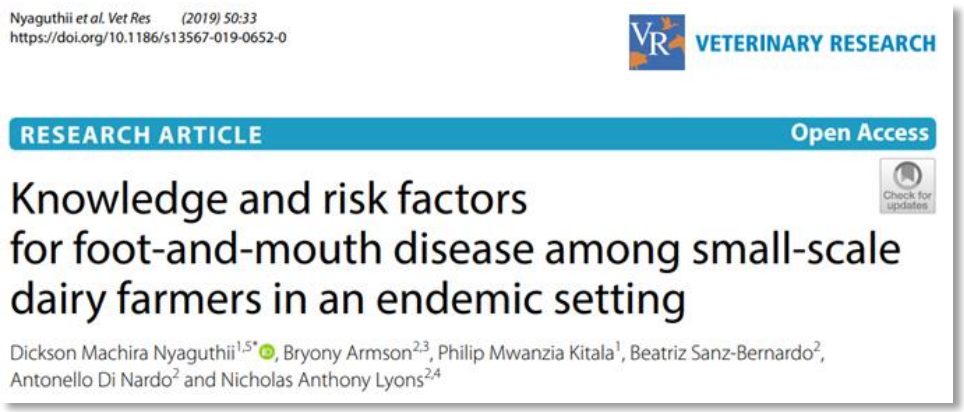
With these considerations in mind, experiments performed in this chapter further support the utility of pooled milk as an alternative sample for FMD surveillance, especially when samples may need to be shipped long distances for FMDV testing.

CHAPTER 5

Utilising milk from pooling facilities as a novel approach for foot-and-mouth disease surveillance

Data presented in this chapter has been published in *Transboundary and Emerging Diseases:* https://onlinelibrary.wiley.com/doi/10.1111/tbed.13487

Additionally, description of the study area and sample size calculations for Survey 1 have been described in the following research article, published in *Veterinary Research* (See Appendix III, Figure 8.2): https://doi.org/10.1186/s13567-019-0652-0.



Acknowledgements:

Fieldwork: Nick Lyons (NL) led the study design including sample size calculations, as the study was performed in conjunction with Dickson Nyaguthii's Master's project to investigate farmer knowledge and risk factors for clinical FMD. The milk sample collection protocol was designed by Bryony Armson (BA) and NL. Milk samples were collected throughout the study period by Samson Kosgey and Peter Othiambo, and their storage was accommodated by Hamish Grant and Leah Chege. BA, NL, Antonello Di Nardo (ADN), Beatriz Sanz-Bernado, Dickson Nyaguthii, Peter Ngugi and Gilbert Rono carried out small-holder farmer surveys, with assistance from the animal health assistants and drivers. Thanks also go to the milk pooling facility managers, the county and sub-county veterinary officers, and village chiefs for their support and cooperation of the project.

Laboratory work and analysis: Milk samples were shipped to The Pirbright Institute and tested by BA, who also carried out data analyses, interpretation of results and writing. Simon Gubbins and ADN provided additional guidance on statistical analysis.

5.1 Summary

Surveillance systems for foot-and-mouth disease (FMD) have several limitations and biases, and are often not effective in resource-limited countries. Using milk for the detection of FMD virus (FMDV) as a non-invasive, routinely-collected and cost-effective surveillance tool could address some of these limitations. This chapter aimed to investigate the potential of pooled milk sampling for FMD surveillance using real-time RT-PCR (rRT-PCR) which was compared with reports of household-level incidence of FMD in Nakuru County, Kenya. Pooled milk samples were collected weekly from five pooling facilities that were supplied by smallholder dairy farmers. Alongside this, periodic cross-sectional surveys of smallholder farmers were performed which were powered to detect a threshold household-level FMD incidence of 2.5%. Information on trends in milk production and sales was also collected, where it was observed that up to 26% of the smallholder farmers surveyed were contributing milk to pooling facilities. FMDV RNA was detected in only 9/219 pooled milk samples, with SAT 1 identified in 3 out of the 9 positive samples using a type-specific rRT-PCR. This finding was consistent with the laboratory confirmed serotype responsible for outbreaks in the study area at the time of milk sampling. Milk samples were positive for FMDV RNA on 4/21 half-month periods when at least one farmer reported observing FMD on their farm, i.e. the clinical FMD incidence at the household level was above a threshold of 2.5%. This indicates that the pooled milk surveillance system can detect FMD household level incidence at a threshold of 2.5%. Additionally, milk samples were positive for FMDV RNA on 5/21 half-month periods when there were no reports of FMD by farmers during the surveys, indicating that this surveillance system may be able to reveal the presence of FMD at even lower levels of infection in the population (i.e. below a threshold of 2.5%), or when conventional disease reporting systems fail. This pilot study highlights that surveillance based on molecular detection of FMDV in pooled milk samples has the potential to address some of the existing limitations of traditional surveillance methods. However, to fully evaluate the reliability of this surveillance approach in FMD endemic settings, further studies are required aimed at establishing a more precise correlation with estimates of household-level clinical incidence.

5.2 Introduction

Foot-and-mouth disease (FMD) has been described as a high impact disease among pastoralists in East Africa (Onono et al., 2013; Nthiwa et al., 2019). According to recent studies and reports by the OIE/FAO World Reference Laboratory for FMD (WRLFMD) four serotypes are currently known to circulate in East Africa, and within each serotype a number of topotypes/lineages also exist (Bachanek-Bankowska et al., 2016; Casey-Bryars et al., 2018; WRLFMD, 2018c, 2019). However, there are still major knowledge gaps about the distribution and epidemiology of circulating FMD viruses (Brito et al., 2015). This is particularly the case in areas where surveillance is dependent upon the recognition and reporting of clinical cases by farmers and livestock workers (Bates et al., 2003; Picado et al., 2011; Machira and Kitala, 2017) due to under-reporting. In these settings, limited capacity to undertake outbreak investigation and collection of clinical specimens following reports renders laboratory confirmation problematic (Kasanga et al., 2012; Namatovu et al., 2013). As a result, evidence-based strategies to respond to outbreaks are often not implemented, which reduces farmers' willingness to report even further. It is therefore difficult to determine the true incidence of the disease (Vosloo et al., 2002; Knight-Jones et al., 2016). Although targeted case finding or serological surveys are valuable, they are generally infrequent due to the costs and labour involved (Hadorn and Stark, 2008; Kasanga et al., 2012). Consequently, the requirement exists for a simple, cost effective approach for the surveillance of FMD that does not rely on farmer reporting.

Pooled milk is a routinely collected, non-invasive sample type that has the potential to be utilised for the herd-level surveillance of FMDV, as demonstrated by the surveillance of a number of other diseases including bovine viral diarrhoea (Dubovi and Section, 1995; Drew et al., 1999; Hill et al., 2010), brucellosis (Hamdy and Amin, 2002; Chand et al., 2005), and Q fever (Kim et al., 2005; Bauer et al., 2015). Limit of detection studies have highlighted the potential of identifying one acutely-infected milking cow in a herd of up to 1000 using pooled milk sampling (Armson et al., 2018, see Chapter 2). Additionally, simulation modelling suggested the earlier detection of FMDV by RT-PCR screening of pooled milk samples compared with farmer reporting, and encouraged

empirical studies to investigate the use of pooled milk for regional FMD surveillance (Thurmond and Perez, 2006; Garner et al., 2016; Kompas et al., 2017).

This chapter therefore describes a proof-of-concept pilot study performed in Nakuru County Kenya to explore the use of pooled milk as a non-invasive alternative sample matrix for the surveillance of FMD. Clinical FMD has been frequently reported in this region, and confirmed during outbreak investigations as part of the 'real-time' training courses organised by the European Commission for the Control of Foot-and-Mouth Disease (EuFMD) (Machira and Kitala, 2017; Nyaguthii et al., 2019). Indeed, in the six months prior to the commencement of this study, 13/220 smallholder farmers surveyed in Nakuru County reported having a case of FMD in at least one animal on their farm, as described by Nyaguthii et al. (2019) (Figure 5.1)

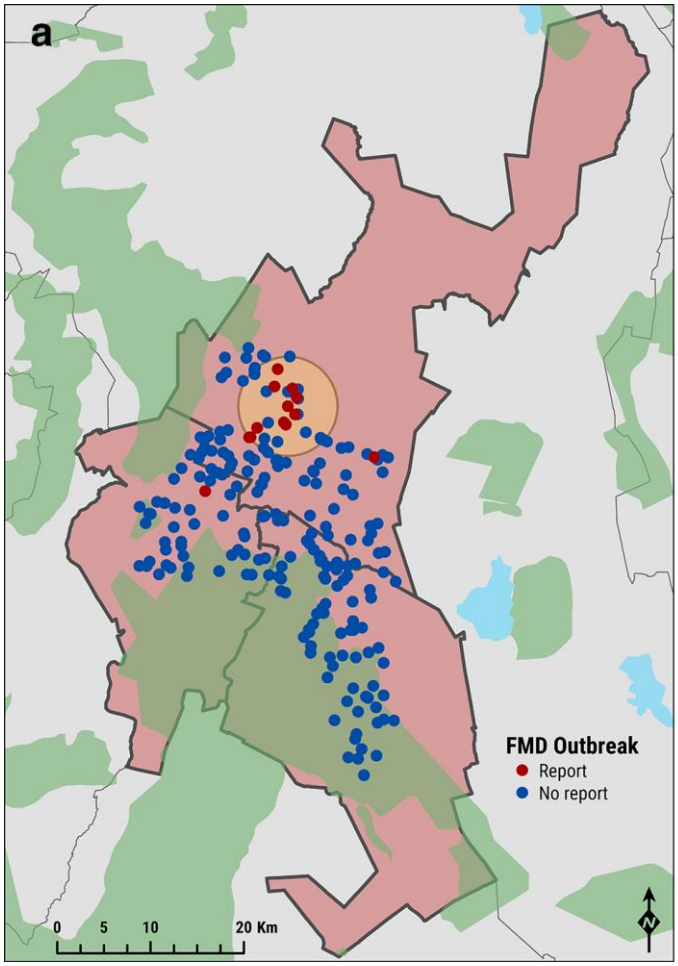


Figure 5.1. Map of occurrence of FMD cases in Nakuru County, Kenya in the six months prior to the study described in this chapter. Reports are according to cross-sectional smallholder farmer surveys performed by Nyaguthii et al. (2019) (Survey 1 – see section 5.3.3). Farmer-reported FMD outbreak locations within the study area are represented in red. Adapted from Figure 3 in Nyaguthii et al. (2019).

Although several studies have examined the impact of FMD outbreaks on large-scale farms in Kenya (Mulei et al., 2001; Kimani et al., 2005; Lyons et al., 2015), the majority of livestock is owned by smallholder dairy farmers, who contribute to an estimated 70-80% of all milk sold to the dairy production chain. This milk directly or indirectly supplies consumers, milk pooling facilities or private processors (Omore et al., 1999; Karanja, 2003; Rademaker et al., 2016). Generally, a small amount of milk produced by smallholder farmers is retained at home, while the rest is traded either to local shops, hotels or neighbours, or sold to milk pooling facilities directly or indirectly via hawkers (Karanja, 2003; TechnoServe Kenya, 2008; Muriuki, 2011) (Figure 5.2). Therefore, for the study described in this chapter it was anticipated that milk supplied by smallholder farmers and collected from pooling facilities could represent a useful resource for FMD surveillance in this endemic region.

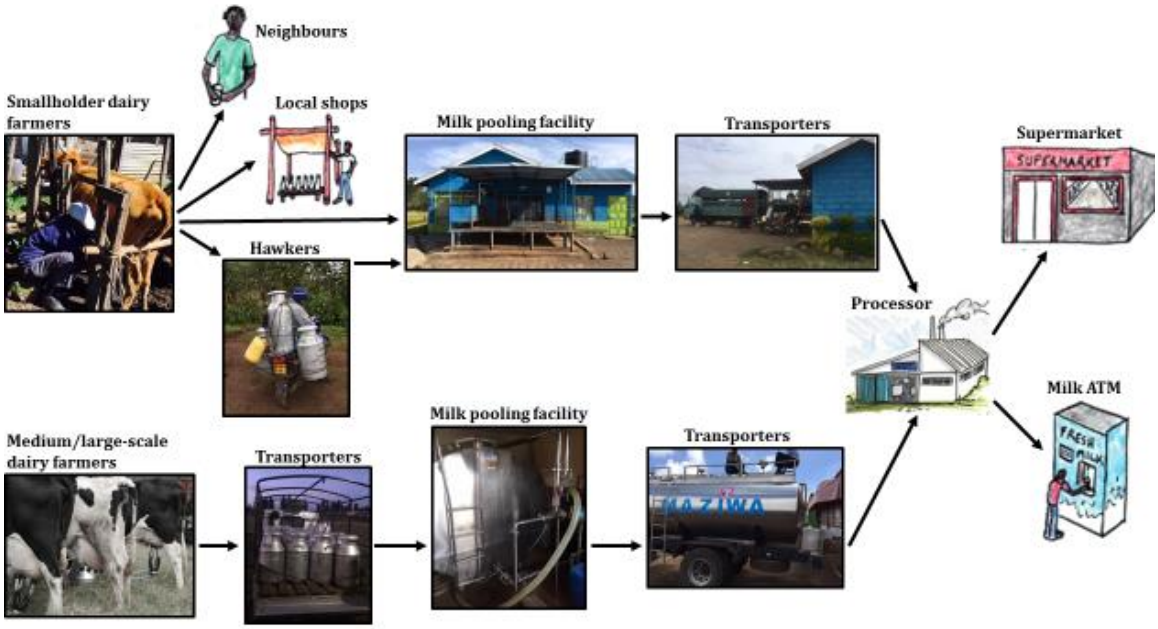


Figure 5.2. The dairy value chain in Kenya for milk supplied by smallholder and medium/large-scale dairy farmers. Based on data from Karanja (2003); TechnoServe Kenya (2008); Muriuki (2011). Adapted from Recheis (2019).

The aim of this study was therefore to (i) validate the use of milk collected from pooling facilities as a sample matrix for FMDV detection and characterisation and (ii) assess the usefulness of pooled milk as a simple, non-invasive alternative for FMD surveillance in Kenya whilst improving our knowledge on milk production and selling trends. To

achieve this, results obtained by FMDV rRT-PCR of milk samples collected from pooling facilities were tested for correlation with reports of clinical disease from surveys of smallholder farmers.

5.3 Materials and Methods

5.3.1 Study area and population

The study area is outlined by Nyaguthii et al. (2019). Briefly, the study area consisted of neighbouring catchment areas of five milk pooling facilities that were recruited for sample collection, located within Molo, Njoro and Rongai sub-counties of Nakuru County, Kenya (Figure 5.3). This area was selected due to the large numbers of FMD-susceptible livestock present, regular outbreaks of FMD, and the presence of a large number of smallholder dairy farmers. The milk pooling facilities were approached and informed consent acquired prior to participation in the study. Catchment areas were constructed with the guidance of facility managers using Google Earth (Google Inc., USA), and as some of the catchment areas bordered or overlapped each other, a single spatial polygon layer was created using QGIS version 2.18.10 (QGIS Development Team, Las Palmas, USA) to define the entire study area (Figure 5.3).

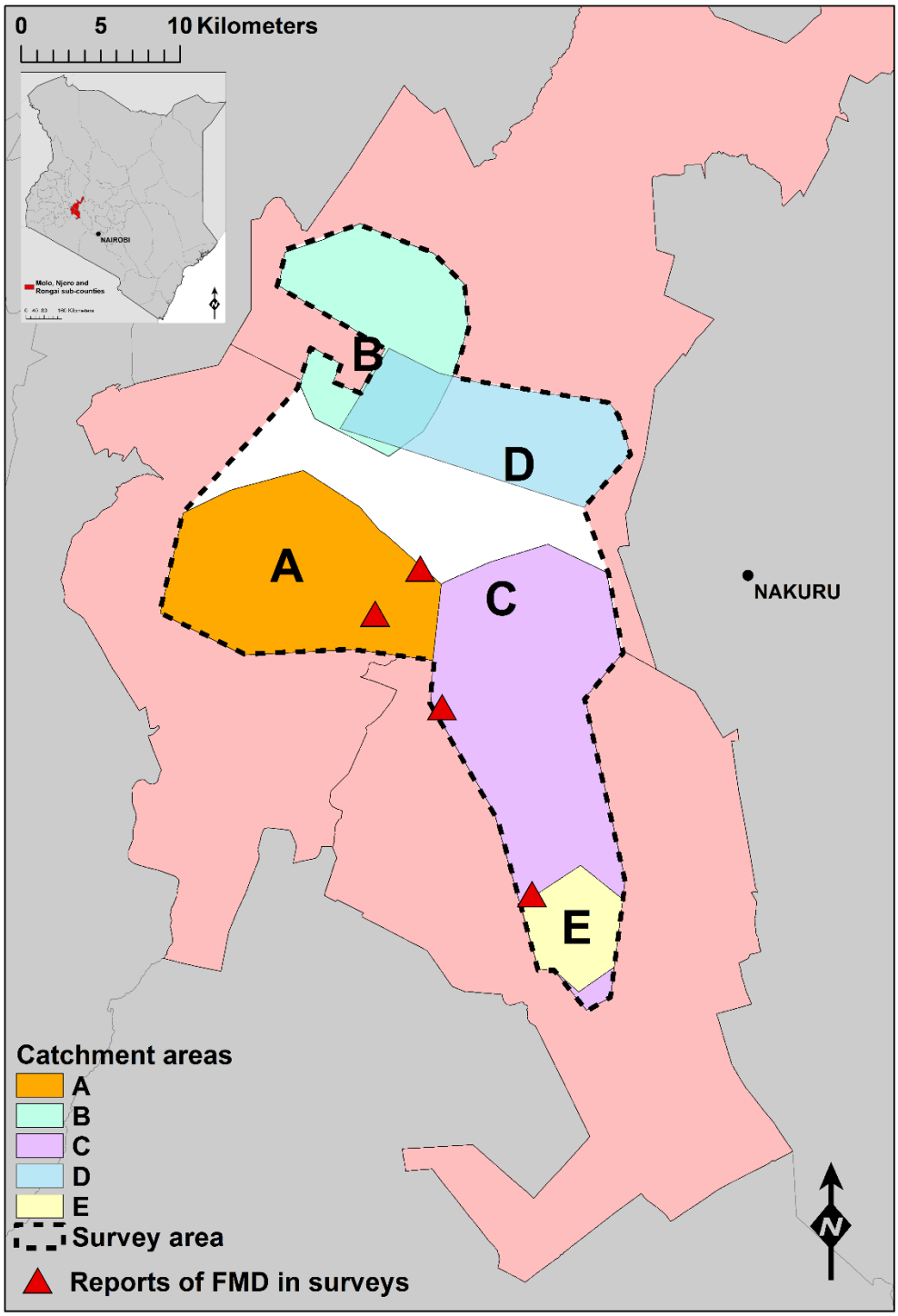


Figure 5.3 Map of the study area located in Molo, Rongai and Njoro sub-counties of Nakuru County, Kenya. Capitalised letters indicate the location of each milk pooling facility (A-E). Catchment areas for each facility are colour coded. Catchment area D overlaps that of B. Additionally, catchment area E overlays C. The survey area is bordered with a dashed black line, and the white area indicates parts of the study area that were not within the catchment areas of any of the milk pooling facilities. Red triangles indicate the locations of smallholder farms where FMD was reported during Survey 2 (see section 5.3.3).

5.3.2 Study objectives

The primary goal of the study was the detection of viral RNA by rRT-PCR in pooled milk samples collected from the milk pooling facilities described above to be compared with the presence of clinical FMD (as reported by smallholder farmers) above a defined threshold in the entire study area. In order to achieve this objective, two concurrent studies were performed:

(i) Repeat cross-sectional surveys of smallholder dairy farmers - to determine FMD incidence in the entire study area, and improve knowledge on milk production and selling trends in the catchment areas of the five milk pooling facilities.

(ii) The collection of pooled milk samples from the five milk pooling facilities, at 45 weekly time points - for subsequent FMDV detection by rRT-PCR.

5.3.3 Cross-sectional surveys for clinical disease

Three cross-sectional smallholder farmer surveys (S) were carried out for the study in this chapter.

For all surveys the eligibility criteria for the study population to be interviewed was smallholder dairy farmers described as those that owned at least one, but no more than fifty dairy cattle, and had cattle located within the premises. A systematic set of spatial points was randomly generated within the study area polygon using QGIS version 2.18.10 (QGIS Development Team, Las Palmas, USA). During the field surveys, the closest smallholder dairy farm to a randomly generated coordinate that fitted the eligibility criteria was surveyed. This was considered the optimal approach in the absence of a sampling frame or recent census data (with the last being done in 2009). The limitation of this approach is the assumption that smallholder farmers were evenly distributed throughout the study area. The spatial coordinates of surveyed farms were assigned to a facility catchment area using ArcGIS version 10.6.1 (Environmental Systems Research Institute, Inc.) based on approximate descriptions of the catchment areas from facility managers (Figure 5.3). Questionnaire data were collected using the EpiCollect+ mobile phone application (Aanensen et al., 2009).

Survey 1 (S1) was a baseline survey conducted between 16th November and 1st December 2016 to provide information on herd size, milk production and milk sales

only, as results on FMD clinical incidence from this survey do not correlate with milk sampling periods. This survey is described in more detail (including sample size calculations) by Nyaguthii et al., (2019) (see Appendix III, Figure 8.2). Briefly, based on an estimated prevalence of 15%, a total of 237 GPS coordinates were assigned, which included accounting for non-responsiveness and the potential inaccessibility of some farms.

Two subsequent smallholder farmer surveys (S2 and S3) were carried out during 23rd - 29th March 2017 (S2) and 20th - 26th September 2017 (S3), to provide further information on herd size, and the temporal trends of milk production and milk sales throughout the study period. During S2 and S3 farmers were asked how many cattle they owned, how many were lactating, how much milk their cows produced, how much milk they sold, and where they sold it (e.g. neighbours, ID of pooling facility). The full questionnaire is included in Appendix III, Figure 8.3. Additionally, epidemiological information was also obtained, including determination of the FMD clinical incidence in the study area since the previous survey. Farmers were asked if they had observed FMD on their farm sequentially since the time of the last survey (i.e. since S1 for S2, and since S2 for S3), and to provide an indication of the time of the outbreak (either the first half or second half of the month). This allowed FMD household-level clinical incidence to be estimated in two-week blocks of time (see statistical analysis section for further details). The case definition for FMD was defined as farmers observing at least two of the clinical signs listed by the African Union – Inter African Bureau for Animal Resources (AU-IBAR, 2014) in at least one of their animals. Due to a limitation on resources, S2 and S3 surveys were powered to detect a threshold household-level FMD incidence of 2.5% based on perfect sensitivity and specificity, a 95% confidence interval and an infinite study population. Based on these parameters, using the online epidemiological calculator EpiTools (http://epitools.ausvet.com.au) (Sergeant, 2019), the required number of households for each survey was 120. Consequently, 120 spatial points were randomly generated within the study area for each survey, as described above.

5.3.4 Pooled milk samples

Milk samples were collected once per week for 45 weeks (16th November 2016 – 20th September 2017) from each of the five recruited milk pooling facilities (denoted A – E). Milk was collected in 15 mL sterile Falcon tubes directly from the pooling tank for facilities A and B (Figure 5.4). A pooling tank was either not available or in use for facilities C, D and E. and so milk was pooled into 50 litre cans (<25 cans per facility). Therefore, to obtain a representative milk sample at these facilities, 3 mL of milk was taken from each can, pooled in a jug, and mixed. A 15 mL aliquot was then taken (Figure 5.4). At each collection the total volume of milk in the tank/cans, and the number of farmers contributing was recorded. Sample collection was organised so that one person could visit all facilities within a few hours. Immediately upon collection, all milk samples were stored on ice during transportation to a local -20°C storage facility that was equipped with a temperature monitor. Milk samples were shipped on dry ice to The Pirbright Institute (TPI) for subsequent laboratory analysis. Additionally, at the time of milk sample collection, information was obtained from the pooling facility regarding the tank volume and number of farmers supplying milk.

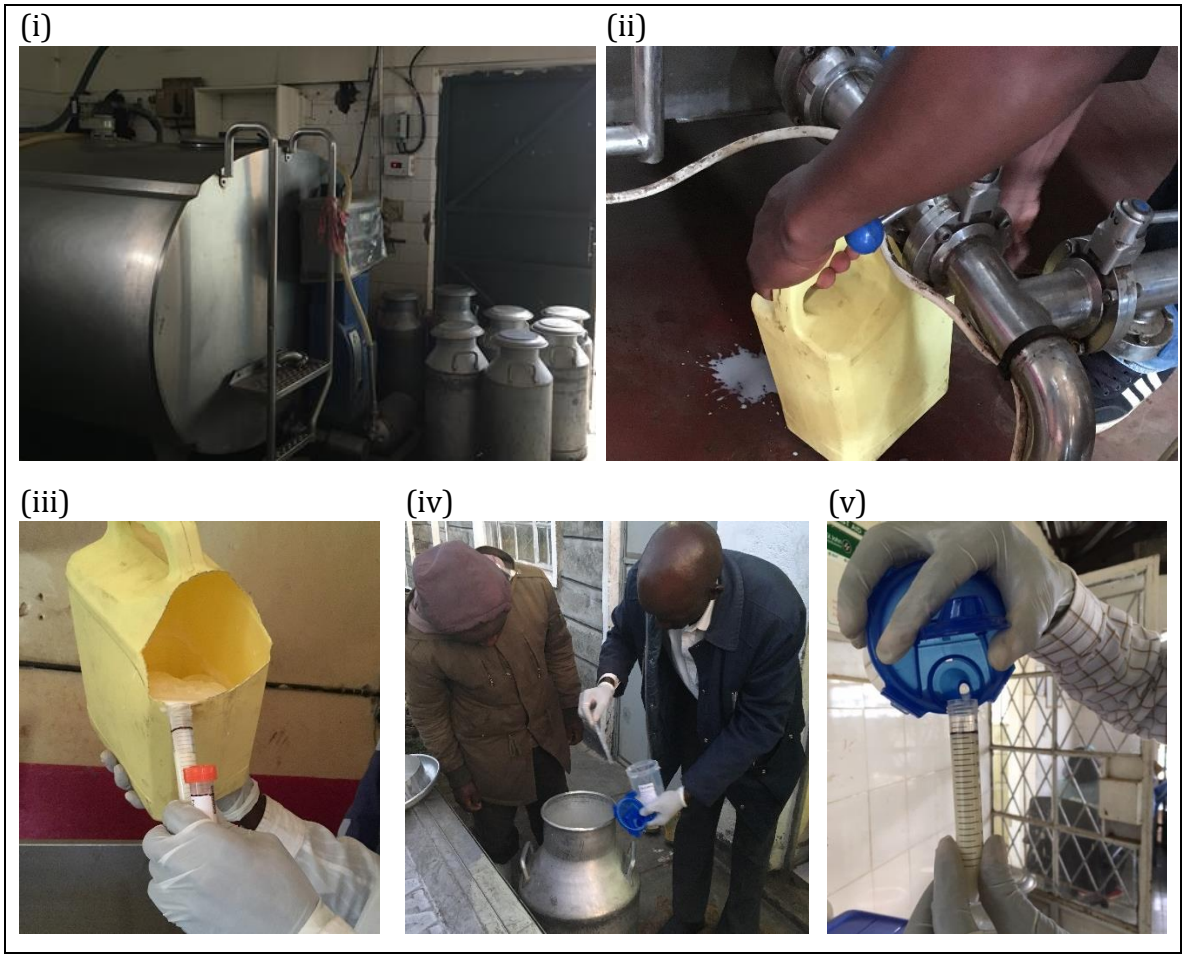


Figure 5.4 Milk sample collection from tanks (i, ii, iii) and 50L cans (iv, v) at milk pooling facilities in Nakuru County, Kenya.

5.3.5 Laboratory testing of pooled milk samples

RNA extraction and the pan-serotypic rRT-PCR assay were carried out in duplicate on all pooled milk samples using an optimised method as previously described (Armson et al., 2018, see Chapter 2). Briefly, RNA was extracted from whole milk samples using the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems®) on a MagMAX™ Express 96 Extraction Robot (Applied Biosystems®). rRT-PCR assays were performed using the reagents, parameters and thermal cycling conditions previously reported (Shaw et al., 2007) , with primers and probes targeting the conserved 3D region of the FMDV genome (Callahan et al., 2002). Any milk sample with a C_T value of ≤ 50 was considered positive, and was subsequently tested by the East Africa (EA) typing rRT-PCR assays [O, A, Southern African Territories (SAT) 1 and SAT 2]. These assays were designed to

detect FMDV lineages currently circulating in East Africa, namely: topotype EA-2 and EA-4 (serotype O); topotype AFRICA, lineage G-I (serotype A); topotype I (NWZ) (serotype SAT 1); and topotype IV (serotype SAT 2) (Bachanek-Bankowska et al., 2016). However, the fluorophores used on each probe were modified to: A – Cy5, SAT 1 – JOE™, SAT 2 – TAMRA™. This modification does not affect the sensitivity of the rRT-PCR assays. Positive samples for the EA rRT-PCR typing assays were also defined as those with a C_T value of ≤ 50.

FMDV cell culture isolates (isolated once in primary bovine thyroid [BTY] cells) were obtained from archival stocks held in the FAO/OIE World Reference Laboratory for foot-and-mouth disease repository (WRLFMD), TPI, UK. Cell culture isolates O/SAU/1/2016, O/TAN/39/2012, A/TAN/6/2013, SAT1/KEN/72/2010, and SAT2/TAN/19/2012 were used to prepare positive control material for the pan-serotypic and EA-O, EA-A, EA-SAT 1 and EA-SAT 2 rRT-PCR assays, respectively, using a 10^{-2} dilution spiked into unpasteurised whole milk from Jersey cattle. Two negative extraction controls consisting of unpasteurised whole milk were also included on each plate.

5.3.6 Statistical analysis

Descriptive and statistical analyses were carried out using R 3.5.3 (R Core Team, 2019) within RStudio IDE (RStudio Team, 2019). Paired t-tests were performed to compare information on milk yield and milk supply data between each survey.

Analyses tested the degree of association between the incidence of clinical FMD in the entire study area (either above or below the 2.5% household-level FMD incidence threshold) and the rRT-PCR testing, both at the study area level and within the individual milk pooling facility catchment area level. Mixed effect logistic regression analysis was performed including the milk pooling facility variable as a random effect on the intercept. This was to examine associations between the binary outcome of the rRT-PCR for weekly testing of the pooled milk samples in the entire study area (i.e. FMDV RNA detected yes/no), and the following explanatory variables: (1) clinical FMD incidence, (2) tank volume, (3) number of farmers contributing to the facility, (4) the

average number of adult female cows per farm, (5) the percentage of famers selling to a milk pooling facility, and (6) the average milk yield per cow per day. Variables 2 and 3 utilised weekly data collected from the pooling facility at the time of milk sampling, using increments of 1000L for tank volume (variable 2) and 100 for the number of farmers contributing to the facility (variable 3). For FMD incidence (variable 1), farmers were asked if they observed FMD on their farm, and when it occurred (in which half of the month) to create a binary variable. This was applied to each week of that half month, to enable comparison with the weekly rRT-PCR of pooled milk (i.e. if FMD was identified on a farm in the second half of January, both weeks in this half month period were assigned as positive for clinical FMD). Variables 4, 5 and 6 utilised data from smallholder farmer surveys, so there were only three data points for weeks 3 (S1), 20 (S2) and 45 (S3). Therefore, for each variable, data points for the unrecorded weeks were predicted by linear interpolation. A backward stepwise regression was performed to fit a final multivariate model, based on the results of a likelihood ratio tests to remove variables with a p value higher than 0.05.

5.4 Results

5.4.1 Pooled milk

5.4.1.1 Milk supply to pooling facilities

The average volume of milk recorded weekly in the tanks/cans over the entire study period was 3019.0, 1469.0, 237.5, 473.1 and 176.5 litres for milk pooling facilities A, B, C, D, and E, respectively. Variabilities in milk supply were observed over the study period, as shown in Figure 5.5 and Table 5.1. This was likely influenced by the number of farmers contributing to the milk pools, which also varied with a similar pattern. Results for individual milk pooling facilities are shown in Appendix III (Figures 8.4 and 8.5). The average number of farmers contributing milk to A, B, C, D, and E was 915 (range [min-max]: 450 – 1500), 29 (17 – 50), 25 (10 - 60), 42 (11 – 57) and 22 (10 - 33), respectively. The average volume of milk sold to a pooling facility per farmer was 14.1 litres (range [min-max]: 0.0 – 55.0) for the entire study area during the study period.

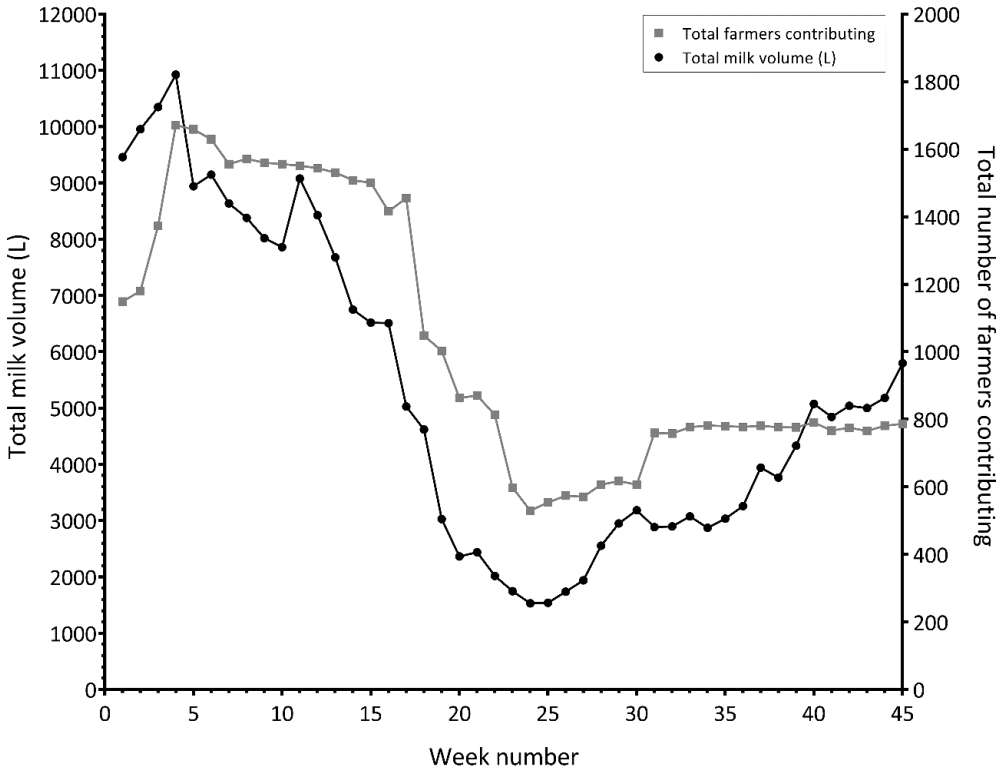


Figure 5.5 Temporal trend of the total volume of milk and the total number of farmers contributing to all milk pooling facilities on the dates sampled (week 1 – 16/11/2016, week 45 – 20/09/2017).

5.4.1.2 FMDV rRT-PCR results of pooled milk

A total of 219 pooled milk samples were collected from five facilities and tested using the pan-serotypic rRT-PCR. Milk samples were not collected on weeks 41 - 45 from facility B, due to a lack of milk supply, and on week 1 for facility E, as it was recruited a week later than the others. FMDV RNA genome was detected in 9/219 (4.11%) milk samples, 6 samples from facility A, and 1 sample from each of facilities B, C and D (mean C_T value: 40.57, range [min-max]: 36.15 – 46.74) (Figure 5.6). Additionally, 3/9 samples (collected at facility A) with the strongest C_T values (<39) were also positive by the EA SAT 1 rRT-PCR typing assay. No other serotypes were detected by the EA rRT-PCR typing assays in the positive milk samples. The detection of SAT 1 in milk was concordant with results from clinical lesion material collected within the study area on the 27/01/2017 and submitted to the WRLFMD for confirmatory diagnostics, sequencing and phylogenetic analyses (WRLFMD, 2017), although these positive milk

samples and the clinical sample were collected from different catchment areas (A and D respectively).

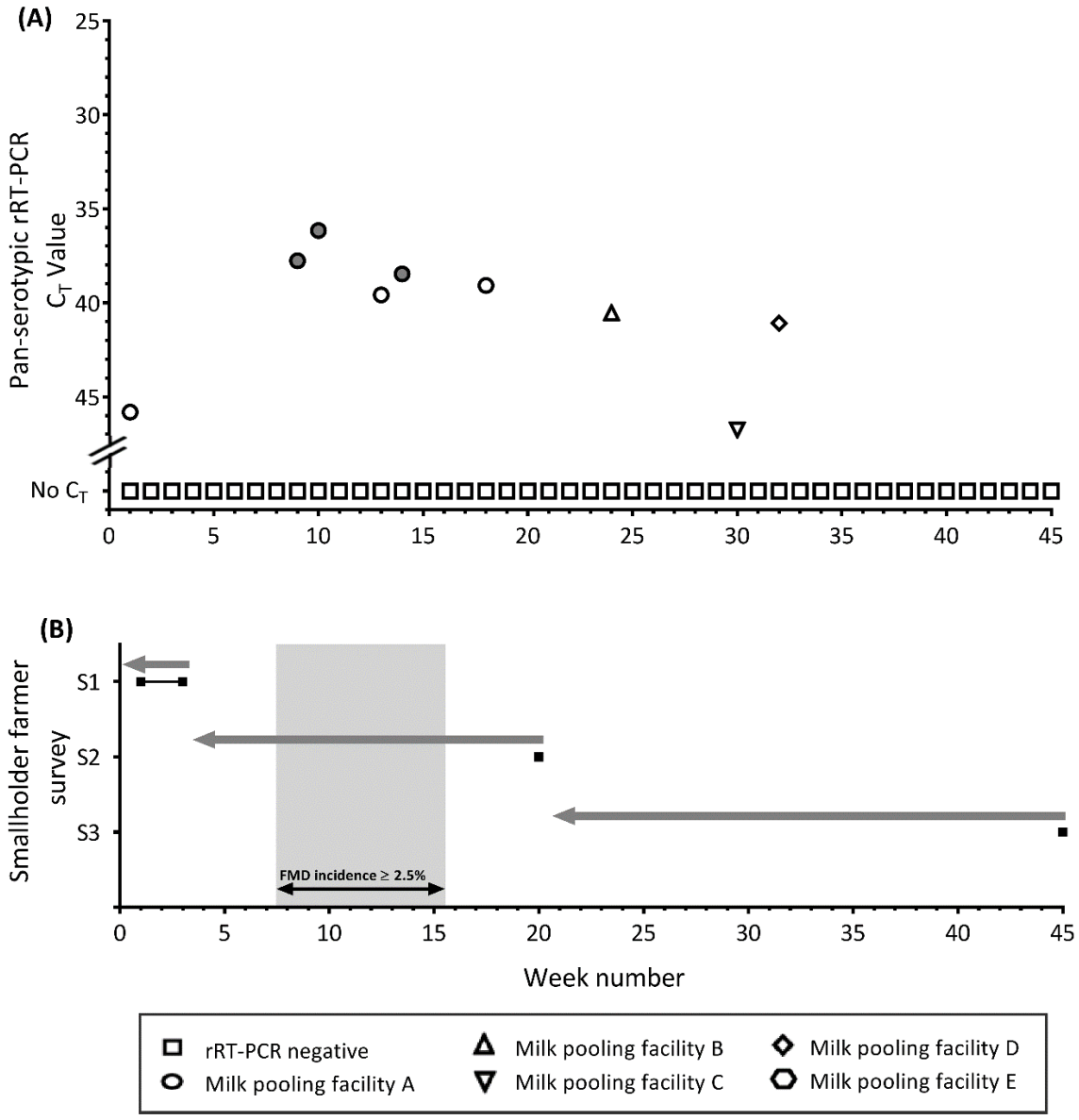


Figure 5.6 (A) Pan serotypic rRT-PCR C_T values from pooled milk samples collected from milk pooling facilities A, B, C, D and E in Nakuru County, Kenya, over the 45-week study period. Points with a grey centre represent samples that were also positive by the SAT 1 serotype-specific assay (C_T value displayed is for the pan-serotypic rRT-PCR assay). (B) Black squares indicate time points of smallholder farmer surveys 1, 2 and 3. Grey arrows indicate the time period for which FMD incidence questions where based for each survey. Grey shading indicates time points where the FMD incidence was ≥2.5% in the entire study area (as reported by smallholder farmers during the household surveys).

5.4.2 Cross-sectional surveys

Descriptive data from each survey is detailed in Table 5.1 for the entire study area, and also for the catchment areas of the individual milk pooling facilities. The number of smallholder farmers that took part in S1, S2 and S3 was 220, 117 and 119, respectively. Due to compilation of a combined catchment area and randomisation of the spatial coordinates generated for surveying smallholder farms throughout the study area, some farms were located outside the boundaries of the individual catchment areas of any milk pooling facilities (Figure 5.3). Therefore, data from 47, 15, and 18 smallholder farms (for S1, S2 and S3, respectively) were not included in the descriptive data for individual catchment areas, and only included in the descriptive data and regression analysis generalised to the entire study area (Table 5.1). Due to the overlap of some of the catchment areas, some farms were also included in the analysis for more than one individual catchment area.

The number of farmers in the entire study area that reported FMD on their farm during the 45-week study period was 4/456 (0.88%). All of the reported cases of FMD were in S2, when 4/117 (3.42%) farmers reported disease in their animals during either January or February (weeks 8-15), representing a household level incidence above 2.5% during those times (Figure 5.6). Additionally, reactive FMD vaccination was reported by farmers during the study period, where 48.7% of farmers interviewed reported vaccination of their cattle during S2, when there were reports of FMD in the study area (Table 5.1). During S3, there were no reports of FMD vaccination.

The average volume of milk yield daily per farm was higher for S1 (11.0 litres, 95% CI 9.0 – 13.1, p = 0.117) and S3 (13.8 litres, 95% CI 11.1 – 16.5, p = 0.003) compared to S2 (8.9 litres, 95% CI 7.0 – 10.7), consistent with the milk supply trends to facilities and is likely related to a prolonged drought that took place during this time (Figure 5.5). For the entire study area, the largest percentage of farmers that sold to a facility at any time during the study period was 25.5% (S1), which was found to be lower during the subsequent surveys (S2 12.0%, p = 0.04; S3 15.1%, p = 0.20). The same pattern was observed for the individual catchment areas, where the largest number of farmers contributing at any time was in catchment area B (47.5%) recorded in S1. In the

catchment area of facility E none of the farms surveyed sold their milk to a pooling facility throughout the study period.

5.4.3 Correlation between FMDV RNA in milk samples from all pooling facilities, and clinical FMD in the entire study area.

Results from the rRT-PCR were cross-tabulated with the household-level FMD clinical incidence in the entire study area (where FMD incidence above the 2.5% threshold was defined as the gold standard) (Table 5.2). As each parameter was measured in different time periods (i.e. pooled milk was collected weekly, whereas the FMD incidence was recorded in half-month increments), half-month time periods were employed. Consequently, a binary variable was created for the results of the rRT-PCR where a half-month period was assigned as positive if there was a positive rRT-PCR result in at least one week of that period. Therefore, by using the half-month periods and taking the clinical incidence as reported by farmers in the whole study area as the gold standard, the pooled milk surveillance system had a sensitivity of 100% (95% CI 51.0% – 100%) and specificity of 70.6% (95% CI 46.9% - 86.7%) (Table 5.2). FMDV RNA was identified in four pooled milk samples collected during the period from January to February 2017 (weeks 8-15) when the clinical incidence at household level across the entire study area was ≥2.5% (3.42%) (Figure 5.6). There were also instances where FMDV RNA was detected in the milk samples, but there were no corresponding reports by farmers (i.e. the household-level incidence of FMD was not above 2.5%).

Table 5.1 Descriptive summary of the study population.

Milk pooling facility catchment area	N‡	Mean number of cows per farm (range: min-max)	Mean number of female cows > 2yrs per farm (range: min-max)	Mean volume of milk (L) yield daily per farm (range: min-max)	Number of farms that sell to a milk pooling facility (%)§	Mean volume of milk (L) per farm sold to a milk pooling facility (range: min-max)§	Number of farmers reporting FMD cases (%)¶	Number of farms vaccinated for FMD (%)¶
Survey 1†								
A	45	4.4 (1.0 – 11.0)	2.7 (0.0 – 7.0)	8.5 (0.0 – 22.0)	13 (28.9%)	6.8 (0.0 – 15.0)	N/A	N/A
B	40	7.9 (1.0 – 30.0)	3.9 (1.0 – 10.0)	11.6 (0.0 – 50.0)	19 (47.5%)	13.4 (2.0 – 50.0)	N/A	N/A
C	74	4.9 (1.0 – 24.0)	2.2 (0.0 – 15.0)	9.7 (0.0 – 120.0)	10 (13.5%)	7.9 (1.5 – 25.0)	N/A	N/A
D	35	6.3 (1.0 – 30.0)	3.4 (1.0 – 10.0)	13.4 (0.0 – 60.0)	9 (25.7%)	18.9 (3.5 – 50.0)	N/A	N/A
E	8	3.5 (1.0 – 7.0)	1.9 (1.0 – 3.0)	6.6 (0.0 - 11.5)	0 (0.0%)	0.0 (0.0 – 0.0)	N/A	N/A
Total study area	220	5.5 (1.0 – 42.0)	2.9 (0.0 – 15.0)	11.0 (0.0 – 140.0)	56 (25.5%)	12.1 (0.0 – 55.0)	N/A	N/A
Survey 2								
A	39	5.5 (1.0 – 46.0)	2.8 (0.0 – 12.0)	6.8 (0.0 – 46.0)	7 (18.0%)	11.0 (2.0 – 46.0)	2 (5.1%)	19 (48.7%)
B	16	10.6 (1.0 – 40.0)	3.7 (0.0 – 16.0)	7.4 (0.0 – 35.0)	2 (12.5%)	10.0 (3.0 – 17.0)	0 (0.0%)	5 (31.3%)
C	35	6.5 (2.0 – 44.0)	2.9 (0.0 – 23.0)	8.6 (0.0 – 50.0)	1 (2.9%)	50.0 (50.0 -50.0)	2 (5.7%)	7 (20.0%)
D	15	9.8 (2.0 – 40.0)	5.1 (0.0 – 16.0)	11.0 (0.5 – 35.0)	2 (13.3%)	10.0 (3.0 – 17.0)	0 (0.0%)	1 (6.7%)
E	4	3.5 (2.0 – 5.0)	1.5 (1.0 – 2.0)	4.0 (2.0 – 6.0)	0 (0.0%)	N/A	0 (0.0%)	0 (0.0%)
Total study area	117	7.3 (1.0 – 46.0)	3.4 (0.0 – 26.0)	8.9 (0.0 – 50.0)	14 (12.0%)	14.8 (2.0 – 50.0)	4 (3.4%)	42 (35.9%)
Survey 3								
A	28	5.5 (1.0 – 17.0)	2.9 (0.0 – 10.0)	11.7 (0.0 – 45.0)	7 (25.0%)	15.7 (5.0 – 40.0)	0 (0.0%)	0 (0.0%)
B	15	7.2 (1.0 – 30.0)	2.7 (1.0 – 5.0)	12.5 (0.0 – 77.0)	1 (6.7%)	7 (7.0 -7.0)	0 (0.0%)	4 (26.7%)
C	41	6.6 (2.0 – 44.0)	3.1 (1.0 – 14.0)	16.0 (3.0 – 48.0)	2 (4.9%)	23.5 (12.0 – 35.0)	0 (0.0%)	0 (0.0%)
D	17	4.2 (1.0 – 16.0)	2.3 (1.0 – 8.0)	15.2 (0.0 – 95.0)	2 (11.8%)	6.5 (5.0 – 8.0)	0 (0.0%)	2 (11.8%)
E	8	6.9 (3.0 – 14.0)	3.1 (1.0 – 6.0)	20.3 (4.0 – 32.0)	0 (0.0%)	N/A	0 (0.0%)	0 (0.0%)
Total study area	119	6.1 (1.0 – 44.0)	3.0 (0.0 – 16.0)	13.8 (0.0 – 95.0)	18 (15.1%)	15.5 (4.0 – 40.0)	0 (0.0%)	7 (5.9%)

†S1 was a baseline survey and results are described previously by Nyaguthii *et al. (2019)*. ‡Number of farms surveyed. §Farms may sell to any milk pooling facility. ¶S2 and S3 since the previous survey. N/A – not applicable as this occurred outside of the study period.

Table 5.2 Comparison of the FMDV pan-serotypic rRT-PCR results of samples from all milk pooling facilities, with FMD incidence by farmer reports for the entire study area.

		FMD incidence ≥ 2.5%		
		Yes	No	Total
Pan-serotypic rRT-PCR of pooled milk samples	Positive†	4	5	9
	Negative	0	12	12
	Total	4	17	21

Half-month periods were assigned to the results of the rRT-PCR of the pooled milk and deemed positive if there was a positive rRT-PCR result in at least one week of that period. There were a total of 21 half-month periods for the duration of the study. †A positive pan-serotypic rRT-PCR result is defined as a C_T value of ≤50 in any week of the half-month time period.

Both univariable analyses and the multivariable mixed effect logistic regression models were used to determine whether there was any association between the household-level incidence being above 2.5% in the entire study area and the FMD rRT-PCR results from pooled milk, further incorporating other predictors listed in Table 5.3. Based on the univariable analysis, there was a higher odds of observing a FMD positive rRT-PCR result when the clinical incidence in the whole study area was ≥2.5% (OR = 4.21, 95% CI = 1.02 – 17.30, p = 0.046), the volume of milk supplied to the facility increased (OR = 1.78 for each additional 1000 litres supplied, 95% CI = 1.25 – 2.54, p = 0.002), and when the number of farmers contributing to the facility increased (OR = 1.27 for each additional 100 farmers, 95% CI = 1.12 – 1.43, p < 0.001). During multivariate model selection, after model simplification by removing non-significant terms (p > 0.05), only the number of farmers contributing to the facility (3) was retained as significant (OR = 1.27, 95% CI = 1.12 - 1.43, p < 0.001), and there was no longer any association between the household-level incidence being above 2.5% in the study area and the FMD rRT-PCR result in the pooled milk.

Univariable and multivariable logistic regression analysis was also carried out using the data for each individual catchment area only (data not shown). The only catchment area with any significant associations was for facility A, where there was a higher odds of observing an FMD positive rRT-PCR result when there was an increase in the number of farmers contributing to the tank (OR = 1.38 for each additional 100 farmers,

95% CI = 1.03 – 1.85, p = 0.031). Significant associations were not observed for any of the other catchment areas.

Table 5.3 Univariate mixed effect logistic regression analysis for association with a positive FMD rRT-PCR result for the total study area.

Variable	Type of variable	Odds Ratio (95% CI)	*P* value
1. FMD incidence	Categorical	4.21 (1.02 - 17.31)	0.046
2. Tank volume (per 1000 litres)	Continuous	1.78 (1.25 - 2.54)	0.002
3. Number of farmers contributing to the facility (per 100 farmers)	Continuous	1.27 (1.12 - 1.43)	< 0.001
4. Average number of adult female cows per farm	Continuous	0.99 (0.26 - 3.78)	0.990
5. Percentage of farmers selling to a milk pooling facility	Continuous	1.04 (0.96 - 1.11)	0.358
6. Average milk yield per cow	Continuous	0.48 (0.15 - 1.49)	0.203

5.5 Discussion

Previous studies have demonstrated the potential of using milk from individual animals as an alternative sample type for FMDV detection and surveillance (Armson et al., 2019, Chapter 3), and that it is possible to detect FMDV in highly diluted milk samples from individual clinical cases (Armson et al., 2018, Chapter 2). This pilot study aimed to expand on this work and explore the use of milk collected from pooling facilities supplied by smallholder farmers as a simple, non-invasive alternative sample matrix for the surveillance of FMD. In order to achieve this, the household-level incidence of clinical disease was compared with FMDV RNA detection by rRT-PCR from pooled milk facilities in an endemic region of Kenya.

According to data collected by milk pooling facilities, the number of farmers contributing milk fluctuated throughout the study period, with a similar pattern observed for the volume of milk supplied. There was a decrease in the output of milk supplied to all facilities in or after March 2017, likely due to the effects of a drought that occurred in the first quarter of 2017 (World Food Programme Kenya, 2017). This corresponded with data collected from the smallholder farmer surveys, demonstrating

the lowest milk yield per farmer, and the lowest percentage of farmers supplying milk to a facility (12.0% for the entire study area) occurring at this time. The largest percentage of farmers that supplied milk to a facility at any time was 25.5% for the entire study area, or 47.5% for an individual catchment area.

FMDV RNA was detected in milk samples collected from pooling facilities with tanks containing up to 5000 litres. Additionally, typing assays confirmed the presence of SAT 1, which was concurrent with reports from clinical samples collected from reported outbreaks for confirmatory diagnostics (WRLFMD, 2017). The average C_T values obtained for the positive milk samples were high (>36), likely due to the dilution factor of the samples, as some were collected from large pools (up to 5000 litres). This corresponds with previous limit of detection studies (Armson et al., 2018) that predicted similar C_T values (>30) for pools of this size. This study observed that an increase in tank volume was correlated with an increase in the number of farmers contributing milk, and consequently it is probable that the likelihood of an FMD infected cow that supplied milk to one of these pools is increased, contrary to what might be expected based on rRT-PCR test sensitivity. Univariable analysis supported this, suggesting a positive association between an FMD positive rRT-PCR result in the pooled milk and the number of farmers contributing to the facility, and also with the volume of milk in the tank/cans at the time of sample collection. Based on these results, the likelihood of FMDV detection, and therefore surveillance efficiency may be optimised by targeting sampling on large milk pooling facilities that have milk supplied from a large percentage of farmers in their catchment area.

During the study period, throughout the entire study area, there were four reports of FMD in the smallholder farmer surveys in facility catchment areas A and C, all during January and February 2017. As there was at least one FMD report in each half-month period during these two months, the household-level incidence in the whole of the study area was significantly ≥2.5%. This correlated with FMDV detected in a milk sample collected from at least one of the facilities in the study area in each of these half-month periods, therefore it could be assumed that the pooled milk surveillance system might be able to detect FMDV when the household-level incidence is ≥2.5%. This was supported by univariable analysis which indicated a positive association between an FMD positive rRT-PCR result when the clinical incidence in the entire study area was

≥2.5%. Although the sensitivity was 100% when using clinical reports from farmers as the gold standard, the authors acknowledge the limitations of using these half-month time steps for this comparative analysis. These half-month time periods for FMD reporting were used to simplify data recording which was based on farmer recall in the absence of written records.

FMDV RNA was also detected in five pooled milk samples that were collected when there were no clinical FMD reports. As there were negative extraction controls and a high number of 'negative' samples where no amplification was observed on the rRT-PCR assay, it is unlikely that non-specific amplification of other template (e.g. from other organisms) present in milk occurred. The laboratory methodology used in this study has been shown to be highly specific, however, inter/intra-assay contamination was observed in the negative cohort tested in Chapter 4 and therefore laboratory contamination cannot be excluded, even though measures were implemented to minimise the likelihood of this occurring. There may also be further alternative explanations which are discussed below.

It is possible that the surveys conducted for this study were underpowered, due to limited resources available, and therefore the clinical disease threshold of 2.5% was too high to robustly assess specificity. In future studies, a more precise evaluation of sensitivity and specificity of the pooled milk detection system may be achieved if surveys are powered to detect a lower threshold FMD incidence. Farmers in this region of Kenya had good knowledge of FMD (Nyaguthii et al., 2019), which was demonstrated by the descriptions of clinical signs by farmers corresponding with the case definition. However, it is possible that mild clinical signs or sub-clinical infection could reduce the likelihood of farmer reporting and provide explanation for instances where there were positive milk samples but no farmer reports of disease. Further investigation is required to determine the incidence of sub-clinically infected animals in this region, for example by using serological surveys, and whether virus particles may be present in the milk of sub-clinically infected animals (Sutmoller and Casas, 2002). Further investigation is also required into the impact of vaccination on FMDV excretion in milk. During the study period, vaccination was carried out in response to an outbreak. Whether vaccination in these herds may increase the likelihood of sub-clinical infection is unknown, although there have been reports of sub-clinical infection in vaccinated

animals (Donaldson and Kitching, 1989; Hutber et al., 1999; Lyons et al., 2017), and virus excretion in the milk of apparently healthy vaccinated animals (Ahmed et al., 2017).

Of the milk samples positive by the pan-serotypic rRT-PCR assay, 3 were identified as SAT 1, but no amplification was observed in any of the EA typing rRT-PCR assays for the other samples. Outbreaks due to the circulation of type O outside of the study area were reported in August 2017 (WRLFMD, 2017). It is possible that these samples were at the limit of detection for the EA-O rRT-PCR typing assay, as this assay has been shown to have a slightly reduced analytical sensitivity compared with the pan-serotypic rRT-PCR assay (Bachanek-Bankowska et al., 2016). It is also possible that another lineage of FMDV was also circulating in the region that cannot be detected by the EA typing assays used.

Several methodological issues arose during this study that may have affected the results of FMD clinical incidence and therefore the sensitivity and specificity estimations of the pooled milk surveillance system. The original aim of the study was to undertake smallholder farmer surveys within the catchment areas of the milk pooling facilities. Catchment areas were approximated by facility managers, and as some of the catchment areas either bordered or overlapped each other, a single spatial polygon was created to define the whole study area. It is unclear how precise these catchment areas were, as in some cases farmers from one catchment area reported supplying milk to a neighbouring catchment area. This may explain cases where there was a positive report of FMD by a farmer in one catchment area, but there were no rRT-PCR positive milk samples from the area's pooling facility in that time period (for example catchment area C). Additionally, some of the surveyed farms were located in more than one catchment area (due to overlap of the catchment areas), or none of the catchment areas (due to being between catchment areas), which may have led to bias in the descriptive data and analysis. Due to the absence of an available sampling frame, it was assumed that smallholder farmers were evenly distributed throughout the whole study area. This was a reasonable assumption based on the knowledge of the authors and animal health assistants in the area, although any disparity may have led to an inaccurate estimation of household-level incidence. In addition, the intention of the study was to recruit milk pooling facilities that stored milk in bulk tanks for the

collection of milk samples. However, three of the facilities either did not have, or were not using their bulk tanks, and instead pooled milk in 50 litre cans. The reasons for not using an existing bulk-tank included a low milk supply and not being fully functional. Consequently, a small volume of milk from each can was pooled and mixed in order to obtain a sample representative of the whole milk pool from this facility. The authors recognise the limitations in this approach and further sampling methodologies for facilities using cans should be explored.

This pilot study describes the rRT-PCR testing of milk samples from milk pooling facilities as a simple surveillance approach for FMD in this endemic region of Kenya. Based on data from the entire study area, by utilising the weekly collection of milk samples, it was possible to detect and type FMDV RNA by rRT-PCR from milk pools of up to 5000 litres, when the FMD clinical incidence was ≥2.5%, and when fewer than 25% of farmers were selling their milk to these pooling facilities. Based on the encouraging results obtained in this study, further investigation is required to obtain a more precise correlation of household-level incidence with pooled milk sample results, to fully assess the usefulness of this novel surveillance approach. With more resources available, this could be achieved by combining clinical surveys of FMD infection at the individual animal level and serological surveys with sufficient statistical powers to detect a low incidence of infection or disease. Additionally, the collection of pooled milk samples should be focussed on larger facilities, which have a large number of contributing farmers from the surrounding area. Furthermore, pooling systems higher up the dairy production chain should also be explored as a target for FMD surveillance, although the possible reduced ability in detecting FMDV RNA from milk samples after pasteurisation. Follow-on studies should also investigate the establishment of sentinel systems in the epidemiological surveillance of FMD, and how geographical limits that may encompass different farming practices may affect this solution.

In conclusion, this pilot study highlights that this novel, simple surveillance approach has the potential to address some of the well-recognised limitations of more traditional surveillance methods in resource-limited countries where there are a high number of smallholder dairy farmers, and to improve the capacity for surveillance which could contribute to informing and evaluating disease control policies in these endemic regions.

CHAPTER 6

Pooled milk for foot-and-mouth disease surveillance on large-scale dairy farms in endemic settings

Data presented in this chapter have been submitted as an original research article to *Frontiers in Veterinary Science* (January 2020).

Acknowledgments: Milk and clinical sample collection in Saudi Arabia was performed by farm veterinarians and workers. Gonçalo Lucena and Scott Waight provided epidemiological information from the farm.

Milk samples were shipped to The Pirbright Institute and tested by Bryony Armson, who also carried out data analysis and interpretation, and writing. Simon Gubbins and Nick Lyons provided technical assistance and experience when performing predictive modelling and analysing the data. Thanks go to Valérie Mioulet and colleagues in the WRLFMD for the supply of original lesion material and cell-culture isolates originating from the farm.

6.1 Summary

Pooled milk has been exploited for the surveillance of several diseases of livestock. Previous studies have demonstrated that foot-and-mouth disease virus (FMDV) RNA can be detected in the milk of infected animals at high dilutions, suggesting that the collection of pooled milk samples from large-scale dairy farms could be used to enhance FMD surveillance. The aim of this study was to evaluate pooled milk collected via proportional in-line samplers for FMDV surveillance on a regularly vaccinated, large-scale dairy farm in Saudi Arabia. During the six-month sampling period, the farm experienced two FMD outbreaks caused by strains within the A/ASIA/G-VII and O/ME-SA/Ind-2001d lineages. FMDV RNA was detected in 5.7% of the 732 pooled milk samples, and typing information was concordant with viral isolates obtained from animals with clinical disease. The FMDV positive milk samples were temporally clustered around reports of new clinical cases, but with a wider distribution. To investigate this further, a model was established to predict C_T values using individual cattle movement data, clinical disease records and virus excretion data from previous experimental studies. These predictions explained some of the instances where positive results by rRT-PCR were observed, but no new clinical cases and suggested that subclinical infection occurred during the study period. The results from this study indicate that testing pooled milk by rRT-PCR may be valuable for FMD surveillance and suggest probable subclinical virus circulation in vaccinated herds that may play a role in the epidemiology of FMD in vaccinated populations. Further studies are required to investigate the effect of vaccination on the detection of FMDV in milk and to evaluate more representative sampling methods.

6.2 Introduction

Milk has been exploited for the surveillance of several pathogens of livestock including bovine viral diarrhoea virus (Renshaw et al., 2000a; Hill et al., 2010), Schmallenburg virus (Daly et al., 2015), *Coxiella burnetti* (Kim et al., 2005), bovine respiratory syncytial virus (BRSV) (Elvander et al., 1995), and neospora (González-Warleta et al., 2011). The use of pooled milk samples has also been validated as a rapid, cost-effective approach for the routine surveillance of diseases such as brucellosis (DEFRA, 2015a) and mastitis caused by Mycoplasma spp (APHIS, 2008).

Previous experiments have shown that the mammary gland is an organ that is highly susceptible to foot-and-mouth disease virus (FMDV) replication, and FMDV can be detected in milk from experimentally infected animals before, during and after the appearance of clinical signs (Nardelli et al., 1968; Burrows et al., 1971; Blackwell and McKercher, 1982; Reid et al., 2006; Armson et al., 2018, see Chapter 2). Additionally, FMDV can be detected and typed by real-time reverse transcription polymerase chain reaction (rRT-PCR) assays in milk from naturally infected cattle in outbreak and endemic scenarios (Armson et al., 2018, 2019, see Chapters 2 and 3). Previous studies (Reid et al., 2006; Armson et al., 2018, see Chapter 2) have suggested that it could be possible to identify one acutely-infected milking cow in a typical-sized dairy herd (100–1000 individuals) using milk from bulk tanks or milk tankers, based on the detection of FMDV RNA in highly diluted milk samples from infected cattle. Simulation modelling using these data (Reid et al., 2006; Thurmond and Perez, 2006; Garner et al., 2016) support the requirement for further research to assess the use of pooled milk as a useful tool to enhance FMD surveillance.

Sampling of milk at the herd level could potentially offer a representative framework for FMD surveillance on large-scale dairy farms in endemic countries. Indeed, milk is routinely collected, and has several advantages over vesicular material or serum by being non-invasive and potentially less susceptible to selection bias in targeted (risk-based) surveillance. For example, the use of milk does not rely on disease reporting by farmers or veterinary professionals, and may detect sub-clinically circulating viruses (Armson et al., 2019, seee Chapter 3) which may be under-represented, particularly in vaccinated populations (Knight-Jones et al., 2016).

These results motivated further studies using pooled milk from different production systems in endemic settings. Saudi Arabia is an FMD-endemic country in which a range of production systems exist, including nomadic and small-scale herds containing small ruminants and cattle, and large-scale dairy production systems (Asghar et al., 2016). Large-scale dairy farms can house up to 20,000 cattle, and often keep detailed records of individual cattle health, movements, milk yields and vaccination status (Hutber et al., 1999; Lyons et al., 2017; Gomaa Hemida et al., 2018). Recently, Saudi Arabia has experienced outbreaks due to viral lineages that are not normally present in this region, including the A/ASIA/G-VII and O/ME-SA/Ind-2001 lineages (Knowles et al., 2015; Bachanek-Bankowska et al., 2018). These FMD outbreaks also affected large-scale dairy farms, despite regular vaccination and strict biosecurity practices (Lyons et al., 2017; Gomaa Hemida et al., 2018).

The aim of this study was therefore to validate the use of pooled milk for the surveillance of FMD in these large-scale production systems. The goal was to provide a representative model for cost-effective and efficient surveillance to rapidly detect infected herds during outbreaks in endemic countries. Similarities that exist in the production systems of FMD-free countries mean that this approach could support targeted/risk-based surveillance in response to an outbreak in a disease-free country. The specific objectives were to (i) validate the use of pooled milk collected from a large scale dairy farm in Saudi Arabia for the detection and characterisation of FMDV by real-time rRT-PCR; (ii) compare the results obtained by FMDV rRT-PCR with clinical incidence; (iii) model the predicted C_T values of pooled milk samples based on detailed epidemiological data available from the farm; (iv) estimate the sensitivity and specificity of this surveillance approach to assess the usefulness of pooled milk as a cost-effective, non-invasive surveillance tool.

6.3 Materials and Methods

6.3.1 Study site and population

The study site was a large-scale dairy farm located in central Saudi Arabia. The farm housed approximately 4,000 Holstein Friesian cattle and was organised into management houses (H) with lactating groups of up to 240 cows that were milked four times a day. The farm had a fenced outer perimeter and there were no other FMD susceptible livestock or wildlife present on the farm. The study population comprised all cattle on the farm that were in lactating groups during the study period (10/09/2015 to 25/02/2016). The farm had electronic recording systems for monitoring individual animal health and movements. Lactating cattle were vaccinated approximately every three months with a high potency (≥6.0 PD_{50}), killed, aqueous adjuvanted (aluminium hydroxide and saponin), non-structural protein (NSP) purified FMD vaccine (containing O Manisa, O-3039, O-PanAsia2, A Iran-05, A Saudi-95, Asia-1 Shamir and SAT-2 virus strains) (Aftovaxpur, Merial Animal Health) (Lyons et al., 2017).

In September 2015, the farm had clinical cases of FMD due to the then emerging A/ASIA/G-VII viral lineage (Bachanek-Bankowska et al., 2018). In February 2016, three months after the last clinical case (on 12/11/2015), new clinical cases were observed and confirmed as serotype O (ME-SA/Ind-2001d lineage), with the last recorded clinical case on 07/03/2016. All recording of clinical cases was done by farm staff supervised by veterinary surgeons employed by the farms. The resulting data were entered into an electronic farm recording system. FMD cases were defined by observation of increased salivation and any of the following additional clinical signs: mouth lesions, feet lesions, teat lesions, fever, reduced feed intake and lameness. The farm policy was to isolate new cases of FMD in a dedicated isolation facility. If the isolation facility was full or the number of observed cases in the group exceeded approximately 5%, cases remained within groups. Animals were moved from isolation back to the main herd either after complete recovery or when sufficiently recovered, depending on available space in the isolation facility.

6.3.2 Pooled milk sampling

As part of routine herd health surveillance, milk samples were collected twice weekly using a proportional in-line milk sampler (Figure 6.1), designed to pull a representative sample from each house, and delivered to the farm laboratory. Throughout the study period (10/09/2015 to 25/02/2016), milk samples (n=732) were collected twice weekly (between 10/09/2015 - 03/12/2015), weekly (between 10/12/2015 – 25/02/2016), or on an ad-hoc basis. Milk samples were collected from each management house containing lactating cows (n=17) and on an ad-hoc basis from two houses containing cows separated due to various diseases (a "sick-cow pen"). All milk samples were labelled with the date and house identification number and were stored in a freezer at -20°C until they were shipped to The Pirbright Institute (TPI, UK) for FMDV detection.

Figure 6.1 Milk sample being collected by the proportional in-line sampler. Photo courtesy of Nick Lyons.

6.3.3 Laboratory testing of pooled milk samples

6.3.3.1 Viral isolates

FMDV cell culture isolates (isolated once in primary bovine thyroid [BTY] cells) were obtained from archival stocks held in the OIE/FAO World Reference Laboratory for foot-and-mouth disease (WRLFMD) repository. O/SAU/1/2016 was diluted 10^{-2} in unpasteurised whole milk and used as a positive control for the pan-serotypic rRT-PCR assay and the serotype specific O (ME-SA/Ind-2001d lineage) rRT-PCR assay. For the

serotype specific A (ASIA/G-VII lineage) rRT-PCR assay, A/SAU/6/2015 was diluted 10^{-4} in unpasteurised whole milk and used as a positive control.

6.3.3.2 FMDV detection assays

RNA extraction and the pan-serotypic rRT-PCR were carried out as previously described using an optimised method (Armson et al., 2018, see Chapter 2). Briefly, RNA extractions were carried out using the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems®) using a sample input of 200 µL on a MagMAX™ Express 96 Extraction Robot (Applied Biosystems®) according to manufacturer's instructions. VetMAX™ Xeno™ Internal Positive Control RNA (Applied Biosystems®) was added prior to extraction. Negative extraction controls consisted of unpasteurised whole milk added to lysis buffer.

The pan-serotypic rRT-PCR assay was performed using the reagents, parameters and thermal cycling conditions previously reported (Shaw et al., 2007) with primers and probes described by Callahan et al. (Callahan et al., 2002). One µL per reaction of VetMAX™ Xeno™ Internal Positive Control LIZ™ Assay (Applied Biosystems®) was also included in the reaction mix. All rRT-PCR assays were performed in duplicate using an Applied Biosystems® 7500 Fast Real-time PCR System. Any milk sample with a C_T value of ≤ 50 was considered positive, and was also tested in duplicate on both lineage-specific rRT-PCR assays for A/ASIA/G-VII (Saduakassova et al., 2017) and O/ME-SA/Ind-2001d (Knowles et al., 2015), using the reagents, parameters and thermal cycling conditions previously reported. Additionally, samples with amplification below the 0.2 fluorescence threshold (which therefore were not considered positive) by the pan-serotypic rRT-PCR assay (termed 'inconclusive' for this study) were also tested using the lineage specific rRT-PCR assays. The reason for this was that lower C_T values have previously been obtained by A/ASIA/G-VII rRT-PCR assay when compared with values on the same samples using the pan-serotypic rRT-PCR assay (Saduakassova et al., 2017).

6.3.4 Development of a model to predict FMD virus concentrations (C_T values) in pooled milk samples

The C_T values of pooled milk samples were predicted using information supplied by the farm and from the literature. These 'predicted' C_T values were then compared with the 'observed' C_T values obtained by the pan-serotypic rRT-PCR assays. The values used for each parameter are described below.

A) Equating C_T value with the number of virus 'units'

The limit of detection of FMDV RNA in milk using the pan-serotypic rRT-PCR assay was based on a previous cattle challenge study (Armson et al., 2018, see Chapter 2), as this was the only study in the literature based on the same rRT-PCR methodology. In the previous study, ten-fold serial dilutions of a whole milk sample from an infected animal gave a limit of detection of 10^{-6} (Armson et al., 2018, see Chapter 2). For this study, a virus unit value of 1 was assigned to this last dilution at which FMDV RNA could be detected (i.e. 10^{-6}) and subsequent virus unit values were assigned to each ten-fold dilution on a log scale (Figure 6.2). Linear regression was applied so that a C_T value could be predicted from the fit when the total virus unit value (V) in the pooled milk was known.

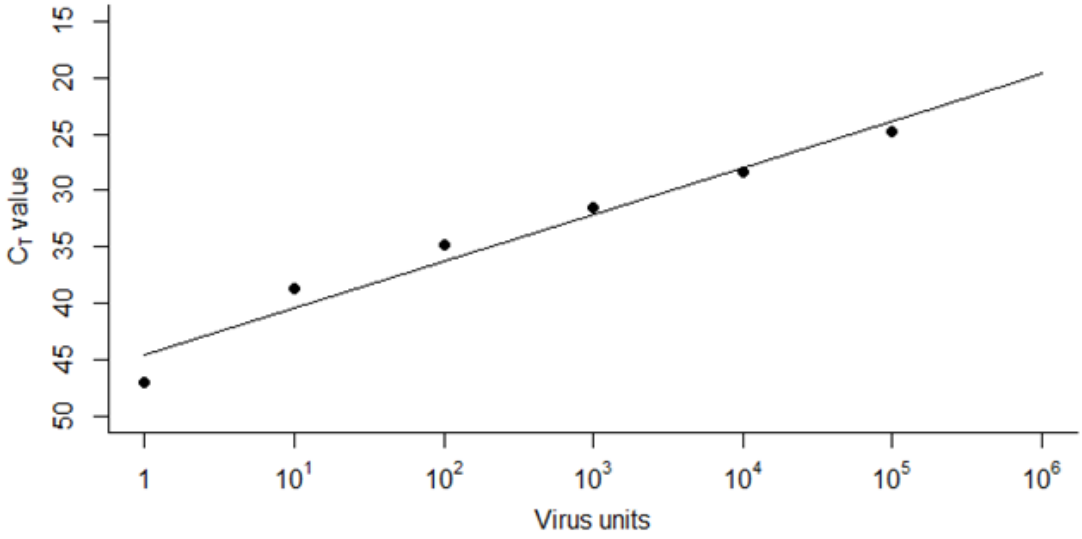


Figure 6.2 Linear regression used to predict C_T values from total virus unit values. Data is taken from limit of detection studies performed in Chapter 2 (Armson et al., 2018).

B) Estimating the number of virus units excreted per cow at each stage of infection (U_i)

Using data from the cattle challenge study performed in the same study (Armson et al., 2018), FMDV RNA could be detected in milk by the pan-serotypic rRT-PCR assay between 3 to 28 days post infection (DPI), while clinical signs were first observed at 4 DPI. As the day of infection for each cow on the large-scale farm in Saudi Arabia was unknown, the model assumed that the day clinical signs were first recorded was day [D] 0. Consequently, an excretion profile was created using the mean C_T values based on data collected from two in-contact animals from the challenge study (Armson et al., 2018, see Chapter 2) between D-1 to D24, subsequently referred to as the 'stage of infection' (*i*) (Figure 6.3). Missing values were interpolated, by retrieving values from the fitted line between the two nearest values. From these C_T values, the virus unit value (U) was predicted for each stage of infection (*i*) using the linear regression model fitted in Figure 6.2.

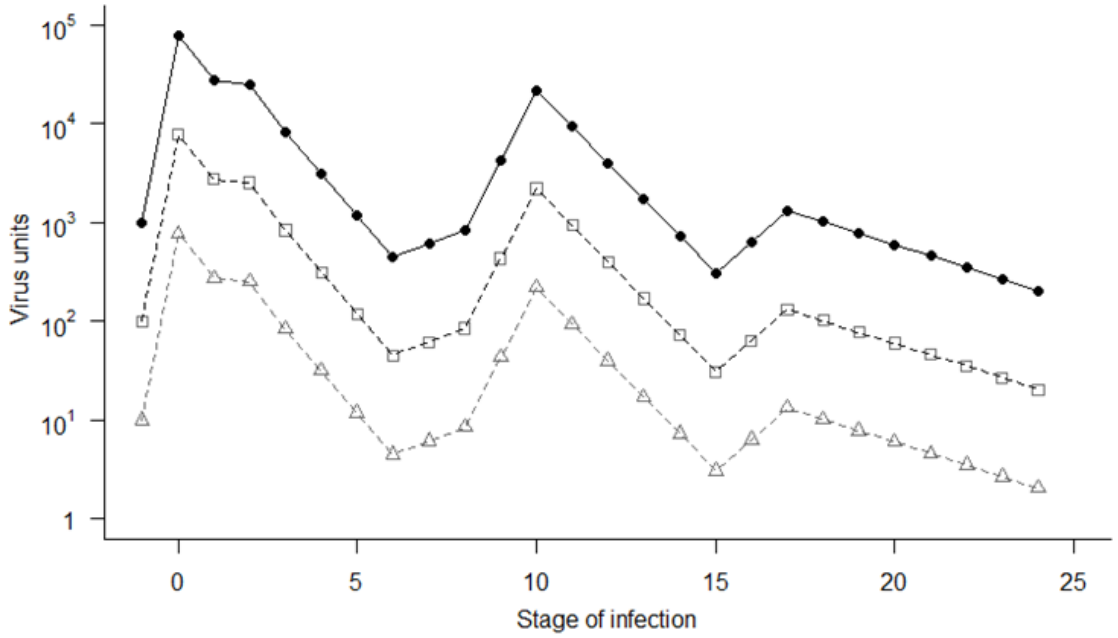


Figure 6.3 Virus unit values (U) assigned to each stage of infection (i) between days -1 and day 24, based on mean C_T values of two animals in the challenge study performed in Chapter 2 (Armson et al., 2018). ● = virus units for '1' (no vaccination), □ = '1/10' virus units, and △ = '1/100' virus units.

Previous studies have described a reduced level of virus excretion in nasal fluid, saliva, and oesophageal–pharyngeal fluid sample types in vaccinated versus non-vaccinated animals (Orsel et al., 2007; Parthiban et al., 2015; Thwiny, 2016). As the effect of vaccination on the duration of excretion or quantity of FMD virus in milk is unknown, additional factors were included to account for this possibility, as milk samples in this study were collected from regularly vaccinated cattle. Data from previous studies were therefore used to inform the model (Orsel et al., 2007; Parthiban et al., 2015; Stenfeldt et al., 2016; Thwiny, 2016), where significantly lower levels of viral excretion (by over 10^2 copies/ml) were observed in vaccinated animals compared with unvaccinated animals. Consequently, in the model prediction for this study three 'levels' of viral excretion were adopted: '1' as described above (no vaccination), and then tenfold reductions of '1/10' and '1/100' (Figure 6.3). In the model prediction, each ('1', '1/10' and '1/100') virus unit value for each stage of infection (i) was used separately to determine the effect this change had on the resulting C_T value in the pooled milk

sample. Additionally, the reduction was assumed to remain constant throughout the course of infection (D-1 to D24).

C) Determining the number of cattle at each stage of infection (N_i) per sampling date (t)

Using records of the onset of clinical signs for each cow and individual movement data available from the farm, the number of cows at each stage of infection (N_i) per sampling date (t) per house was calculated.

D) Determining the reduction in milk yield for infected cattle

The only milk yield data available from the farm was the average milk yield per house for each sampling date. To enable simplification of the model, it was assumed that in each house all lactating cows produced equal volumes of milk (M_u). This was considered a reasonable assumption as cattle were placed into houses on the basis of stage of lactation.

Due to limited studies quantifying the reduction in milk yield during FMDV infection in highly vaccinated cattle, original milk yield data from a large-scale Holstein-Friesian dairy farm in Kenya that reported a FMD outbreak in August 2012 (Lyons et al., 2015a; Lyons et al., 2015b) were used to inform this study. The mean milk yield from 189 cattle was calculated for each 5-day period during infection (D0 to D4, D5 to D9, D10 to D14, D15 to D19, D20 to D24) as a percentage of the mean yield before infection ('normal yield': D-10 to D-1). ANOVA and Welch two sample T-tests demonstrated a significant difference between D5 to D9 and normal yield (p = 0.001). Therefore, a value of 87% of the normal yield (M_i) was employed for each cow at stage D5-D9 of infection when determining the final number of virus units in a pooled milk sample.

E) Determining the final number of virus units in a pooled milk sample per sampling date (F(t))

Using the input parameters calculated in (*A)* to (*D)*, the final number of virus units in a pooled milk sample per sampling date *(F(t))* for each house can be calculated using the following equation:

$$F(t) = \frac{\sum_{i=-1}^{24} M_i U_i N_i(t)}{\sum_{i=-1}^{24} M_i N_i(t) + M_U(H - \sum_{i=-1}^{24} N_i(t))}$$

Where:

- N_i is the number of cows at infection stage *i*
- U_i is the number of virus units excreted per cow at infection stage *i*
- M_i is the amount of milk produced by a cow in infection stage *i*
- M_U is the amount of milk produced by a healthy cow
- *H* is the total number of cows contributing to the milk pool

F) Predicting C_T values for each sampling date (t)

Using the value of *F(t)* for each house the C_T value was predicted from the linear regression model fitted in (*A)* (Figure 6.2).

6.3.5 Statistical analysis

All data analyses were performed using R 3.5.3 (R Core Team, 2019) within RStudio IDE (RStudio Team, 2019). In order to compare the 'observed' C_T values obtained from pooled milk samples with 'predicted' C_T values, values were plotted for visual comparison. For each sampling date (*t*), 'predicted' and 'observed' C_T values were assigned a 0 or 1 for a negative (C_T of >50) or positive (C_T of ≤50) result, respectively. Additional diagnostic cut-off C_T values of 45 and 40 were also investigated. Contingency tables were constructed for each house, and for all houses combined using each virus unit value level (i.e. '1', '1/10' and '1/100'), for which sensitivity, specificity, and the Cohen's Kappa statistic (κ) (Landis and Koch, 1977) were calculated.

6.4 Results

6.4.1 Epidemiology of the FMD outbreak

Throughout the study period, the mean number of lactating cows in each house was 227 (median 237, range 44-240). Details of the farm and clinical incidence for the two FMD outbreaks are shown in Table 6.1. Based on the total number of cattle present on the farm, the overall incidence risk was 2.8% and 0.87% for the two separate outbreaks beginning on 02/09/2015 and 15/02/2016, respectively. The epidemic curves with corresponding sampling periods are shown in Figure 6.4c. Based on movement records, cows affected with suspected FMD were moved into a quarantine house at the start of the outbreak where they continued to be milked (if possible) until they had recovered sufficiently to move back to the same or an alternative lactating house. When quarantine houses reached maximum occupancy or the house level incidence exceeded 5%, this practice was discontinued.

Table 6.1. Summary of outbreak data on the large-scale dairy farm in Saudi Arabia.

Variable		
Total number of lactating cattle during study period (approx.)	4,000	
Number of lactating houses	17	
Number of lactating animals per house[a] (mean, median, range)	227 (237, 44-240)	
Number of lactating houses affected (%)	10 (58.8)[b]	4 (23.5)[c]
Number of clinical cases of FMD[d]	107[b]	33[c]
Overall incidence risk (number of cases/total livestock on farm) (%)	2.8[b]	0.87[c]
Date of index case	02/09/2015[b]	15/02/2016[c]

[a] Calculated on milk sampling days throughout the study period. [b] A/ASIA/GVII outbreak. [c] O/ME-SA/Ind-2001 outbreak. Outbreaks were determined according to reports by WRLFMD. [d] Case definition used by the farm for FMD was any animal seen salivating with any of the following additional clinical signs: mouth lesions, feet lesions, teat lesions, fever, reduced feed intake and lameness.

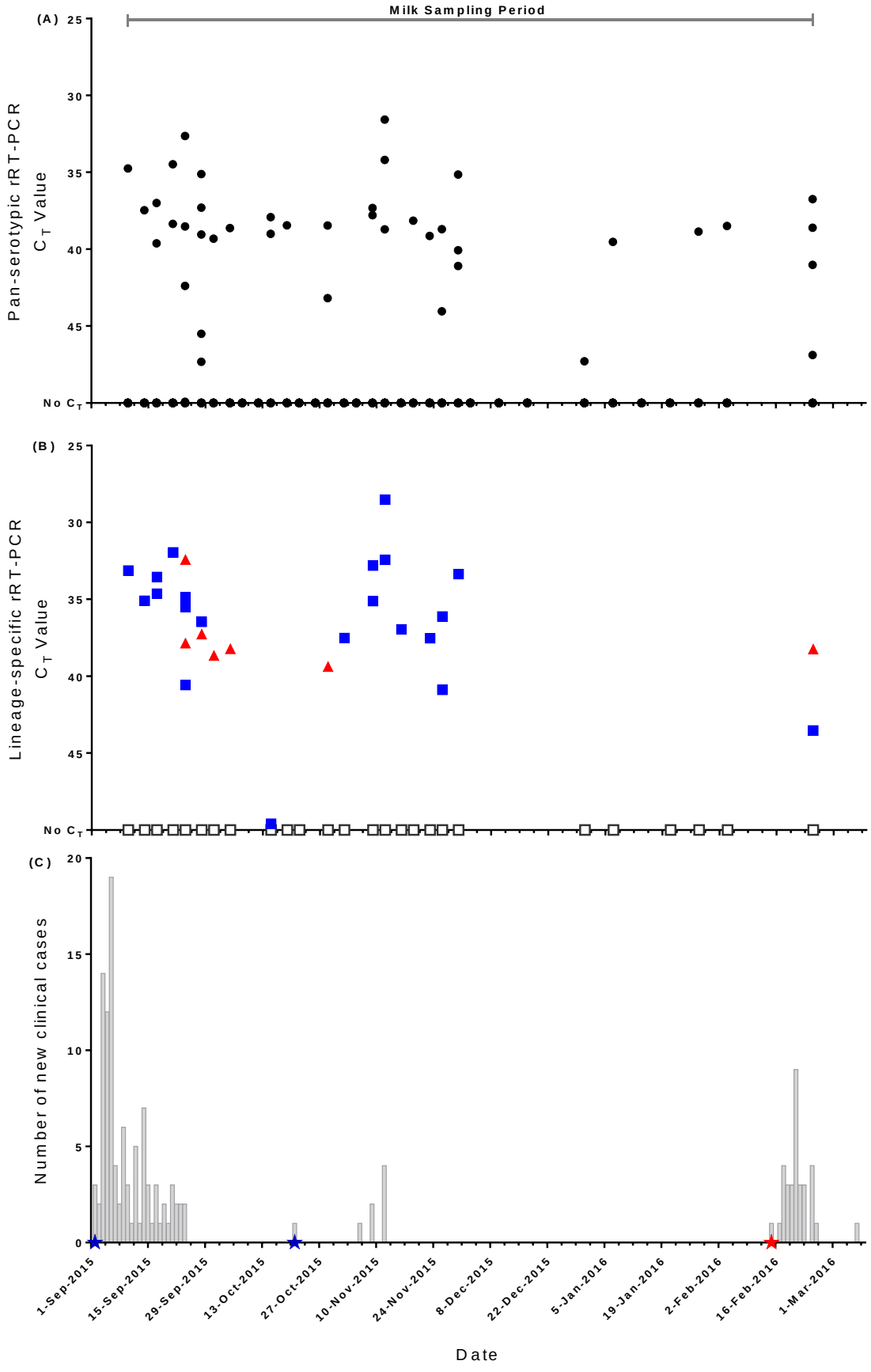

(A)
Milk Sampling Period
Pan-serotypic rRT-PCR
C_T Value
25
30
35
40
45
No C_T
(B)
Lineage-specific rRT-PCR
C_T Value
25
30
35
40
45
No C_T
(C)
Number of new clinical cases
20
15
10
5
0
1-Sep-2015
15-Sep-2015
29-Sep-2015
13-Oct-2015
27-Oct-2015
10-Nov-2015
24-Nov-2015
8-Dec-2015
22-Dec-2015
5-Jan-2016
19-Jan-2016
2-Feb-2016
16-Feb-2016
1-Mar-2016
Date

Figure 6.4 (A) C_T values from the pan-serotypic rRT-PCR assay (●) for pooled milk samples collected from 19 lactating houses in the large-scale dairy farm in Saudi Arabia throughout the study period (n=732). (B) C_T values for each lineage-specific rRT-PCR assay for samples that tested positive ($C_T \leq 50$), or where very low amplification (below the threshold) was observed, in the pan-serotypic rRT-PCR assay. ■: A/ASIA/G-VII. ▲: O/ME-SA/Ind-2001d. □: Sample could not be typed. (C) Epidemic curves of FMD outbreaks on the farm. Stars represent dates where clinical samples (vesicular epithelium/fluid) were collected and submitted to the World Reference Laboratory for Foot-and-mouth Disease (WRLFMD), and reported as ★: A/ASIA/G-VII, ★: O/ME-SA/Ind-2001d.

6.4.2 Pooled milk

During the study period 732 milk samples were collected of which 42 (5.7%) were positive using the pan-serotypic rRT-PCR (Table 6.2, Figure 6.4A). Of the 42 positive samples and those considered 'inconclusive' due to amplification below the 0.2 fluorescence threshold (n=22), 32.8% were positive by the A/ASIA/G-VII rRT-PCR assay, and 9.4% were positive by the O/ME-SA/Ind-2001d rRT-PCR assay (Figure 6.4B). Additionally, 3.1% of the samples tested on the lineage-specific assays were positive for both lineages. Of the samples that were positive on the pan-serotypic rRT-PCR assay, 19/42 (45.2%) could not be typed. Of the samples that were inconclusive on the pan-serotypic assay, 3/22 (13.6%) were positive for A/ASIA/G-VII and 1/22 (4.5%) was positive for O/ME-SA/Ind-2001d.

Table 6.2. Summary of milk sample results for all rRT-PCR assays for the large-scale dairy farm in Saudi Arabia.

Variable	Value
Duration of milk sampling (weeks)	25
Number of houses milk samples were collected from	19
Number of pooled milk samples tested	732
Number positive[a] by pan-serotypic rRT-PCR assay (%)	42 (5.7%)
Number positive[a] by A/ASIA/G-VII rRT-PCR assay (%)	21/64[b] (32.8%)
Number positive[a] by O/ME-SA/Ind-2001d rRT-PCR assay (%)	6/64[b] (9.4%)

[a] Positive results are those with at least one well giving a C_T of ≤50. [b] 22 samples were considered 'inconclusive' (amplification was observed below the fluorescence threshold of 0.2) and were therefore also tested by the lineage-specific rRT-PCR assays.

6.4.3 Correlation between epidemiological data and FMDV RNA in pooled milk

Laboratory results from the pooled milk samples were directly compared against clinical data collected during the FMD outbreaks. The first period of clinical disease was seen in lactating cows between 02/09/2015 and 24/09/2015 (n=99), with two recurrences of clinical disease in a smaller number of cows in mid-October (n=1) and the first half of November 2015 (n=7) (Figure 6.4C). Clinical samples (vesicular epithelium/fluid) were collected from clinically affected animals (n=3) in September and October 2015, and were characterised as belonging to the A/ASIA/G-VII lineage. Further clinical disease was recorded at the beginning of February 2016 (n=33) and a clinical sample identified the strain as from the O/ME-SA/Ind-2001d lineage. Visual comparison of the epidemic curve and temporal representations of rRT-PCR results indicates some clustering of positive pooled milk samples around the occurrence of new clinical cases but with a wider distribution (Figure 6.4). Clustering of lineage A/ASIA/G-VII positive results can also be seen from the commencement of sampling to the end of November, concurrent with reports of this lineage from clinical samples. The clinical incidence in lactating cows over the whole study period was 3.6% (Table 6.1), while FMDV genome was detected in 5.7% of pooled milk samples (Table 6.2). A contingency table was constructed to determine the sensitivity (Se) and specificity (Sp) of the pan-serotypic rRT-PCR, using the number of new clinical cases observed on milk sample collection days for all houses sampled as the gold standard: Se = 42.9% (95% confidence interval (CI): 21.4% - 67.4%), Sp = 95.0% (95% CI: 93.1% - 96.4%) (Appendix IV, Figure 8.6).

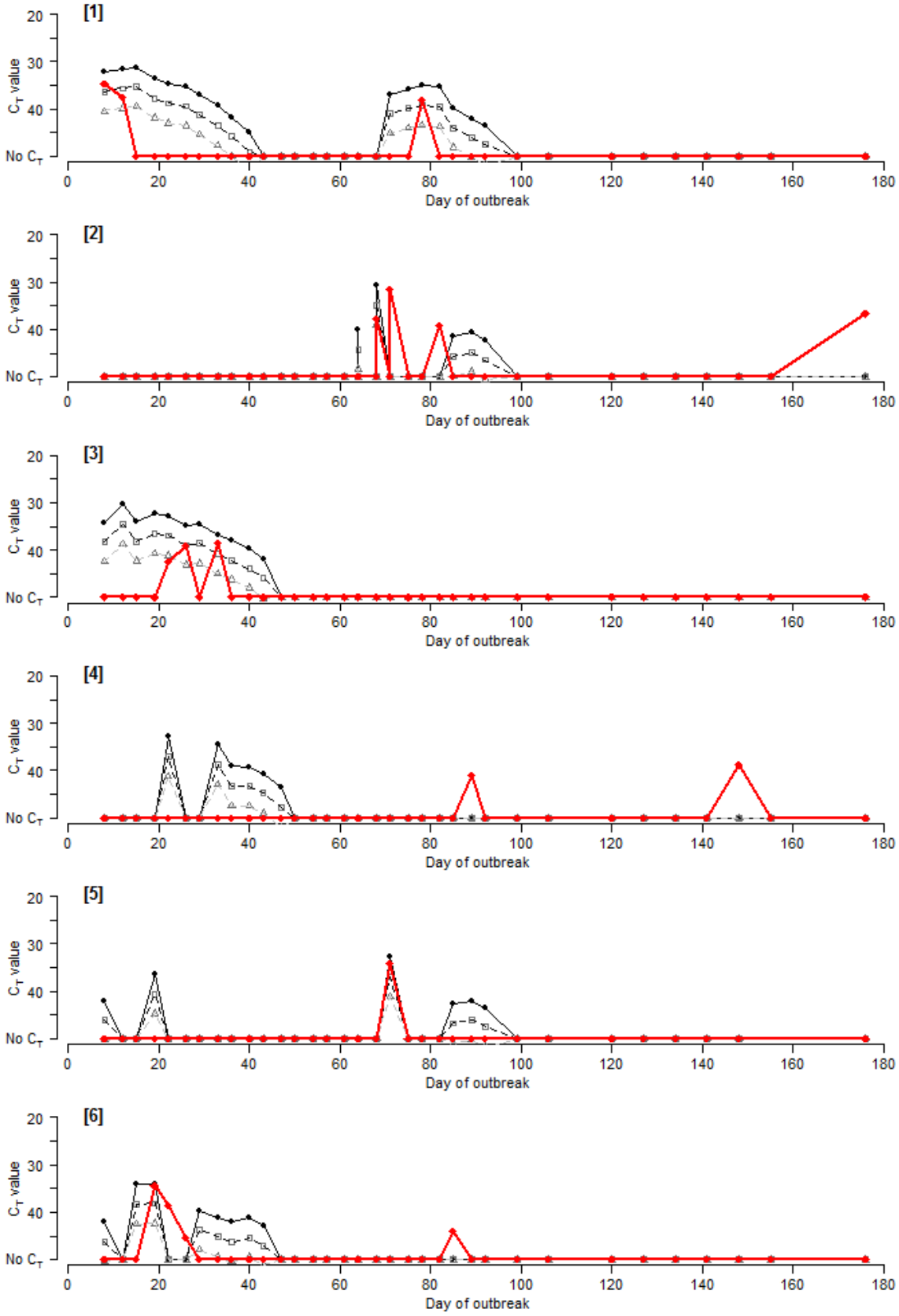
FMDV genome was detected in pooled milk in 17 out of the 19 (89.5%) sampled houses compared to 14/19 (73.7%) houses that reported clinical cases. Of the latter, 13 were PCR positive at some point during the outbreaks (Figure 6.5). Furthermore, four houses were positive by rRT-PCR with no recorded clinical cases at any time during the outbreaks. There were also eight samples taken where the rRT-PCR result was negative but there were new clinical cases observed on that day.

6.4.4 Predicting C_T values in pooled milk

Predicted C_T values were obtained for each house and compared with the observed C_T values from the pan-serotypic rRT-PCR (Figure 6.5). The potential effect of reduced virus excretion that may occur due to vaccination was also investigated, where C_T values were predicted for the different levels of virus excretion to accommodate the possible impact of FMDV vaccination ('1', '1/10' and '1/100') (Figure 6.5). Predicted C_T values were not calculated for some houses due to a lack of available epidemiological data required for the analysis, or because the house was used as a quarantine pen to isolate new cases of FMD at the start of the outbreak, and therefore regular milk samples were not collected (Houses 17 and 18). Additionally, House 12 is not included in figure 6.5 as both the observed and predicted results were all negative.

Visual comparison of observed versus predicted C_T values revealed instances where (i) positive results were obtained for both observed and predicted values, and C_T values were generally comparable, (ii) positive results were obtained for predicted values only, and (iii) positive results were obtained for the observed results only, although this was less frequent than when comparing observed C_T values with new clinical cases (Figure 6.5).

The lowest predicted C_T values (i.e. the highest viral RNA concentration) obtained for '1', '1/10', and '1/100' were 30.4, 34.5 and 38.7, respectively, compared with 31.6 for the observed results. A reduction in viral excretion increased the predicted C_T values and in some instances decreased the duration for which milk samples from a house would remain positive ($C_T \leq 50$). Additionally, applying a diagnostic cut-off value of 45 or 40 decreased the likelihood and duration of predicted positive C_T values. Contingency tables for all houses combined indicated that a virus excretion level of '1/10' with a diagnostic cut-off C_T value of 40 generated results closest to those of the observed rRT-PCR results (Se = 34.6% [95% CI: 19.4% - 53.8%], Sp = 97.2% [95% CI: 95.7% - 98.2%], A_{obs} = 0.95, K = 0.31) (Appendix IV, Figure 8.7). A reduction in sensitivity and increase in specificity was observed when these values were compared with estimates of sensitivity and specificity using records of new clinical cases as the 'gold standard'.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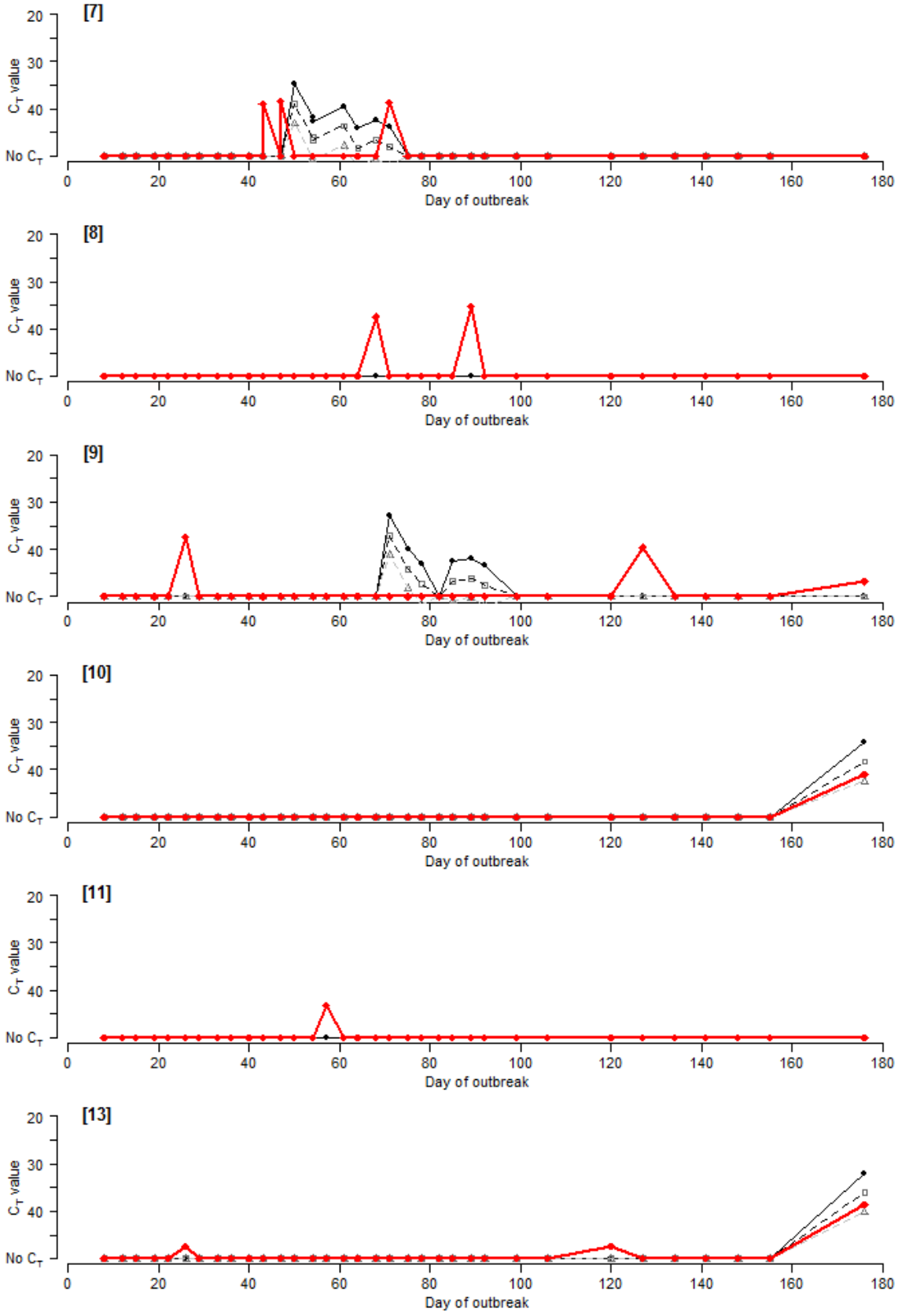
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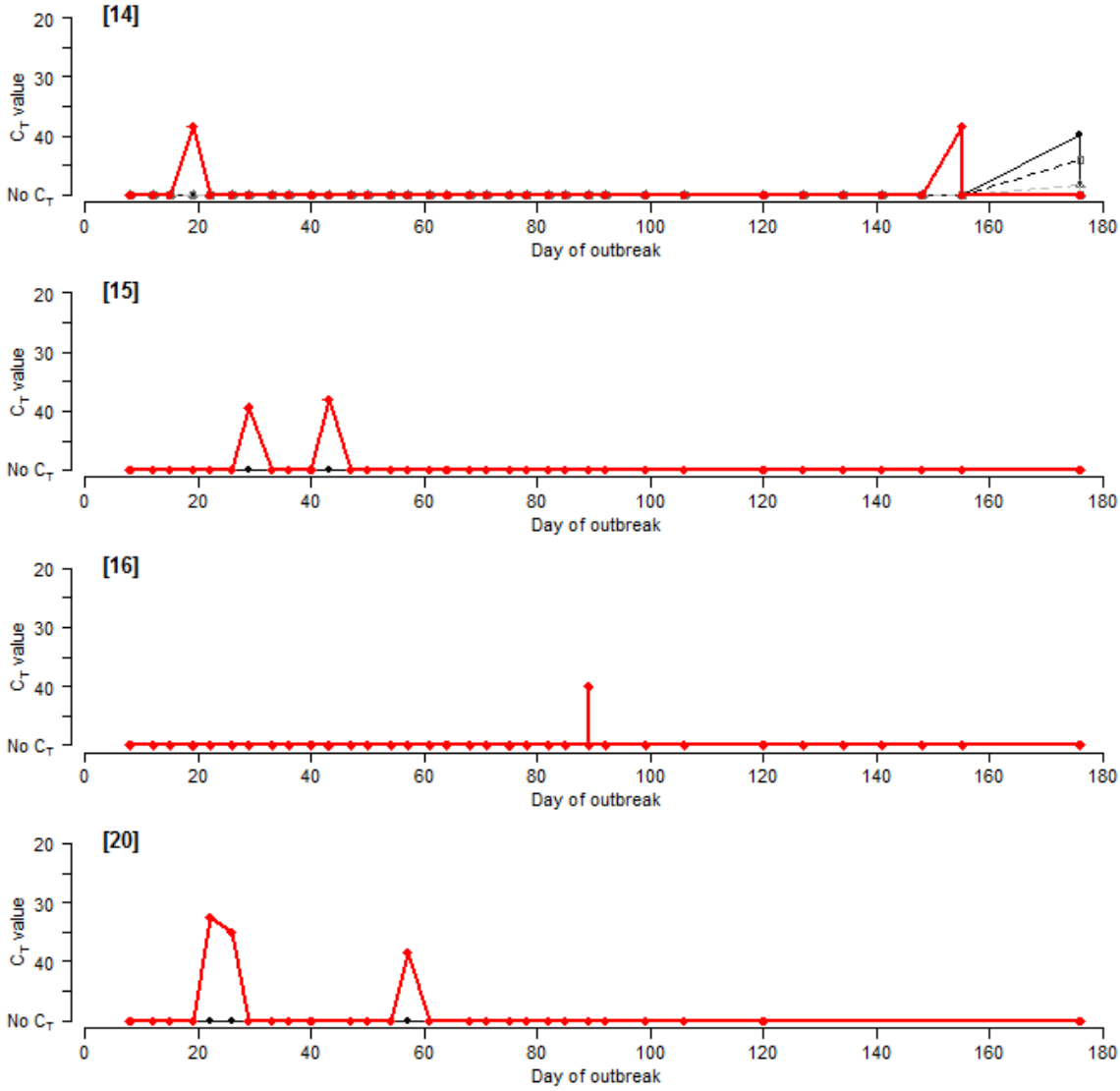

Figure 6.5 'Observed' C_T values for the rRT-PCR of pooled milk samples (◆) vs 'Predicted' C_T values at '1' viral excretion (●), '1/10' (□) and 1'100 (△), for each management house [1-16, and 20]. Houses 12 and 17 are not shown because they were not included in this analysis due to the absence of clinical cases and rRT-PCR positive results in milk. House 18 was an isolation pen and not enough epidemiological data were available for analysis.

6.5 Discussion

This study aimed to expand on previous work to determine the utility of testing pooled milk by rRT-PCR as an alternative approach for FMD surveillance in vaccinated dairy herds. During the six-month study period, 732 pooled milk samples were collected from a large-scale dairy farm housing approximately 4,000 cattle during an FMD outbreak.

The first objective of this study was to determine whether detection and characterisation of FMDV by rRT-PCR was possible from pooled milk samples, and compare these results with epidemiological data recorded during the outbreaks. This is the first study we are aware of showing that FMDV genome can be detected in pooled milk samples from regularly vaccinated cattle collected using a proportional in-line milk sampler on a large-scale dairy farm. The mean C_T values obtained in the pan-serotypic rRT-PCR assay were high (>31), most likely due to the dilution of milk from a relatively small number of infected animals in groups of lactating cattle numbering up to 240 and collectively producing in excess of 10,000 litres per day. These results confirm the hypotheses from previous laboratory and modelling studies that suggested FMDV genome could be detected at these dilutions during outbreaks in field settings (Reid et al., 2006; Thurmond and Perez, 2006; Armson et al., 2018, see Chapter 2).

Lineage-specific rRT-PCR assays (Knowles et al., 2015; Saduakassova et al., 2017) confirmed the presence of the A/ASIA/G-VII and O/ME-SA/Ind-2001d lineages in the pooled milk samples, and this was supported by reports from samples collected from clinical cases that were sent separately for laboratory testing. Reports for these samples demonstrated that the two outbreaks were caused by different FMD viral lineages, the first due to the A/ASIA/G-VII lineage, and the second the O/ME-SA/Ind-2001d lineage, both are which are thought to have emerged recently from the Indian sub-continent (Knowles et al., 2015; Bachanek-Bankowska et al., 2018). The rRT-PCR results from the pooled milk samples suggest that there was a period of co-circulation or even co-infection with FMD viruses from these lineages. Co-infection in clinical samples from individual cattle in Saudi Arabia has been reported previously (Woodbury et al., 1994), although this possibility cannot be confirmed in the present study given that samples were taken and tested from only three clinical cases. Indeed

the collection of a variety of sample types from numerous individual animals during this study (e.g. vesicular lesion material, blood, nasal/oral swabs and milk) may have allowed for the detection of co-infection, and may have also enabled a more thorough validation of the pooled milk surveillance approach.

Although the farm routinely vaccinates with a high-potency, polyvalent FMD vaccine, both potency tests (Fishbourne et al., 2017; Waters et al., 2018) and field studies (Lyons et al., 2017) suggest that the vaccine does not confer complete protection against the A/ASIA/G-VII lineage or the O/ME-SA/Ind-2001d lineage. This may explain why clinical cases still occurred during the study period, albeit with a low overall incidence risk. This is especially the case for, the A/ASIA/G-VII lineage, which was detected in more pooled milk samples compared to O/ME-SA/Ind-2001d, consistent with expected vaccine performance from respective *in vitro* vaccine-matching data and experimental studies (Fishbourne et al., 2017; Waters et al., 2018, A. Ludi, personal communication). The detection of a greater number of milk samples positive for the A/ASIA/G-VII lineage could also be due to the relative performance of the typing rRT-PCR assays. In previous validation studies, lower C_T values for the A/ASIA/G-VII lineage typing assay have been demonstrated compared to the pan-serotypic rRT-PCR assay (indicating an increased sensitivity) (Saduakassova et al., 2017). In contrast, the O/ME-SA/Ind-2001d typing assay has been shown to generate C_T values comparable to the pan-serotypic rRT-PCR (Knowles et al., 2015).

In order to validate the use of pooled milk for the surveillance of FMDV on this large-scale farm, pan-serotypic rRT-PCR results from the pooled milk samples were compared to the clinical incidence of FMD during the study period. At the farm level there were four temporal clusters of clinical cases with gaps of at least 15 days between them. Visual appraisal of the data indicated that FMDV rRT-PCR results were generally correlated to these clusters, although they showed a wider distribution around and in between the clusters of clinical cases. Comparison of the onset of individual clinical cases with assay results on milk sampling days at the house level revealed only 6 occasions when milk samples were positive and a new clinical case was recorded on the same day. There were also occasions when either (i) positive milk samples were obtained when there were no new clinical cases on that day, or (ii) there were new clinical cases occurring but a positive result was not observed in the milk. This resulted

in a low sensitivity and moderate specificity for the pooled milk rRT-PCR assay (42.9% and 95.0% respectively). However, this approach is limited by only comparing the assay results with the onset of new clinical cases on the sampling day which does not account for FMDV genome shedding in pre-clinical, convalescent, or subclinically affected animals.

In order to attempt to account for these limitations, 'observed' C_T values obtained by the pan-serotypic rRT-PCR assays were compared with 'predicted' C_T values for each house based on detailed epidemiological and cattle movement data from the farm, and data from recent literature. Although these results were similar, compared with the onset of clinical cases there was a reduction in sensitivity and an increase in specificity. It is likely that this may be due to the reduced number of sampling points available for the predictive analysis as a result of a lack of epidemiological data available from two of the houses. It is possible that this reduced sensitivity (i.e. instances where there were positive 'predicted' but negative 'observed' rRT-PCR results of the pooled milk) was due to a lower quantity and shorter duration of viral excretion in the milk of these vaccinated infected cattle than was assumed in the model. This theory supports findings by Leeuw et al. (1978) who were unable to detect infectious FMD virus in the milk of vaccinated cattle after challenge. However, this previous study used a homologous vaccine to the challenge strain and focussed on the detection of live virus instead of FMDV RNA genome. As there are no other studies known to have considered viral excretion into the milk of vaccinated cattle, data used to inform the model was based on more recent studies that measured viral excretion from vaccinated and non-vaccinated animals in alternative samples such as nasal fluid, saliva, and oesophageal–pharyngeal fluid (Orsel et al., 2007; Parthiban et al., 2015; Thwiny, 2016). The authors acknowledge the limitation of this approach, particularly since the quantity and duration of viral excretion seemed to have a substantial impact on the likelihood of predicting a positive result in the milk. Consequently, further investigation into the effect of vaccination on viral excretion in milk is required and would enhance the predictive ability of the model.

Management practices on the farm may also have contributed to the low sensitivity of the pooled milk rRT-PCR assay. These include the inconsistent removal of clinical cases and milking practices during the study period in response to the outbreak, with the

potential for increased sensitisation of farmers to disease as the outbreak progressed, resulting in a decreased chance of milk from an infected cow contributing to the milk pool. Additionally, the proportional in-line sampling method may not be truly representative of all cattle in the group, as reported previously (Clarke et al., 1997). Although the in-line sampler is designed to represent the whole milking, it has been demonstrated that this method may terminate sampling early (Clarke et al., 1997) and milk from infected cattle may be excluded from the sample tested leading to false negative results. This may explain the low sensitivity obtained for this FMDV detection system compared with what was predicted in the model. Other methods, for example collecting a sample from the bulk tank after thorough agitation, may be more representative (Barnard, 1977) and could be considered for future studies.

During the study period there were also instances when positive rRT-PCR results observed in milk samples did not correspond to new clinical cases observed, or indeed 'infected' (D-1 to D24) cows present in the house that would excrete virus into the milk pool. The possibility that these 'false positives' were due to laboratory contamination cannot be excluded. However, the laboratory methodology used in this study has been shown to be highly specific (see Chapter 4), and as there were a high number of 'negative' samples it is unlikely that these results are due to either laboratory contamination or non-specific amplification. An alternative explanation for this observation include spill-over of virus between houses as cattle were being milked (i.e. virus from an infected animal in one house may have been carried over to the milk from the subsequent house, generating false-positive results for an otherwise negative house) as there was no milk line disinfection between houses. There is also the possibility of delays in clinical case detection, sub-clinical infections or mild clinical cases that may not have been noticed by farm workers. Subclinical infections in vaccinated animals have been reported previously (Bertram et al., 2018; Farooq et al., 2018; Stenfeldt et al., 2018) and this is a possible explanation for the prolonged period between cases (up to 27 days), although it is unknown whether the outbreaks on this farm were due to prolonged circulation or new virus introductions.

This is the first study to evaluate the use of pooled milk as a surveillance sample for the detection of FMDV on large-scale dairy farms in endemic regions. This study demonstrates that rRT-PCR testing of pooled milk may be utilised for FMD surveillance

and reveal underlying sub-clinical FMD infection. More representative sampling methods should be investigated that may increase the sensitivity of this approach including an exploration of how the dairy value chain may be exploited for FMD surveillance. Subsequently, this methodology could be integrated into FMD surveillance programmes providing significant benefits over conventional surveillance strategies. The similarities in the farming system evaluated in this study and dairy farms in FMD-free countries highlights the potential of this surveillance approach for use in disease-free regions in the event of an incursion of FMDV, to allow rapid identification of infected herds, tracing the source and spread of infection and to screen infected premises to assess disease freedom.

CHAPTER 7

Discussion and future recommendations

7.1 Thesis summary

Effective disease surveillance enables stakeholders to confidently determine the health status of animals, and allows the necessary control methods to be implemented in order to reduce disease impact. Additionally, surveillance output data may help to assess the effectiveness of intervention strategies such as vaccination programmes (Drewe et al., 2011; Falzon et al., 2019). Currently, several limitations exist for foot-and-mouth disease (FMD) surveillance in endemic regions which have been described in more detail earlier in this thesis (see Chapter 1, section 1.5), and include the dependence on disease recognition and reporting by farmers, and the labour and costs involved in collecting and testing invasive clinical samples. Consequently, it is likely that much of the FMD circulation estimated to occur goes undetected (Sumption, Rweyemamu and Wint, 2008; Knight-Jones and Rushton, 2013).

Milk has been utilised for the detection of several pathogens and their specific antibodies, including *Coxiella burnetti* (Kim et al., 2005) and bovine viral diarrhoea virus (Drew et al., 1999; Renshaw et al., 2000b), among others. Additionally, pooled milk systems for herd-level surveillance are in place for diseases such as brucellosis (DEFRA, 2015a) (see Chapter 1, section 1.6). Previous studies have demonstrated FMDV detection from milk samples in experimental and field scenarios (Burrows, 1968; Reid et al., 2006; Ranjan et al., 2016; Ahmed et al., 2017). Additionally, the potential use of pooled milk samples for cost-effective FMD surveillance has also been highlighted (Reid et al., 2006; Thurmond and Perez, 2006).

The principal aim of this thesis was to expand on previous studies to determine the utility of milk for FMDV detection and surveillance. The objectives were to (i) optimise a high-throughput molecular FMDV detection system to be employed throughout the project; (ii) determine the utility of milk samples compared to conventional sample types for FMDV diagnosis from individual experimentally and naturally infected animals; (iii) evaluate the stability of FMDV RNA in milk samples during transportation; (iv) determine the effects of pooling; and (v) assess the use of pooled milk surveillance approaches in different farming systems. By addressing these research gaps, this thesis demonstrates that milk can be used as a non-invasive, simple sample type for FMD surveillance.

7.1.1 The advantages of using milk for FMD surveillance

7.1.1.1 FMDV detection in milk samples

Since it was first demonstrated that FMDV was excreted in the milk of FMD infected animals by Lebailly (1920), many studies have evaluated methodologies for the detection of both FMD virus and antibodies in milk samples. These techniques are summarised in Figure 7.1, and include antibody detection assays, the detection of live virus by isolation onto susceptible cell lines, and various molecular detection methods.

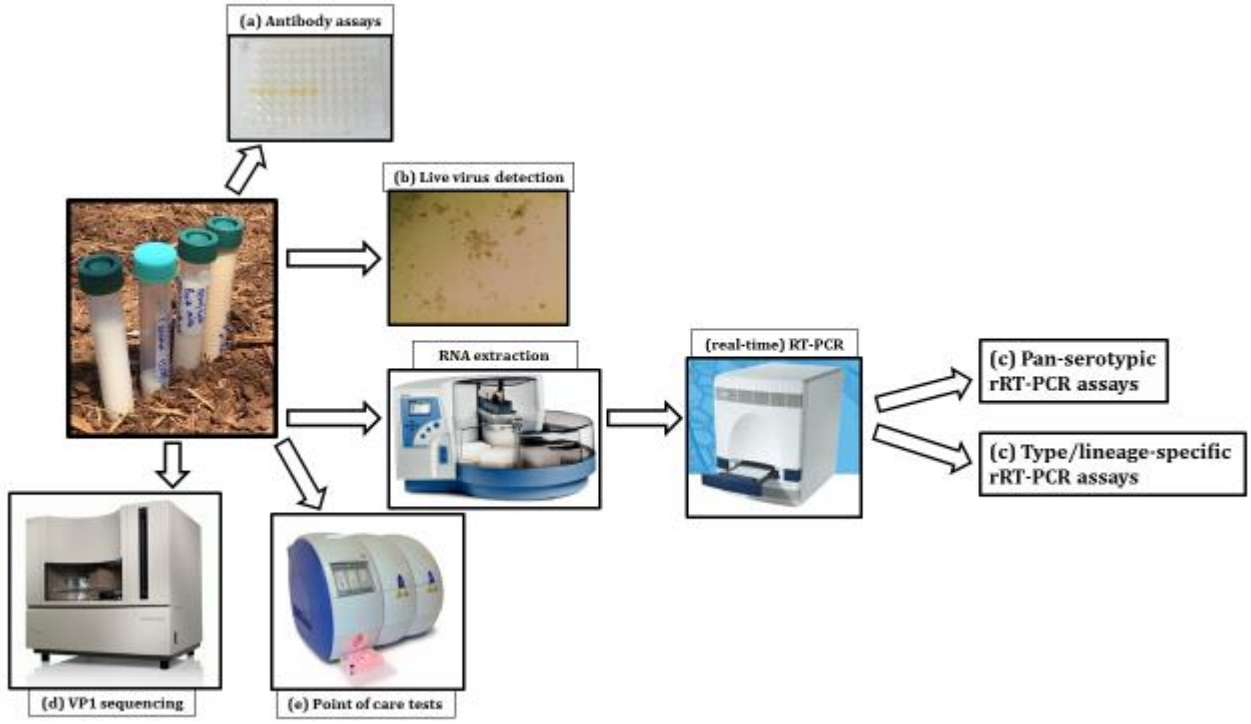

Figure 7.1 Techniques for the detection of FMDV/antibody in milk samples: (a) FMDV antibody assays (Stone and DeLay, 1960; Armstrong, 1997); (b) live virus on susceptible cell-lines (Burrows, 1968; Hedger and Dawson, 1970; Blackwell and McKercher, 1982; Reid et al., 2006; Armson et al., 2018)(Chapter 2); (c) the detection and typing of FMDV RNA genome by real-time rRT-PCR (Reid et al., 2006; Ranjan et al., 2016; Ahmed et al., 2017; Armson et al., 2018, 2019)(Chapters 2 and 3); (d) VP1 sequence data generation (Armson et al., 2019)(Chapter 3); and (e) the detection of FMDV RNA genome by point of care technology such as the Enigma® Mini Laboratory (Goller et al., 2018).

This thesis has expanded on these studies, focussing primarily on the application of molecular methods for the detection of FMDV RNA genome. A highly sensitive and specific real-time rRT-PCR using a high-throughput RNA extraction protocol was optimised and utilised for FMDV RNA genome identification throughout the project (Armson et al., 2018)(Chapter 2). Absolute quantification of the virus stocks used throughout this thesis was not performed and consequently the true analytical sensitivity could not be determined, and compared with previous studies such as that performed by Reid et al. (2006). However, milk samples collected in the experimental study (Chapter 2, Armson et al., 2019) were also tested using the method detailed in Reid et al. (2006) at the time of sample collection. The C_T values observed were higher (weaker) than those observed when using 'Method B' after five years of sample storage. Therefore, the optimised method used throughout this thesis likely has a greater sensitivity than that used by Reid et al. (2006).

Results from the testing of milk from experimentally and naturally infected cattle demonstrated that the FMDV serotype or lineage could be identified using previously developed type-specific or lineage-specific rRT-PCR assays, and VP1 sequence data could also be obtained (Armson et al., 2019)(Chapter 3). Additionally, RNA genome detection, and typing and sequence data obtained from milk samples was generally consistent with that of vesicular epithelium, fluid or serum samples commonly used for FMD diagnosis (Armson et al., 2018, 2019)(Chapters 2 and 3). Further studies should perform more thorough comparisons of these sample types with milk and include additional sample types such as nasal and oral swabs, and OP fluid. Consequently, these findings provide confidence that milk is a sample type from which a range of information may be obtained for FMDV diagnostic and epidemiological purposes.

7.1.1.2 Increasing the window of detection compared to established sampling methods

Utilising an optimised high-throughput screening method, Chapter 2 demonstrated the increased window of FMDV detection by rRT-PCR in milk samples compared to serum samples and as previously described, vesicular epithelium or fluid (Alexandersen et al., 2003; King et al., 2012) (Figure 7.2). Evidence from this thesis supports previous observations that FMDV RNA genome can be detected before and during the

appearance of FMD clinical signs. Further evidence for the detection of FMDV in convalescing cows is also described in Chapter 2 (Armson et al., 2018), which supports previous observations by Reid et al., (2006). However, the termination of the cattle experiment while FMDV RNA genome was still being detected in the milk indicates further work is necessary to determine the true duration of virus excretion in the milk of naïve-infected animals. Additionally, comparison of the predictive modelling results with 'observed' rRT-PCR results (Chapter 6) suggested that the window of virus detection may have been different in these regularly vaccinated cows to that of the naïve cattle during the experimental study. Indeed it is unfortunate that milk was not collected from selected individual cattle during this study period (Chapter 6), so that the window of virus detection may have been defined in the milk of these regularly vaccinated animals. Future studies should therefore aim to elucidate viral excretion patterns in the milk of cattle of different breeds, and those vaccinated/infected with various serotypes/topotypes.

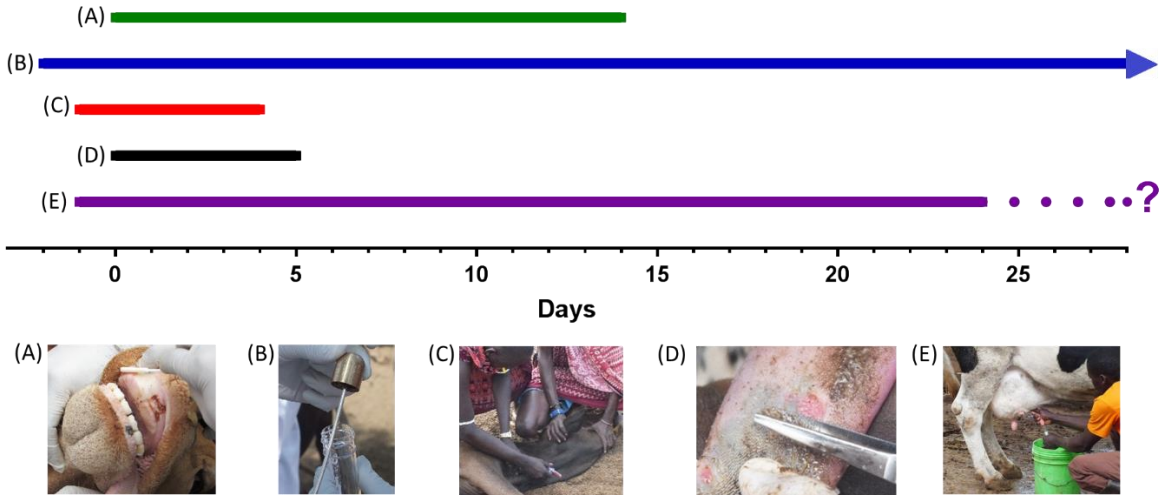

Figure 7.2 Approximate clinical window of FMD virus detection from different sample types: oral swab (A), OP fluid (B), blood (C), vesicular epithelium (D) and milk (E). Day 0 indicates the day vesicular lesions are first noticed. Based on data from Alexandersen et al., 2003; King et al., 2012; Stenfeldt, Lohse and Belsham, 2013; and Armson et al., 2018, Chapter 2. Photographs courtesy of Bryony Armson and Emma Howson, 2016.

7.1.1.3 The detection of subclinical infection

Several studies have identified FMDV infection in animals with no obvious signs of disease (Bertram et al., 2018; Hayer et al., 2018) (see Chapter 1, section 1.5). It is reasonable to assume that FMDV RNA genome may also be excreted in the milk of sub-clinically infected animals. Multiple data sets in this thesis demonstrate the detection of FMDV RNA genome in milk samples in the absence of clinical disease (Chapters 3, 5 and 6)(Armson et al., 2019), with subclinical infection as a possible explanation in some of these instances. These data support observations by Ahmed et al., (2017) where FMDV RNA genome was detected in the milk of apparently healthy vaccinated water buffaloes (*Bubalis bubalis*). It is unknown whether vaccination may increase the likelihood of sub-clinical infection. Investigating FMD viral excretion patterns in the milk of vaccinated versus non-vaccinated cattle is recommended as a research priority if milk is to be utilised as a sample type for FMD surveillance where regular vaccination is practised. For example, viral excretion in the milk of vaccinated animals could be measured as part of FMD vaccine field trials using dairy cattle. Further data from these types of studies may enhance the ability of predictive models and may be used to better inform milk surveillance programs.

7.1.1.4 The detection of FMDV in pooled milk

This thesis aimed to expand on previous studies to investigate whether FMDV RNA genome could be detected in pooled milk samples from outbreak or endemic settings which has not been demonstrated previously.

Initially (A) the ability to detect FMDV from the milk of individual cattle was assessed (discussed above in section 7.1.1.1) (Figure 7.3A), and these data were used to inform pooling studies.

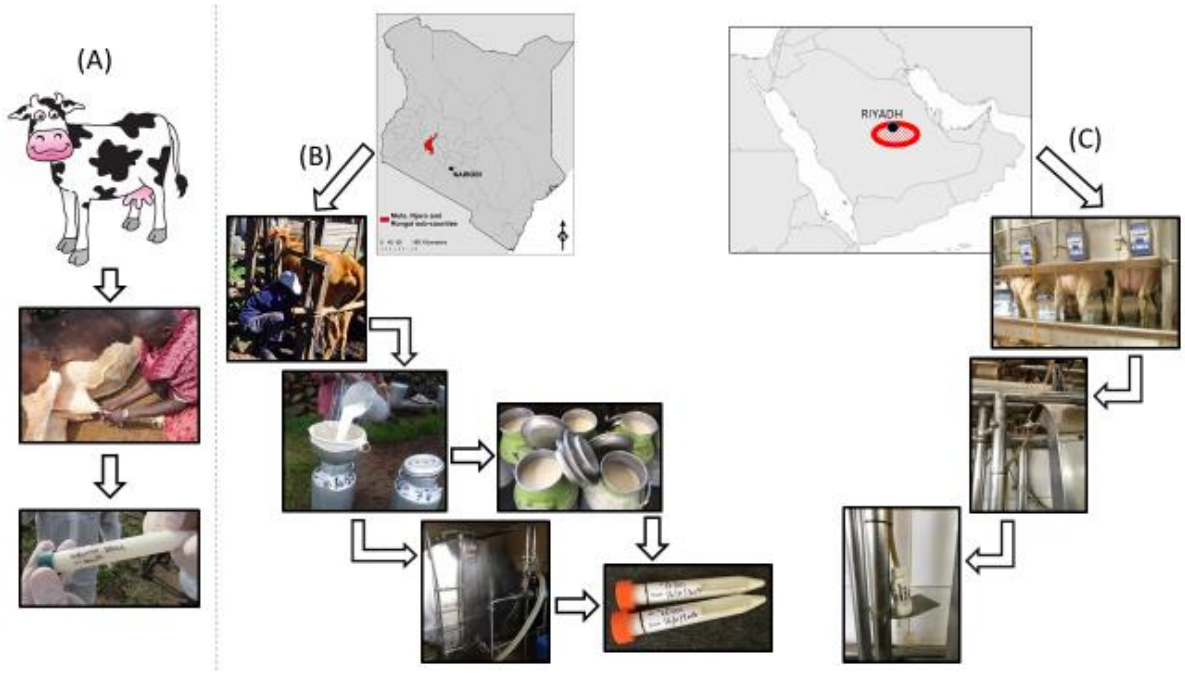

Figure 7.3 Summary of collection of milk from the field. (A) Milk samples from individual cattle in northern Tanzania. (B) Pooled milk samples representing dairy cattle on small-holder farms in Nakuru County, Kenya. (C) Pooled milk samples representing dairy cattle on a large-scale farm in Saudi Arabia. Photographs courtesy of Bryony Armson and Nick Lyons.

Subsequently, proof-of-concept pilot studies were carried out which demonstrated the ability of the rRT-PCR assay to detect FMDV in pooled milk samples collected from two different farming systems: (B) small-holder farmers supplying local milk pooling facilities in Kenya (Figure 7.3B) (Chapter 5); and (C) individual management groups of a large-scale dairy farm in Saudi Arabia (Figure 7.3C)(Chapter 6).

Results demonstrated that FMDV could be detected from milk pools of up to 5,000 litres and 10,000 litres for studies (B) and (C) respectively, even when there were low numbers of clinical cases of FMD. Due to the methodology used for sampling pooled milk for study (B), the number of cows represented by each sample was not known but could be estimated from data obtained from small-holder farmer surveys, suggesting the contribution of milk by over 1000 cattle. For study (C) although the volume of milk per management group was greater, it was supplied by a relatively smaller number of high-yielding cattle, approximately 240 cattle per house. These data support laboratory findings from limit of detection studies using the optimised high-

throughput FMDV rRT-PCR method carried out in Chapter 2 (Armson et al., 2018) and in a previous study that employed a different detection system (Reid et al., 2006), that suggested the assay would be sensitive enough to detect FMDV in pooled milk from large herds, even in the unlikely case that only one animal was infected. Data obtained in this thesis also support previous modelling studies which suggested that it would be possible to detect FMDV from a milk pool supplied by over 1000 cattle (Thurmond and Perez, 2006; Garner et al., 2016; Kompas et al., 2017). However, the milk pool volumes suggested in these studies (up to 20,000 litres) are larger than those tested in this thesis (up to 10,000 litres). Indeed, based on the weak C_T values observed from pooled milk samples throughout this thesis (C_T value > 30), it is unknown whether FMDV could be detected in pooled milk samples collected from larger herds (>1000), or samples collected higher up in the dairy value chain. Although it is assumed possible by limit of detection and modelling studies (Reid et al., 2006; Armson et al., 2018), these methods may not represent the realities of the field, and consequently field studies are required in order to determine the absolute limit of the FMDV detection system for pooled milk samples.

7.1.2 The limitations of utilising milk for FMD surveillance

7.1.2.1 Potential for contamination of the rRT-PCR assay

The high-throughput RNA extraction and real-time rRT-PCR optimised and utilised for FMDV RNA genome identification throughout this project was shown to be highly sensitive, able to detect FMDV RNA genome in large milk pools of up to 10,000 litres (Armson et al., 2018)(see Chapters 2, 5 and 6). The FMDV detection method used throughout this project was also shown to be highly specific (99.66%) when testing a negative cohort of milk samples from UK dairy herds. However amplification was observed in several wells of the rRT-PCR plates during the testing of this negative cohort. Additionally, there were several occasions (see Chapters 5 and 6) where high (weak) C_T values were observed where there were no clinical cases, or where there were no obvious alternative explanations for example viral excretion during convalescence or environmental contamination (such as contamination of the milk line from a previous 'positive' house [see Chapter 6]).

Due to the number of negative milk samples observed from Kenya and Saudi Arabia, it is unlikely that non-specific amplification of other template present in the milk (e.g. from other organisms) occurred, as it would be expected that more/all samples would have been positive. Additionally, the molecular detection method used throughout this thesis was based on the well-established and validated 3D pan-serotypic rRT-PCR assay utilised in reference laboratories that has been shown to be highly specific in a wide variety of sample types collected across the world (Callahan et al., 2002; Goris et al., 2009; Reid et al., 2009). However, as the negative cohort performed in chapter 4 was only collected from select breeds in the UK, to fully validate the specificity of the rRT-PCR assay, further cohorts of known negative milk samples should be tested from various cattle breeds and locations, and from those that may contain organisms exotic to the UK,

It is more likely that inter/intra-assay contamination of the rRT-PCR assay was responsible for the 'false positive' samples observed in the negative cohort of milk samples from the UK (see Chapter 4). The rRT-PCR assay performed throughout this thesis did not employ a cut-off value for positivity because the viral RNA in large milk pools may be highly dilute. A 'positive' result was therefore defined as the observation of amplification above the cycle threshold (C_T) combined with a 'healthy' looking amplification curve, until the end of the run (50 cycles). Indeed, some of the C_T values observed for the milk samples collected from Kenya and Saudi Arabia were higher than those observed for limit of detection experiments (see Chapters 2 and 4), and therefore contamination is a possible explanation. Consequently it is possible that at least some of the high C_T values observed throughout this thesis may have been 'false' positive results.

Due to the sensitive nature of the rRT-PCR assay, it is often impossible to differentiate between a true and a false positive result. Interpretation of the amplification curve may help to identify non-specific amplification, however amplification due to contamination of the sample during collection or during assay set-up cannot be identified in this way. Future work should aim to incorporate the use of negative controls throughout the whole sampling process i.e. from sample collection to testing. Additionally, to define cut-off values for positivity using analytical and epidemiologic approaches (Caraguel et al., 2011), bearing in mind the increased likelihood of higher

C_T values due to potentially highly diluted viral RNA. Additionally, absolute quantification of different serotypes of virus to more accurately determine the analytical sensitivity of the rRT-PCR assay may assist in the definition of these cut-off values. This would improve confidence in the results so that the milk sampling approach may be incorporated into active surveillance plans for FMD-free countries.

To mitigate the risk of contamination in the field, stringent biosecurity measures must be applied, including the use of personal protective equipment where necessary and appropriate disinfection of equipment. Additionally in the laboratory, sample tubes should not be opened unnecessarily and samples from different regions and dates should be processed separately, which was the method used for testing milk samples throughout this thesis. Chapter 4 also highlighted the importance of performing multiple replicates of samples during assay set-up, where resources allow, so that true positive results may more easily be determined.

7.1.2.2 Milk sampling methods

Results presented in this thesis identified several limitations of the milk sample collection methods used in these studies, and have been discussed in more detail in the respective chapters. For example, in Kenya (see Chapter 5), three of the milk pooling facilities did not own, or were not using their bulk milk tank, and therefore an alternative sampling method taking a small amount of milk from all 50 litre cans was employed, resulting in a more labour-intensive sampling process. However, samples collected in this way were assumed to be as representative as collection from a bulk tank, and excluding facilities using this method may bias the surveillance as this appeared to be a common practice. An optimal system could be designed for each of the various levels of pooling that occurs, including those further up the milk production chain (see section 7.2.2). Results from the large-scale dairy farm in Saudi Arabia (see Chapter 6) suggested that the proportional in-line milk sampler may not have always generated a representative sample from the herd, potentially resulting in 'false negative' results, especially if the sample container became full before the last cows of the group had been milked. Additionally, potential virus contamination of the milk line between different management groups may have resulted in 'false positive' results.

Consequently, it may be more suitable to collect milk samples directly from the bulk tank after mixing, that would be supplied by all/more lactating cows on the farm. This may have an additional cost-benefit of capturing more animals in fewer samples, although it is important to ensure that the FMDV detection method is indeed sensitive enough to still enable the detection of virus from one infected animal in this higher dilution of milk.

7.1.2.3 Estimation of FMD clinical incidence

The methods used for the pilot studies performed in this thesis (see Chapters 5 and 6) were appropriate, given that FMD surveillance using pooled milk has not previously been investigated, and therefore there were limited resources available. However, as discussed in more detail in these chapters, the estimation of disease incidence in the respective study populations may not have been truly accurate. For example, although it has been reported that farmers in Kenya had good knowledge of FMD (Nyaguthii et al., 2019), mild clinical signs, subclinical infection or indeed an unwillingness to report disease are likely to have resulted in low incidence estimates (Vosloo et al., 2002; Knight-Jones et al., 2014). Also, although farm staff on the large-scale dairy farm in Saudi Arabia were reported to be familiar with performing individual FMD case detection of individual cattle, it is possible that clinical signs may have been mild in this vaccinated population, and therefore cases may have gone undetected. Additionally, the study performed in Kenya was underpowered, due to the limited resources available for performing cross-sectional surveys of small-holder farmers. Future studies should therefore aim to more precisely estimate the level of FMD clinical incidence so that the milk sampling approach can be more robustly assessed. For example studies performed on large farms could integrate the collection of multiple sample types and milk from individual animals in addition to pooled milk samples. Additionally, those studies investigating small-holder farming systems could employ serological NSP testing of selected farms (discussed further in section 7.2.3).

7.1.2.4 Reduction in milk yield during FMDV infection

Reservations exist as to the ability of FMDV detection in pooled milk due to the many reports of reduced milk yield during FMDV infection (James and Rushton, 2002; Knight-Jones and Rushton, 2013; Ferrari et al., 2014; Jemberu et al., 2014; Bastola, 2015; Ansari-Lari et al., 2017; Casey-Bryars et al., 2018). Indeed, animals that are sick and/or experience a cessation or reduction in milk production may not contribute to the milk pool, and therefore a pooled milk sample may not be representative of FMD virus circulation in the whole herd. Additionally, the milk of uninfected cows may dilute the virus contained in the small amounts of milk produced by infected cattle so that the final virus concentration is beyond the analytical sensitivity of the rRT-PCR assay.

Despite these reservations, data presented in this thesis demonstrated that FMDV RNA genome could still be detected from a pooled milk sample when the FMD incidence rates over each of the study periods were low (Chapters 5 and 6). For the study in Saudi Arabia (Chapter 6), sick or diseased animals were isolated from their management house, although this process was not consistent when isolation pens became full. Virus-laden milk from these animals was therefore not included in the sampled milk pool, yet FMDV RNA could still be detected. It is possible that the regular vaccination of these animals resulted in less severe/no clinical signs in some animals, and consequently a reduced impact on milk yield. For the study performed in Kenya (Chapter 5), farm-level data was not available and therefore it is unknown how many infected animals did not contribute their milk. Although this information is not necessary when performing surveillance of the study population, it would be useful to determine the true sensitivity of the FMDV detection system, and to inform future modelling studies.

7.1.2.5 Sampling bias

Milk samples can only be collected from lactating female animals. Therefore, if surveillance approaches focus exclusively on dairy cattle; males, young stock, breeds designed for meat, and other species such as sheep, goats and pigs may not be represented. It is therefore anticipated that the milk sampling surveillance approach could act as a supplement to current surveillance systems that may be limited to the detection of acute disease. Therefore, it is important that data obtained from the milk

sample collected is representative of the FMDV circulation in the susceptible population.

In FMD endemic regions such as East Africa and the Middle East, there are many nomadic and small-holder farmers that keep small ruminants together with their dairy cattle, and a small number of large-scale dairy farms (Nthiwa et al., 2019; Nyaguthii et al., 2019). It has been suggested that sheep and goats may play a significant role in the transmission of FMDV, and therefore the close proximity likely results in high levels of viral circulation between species (Kitching and Hughes, 2002; Asghar et al., 2016). In FMD-free countries such as Western Europe, dairy farms are also generally widespread (Department of Environment Food and Rural Affairs, 2010). It is likely that dairy cattle may act as sentinels for the surrounding susceptible FMD population, and consequently surveillance systems that focus on dairy cattle are likely to be highly representative of disease circulation in these settings. This was supported by simulation models performed by Garner et al. (2016) who suggested that bulk milk testing could enhance early detection in areas where there are many dairy cattle, even when the outbreak starts outside of a dairy area.

In contrast, a milk sampling surveillance approach may not be appropriate for endemic countries such as Hong Kong SAR, China and Thailand that have large populations of pigs, and less market for dairy products resulting in a lower number of dairy cattle (see Figure 7.4) (Sumption et al., 2008; Robinson et al., 2014).

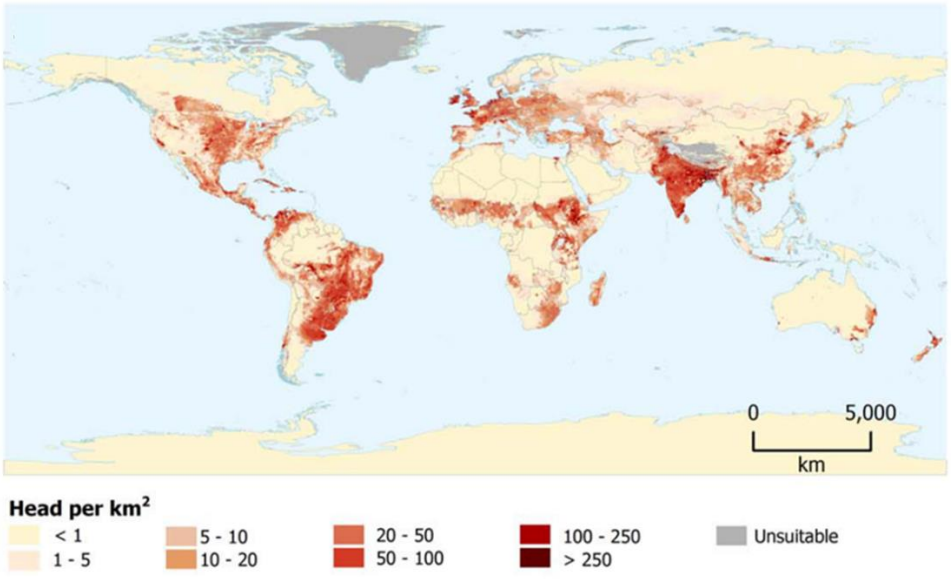

Figure 7.4 The global distribution of cattle. Adapted from Robinson et al. (2014).

7.2 Recommendations for future studies

Data presented in this thesis have demonstrated the benefits and limitations of milk as a sample type for FMDV detection and surveillance, with the potential of implementation into regional surveillance plans alongside existing surveillance schemes. Before integrating pooled milk into FMD surveillance systems, there are several considerations that require further investigation.

7.2.1 Technologies for improved molecular data collection

Although results from Chapter 4 demonstrated that FMDV RNA detection by rRT-PCR was not significantly affected during the long-term storage and simulated transport of milk samples, the use of point of care (POC) technologies would allow for rapid result reporting at source, reducing the need for expensive and logistically challenging transport of samples to national or international FMD laboratories. The detection of FMDV RNA from milk samples using a fully automated cartridge-based real-time RT-PCR diagnostic system (the Enigma® Mini Laboratory) has been described (Goller et al., 2018), and although this system is no longer commercially available, similar systems could be designed that are suitable for use in mobile and local laboratories with limited resources, or on large-scale dairy farms where samples may be tested on-site. It is possible that existing POC technologies that do not include an RNA extraction step may not be suitable for the detection of FMDV RNA from milk samples due to PCR inhibitors such as the proteins and lipids found in milk. Therefore, future work could aim to optimise these or alternative technologies in combination with effective simple sample preparation methods such as those described by Howson et al. (2018).

Furthermore, as results obtained in Chapter 3 highlighted the ability to obtain VP1 sequence data from milk samples, the potential for next-generation sequencing (NGS) should also be investigated. This genomic data could then be used to better understand the epidemiology of FMDV in a region, for example by investigating the viral diversity of pooled milk samples to determine the burden of infection in a region (Walker et al., 2013; King et al., 2016). With the rapid advancement of technology, the potential exists for the application of POC NGS technology such as the MinION nanopore sequencer

(Oxford Nanopore Technologies, Oxford, UK) for real-time, portable genome sequencing of FMDV in milk samples (Hansen et al., 2019). However, it is possible that the low concentrations of viral RNA in a pooled milk sample may be too low to obtain quality sequence data, and milk may not be suitable for rapid field sequencing as prior processing may be required (Logan et al., 2014).

7.2.2 Exploring the milk production chain for representative pooled milk sampling

Pooling studies performed for this thesis have highlighted the importance of simple, representative sampling methods to ensure confidence in the test result. Further work is required to examine the pooling systems employed by different geographical regions or farming settings, and how they may be targeted for effective surveillance. For example, assessment of the ability to detect FMDV at different stages of the milk production chain, from the individual animal, to the farm-level and up to processing centres should be performed (Figure 7.5). Sampling milk at higher levels would capture the contribution of milk by a greater proportion of the dairy cattle population in a region. However, the stage at which pasteurisation occurs should be carefully considered, as it has been demonstrated that FMDV is less likely to be detected following this process (Reid et al., 2006; Aly and Gaber, 2007; Tomasula et al., 2007). Garner et al. (2016) modelled FMDV detection from a transport tanker containing 20,000 litres of milk collected from up to five large-scale dairy farms, and it is important to externally validate these findings based on field data particularly if they are used to inform surveillance policy. Information should also be obtained concerning the cost implications for sampling at each stage, and how representative the sample is of the region of interest, i.e. what percentage of susceptible animals are contributing to the sample. Additionally, FMDV has been previously identified in the milk of sheep and goats (Aly and Gaber, 2007), and therefore it could be investigated whether this approach could be useful for FMD surveillance, as has been demonstrated for the detection of *Brucella* spp. (Hamdy and Amin, 2002).

The adulteration of milk by farmers for financial gain has been reported, especially in underdeveloped countries (Azad and Ahmed, 2016). Substances added to milk may

include milk powder, water or chemicals such as hydrogen peroxide, formalin and salicylic acid. Although pooling facilities often perform tests for these substances, there remains the potential for milk contamination, and it is unknown how these substances may affect any FMD virus contained within the milk. Therefore, experiments should be performed to determine how the presence of these substances in milk samples may affect the ability of the rRT-PCR to detect FMDV RNA genome. Alternatively, milk samples could only be taken for testing once the appropriate quality assessments have been performed.

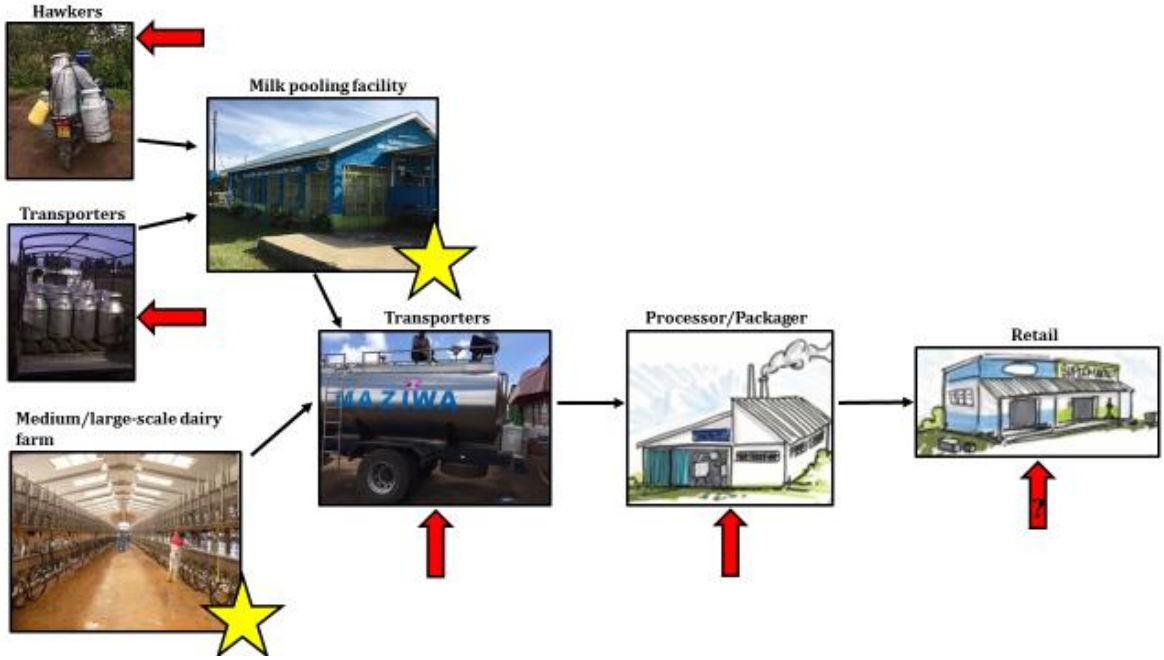

Figure 7.5 Targeting different stages of the milk production chain for cost-effective pooled milk sampling. Yellow stars indicate sampling levels already investigated in this thesis. Red arrows indicate potential sampling levels for future research. Adapted from Recheis (2019)

7.2.3 Improving sensitivity estimates of the pooled milk surveillance system

Proof-of-concept pilot studies performed for this thesis demonstrated the ability to detect FMDV RNA genome from large milk pools. However, the methods used to estimate sensitivity involved comparing pooled milk sample results against reports of clinical disease by farmers. Although this method was appropriate for the pilot studies

performed for this thesis and with the resources available, it may not have provided an accurate representation of the true burden of disease in the population, as described earlier (section 7.1.2.3).

Further studies are therefore required, in order achieve improved estimates of clinical incidence in a region, so that the sensitivity of the pooled milk surveillance system can be more precisely estimated. For example, testing frequently collected serum samples for non-structural protein (NSP) and structural protein (SP) antibodies, and for FMDV RNA genome alongside reports of clinical cases would provide evidence for FMD infection and may identify cases of subclinical infection in small-holder farming systems. Farmer surveys should also be powered to estimate the true incidence of disease if possible, instead of the detection of a specific threshold of FMD incidence, which was employed for this thesis. Additionally, studies performed on large farms could integrate the collection of multiple sample types e.g. vesicular epithelium, serum, nasal/oral swabs, or oesophageal-pharyngeal fluid from suspected infected animals and milk from individual animals throughout the period of infection. This would confirm suspected cases of FMD, provide further information on the window of detection of virus excretion in the milk of these cows, potentially identify/confirm cases of co-infection and consequently would assist in more robustly assessing the pooled milk sample surveillance approach.

7.2.4 FMDV antibody testing

Antibodies to FMDV have been detected in milk, and a significant correlation found with the levels of antibodies found in serum (Armstrong, 1997; Armstrong and Mathew, 2001; Fayed et al., 2013). Consequently, the detection of FMDV antibodies in milk samples could be integrated into surveillance schemes to identify infected cattle/herds, or could be a useful alternative sample type to blood for post-vaccination monitoring (Fayed et al., 2013). Although several assays for the detection of FMDV antibody from milk samples have been developed (Stone and DeLay, 1960; Armstrong, 1997; Armstrong et al., 2000), newer methods may need to be optimised and validated, based on current commercially available tests for serum. Indeed, it is possible that virus-specific antibody testing of milk samples collected for work performed in this

thesis could provide additional information, for example to support suggestions of sub-clinical infection, or provide support for results that could be interpreted as 'false positives'.

7.3 Potential applications of the pooled milk surveillance system

Once further validation of the pooled milk surveillance system has been performed, it is anticipated that it may be valuable for targeted/risk-based surveillance alongside existing surveillance systems to facilitate improved knowledge of FMD epidemiology, or for use in FMD contingency plans.

7.3.1 FMD endemic regions

Data presented in this thesis have demonstrated the potential use of milk to improve knowledge on FMD epidemiology in endemic regions. It is anticipated that this approach could be utilised to answer specific research questions, such as:

(i) Estimation of FMD incidence and circulating serotypes/lineages. The burden of infection could be predicted based on C_T value, or through sequence diversity data;
(ii) Estimation of the level of subclinical infection, for example in vaccinated vs. non-vaccinated populations;
(iii) Assessment of control strategies, such as the effectiveness of vaccination programmes.

Although further research is required to fully validate the use of milk for FMD surveillance in different scenarios, it is possible that a simple, risk-based surveillance approach could be employed in the near future, especially to answer some of the questions that require less precise estimates of disease incidence, such as which serotypes/lineages are circulating in a region. Improved knowledge of the FMD epidemiology in a region may facilitate the progress of a country through the

Progressive Control Pathway for Foot and Mouth Disease (PCP-FMD) (Food and Agriculture Organization of the United Nations (FAO), 2011).

7.3.2 FMD free countries

Farm-level bulk milk sampling has been demonstrated to be effective for FMDV detection (see Chapter 6). Therefore, one speculates whether a similar milk sampling approach to that utilised for brucellosis in the United Kingdom (DEFRA, 2015a, 2015b) could be applied as an early warning indicator of disease, where milk samples are routinely submitted to testing laboratories (see Chapter 1, section 1.6). However, due to the short window of FMDV excretion in milk (<28 days), much shorter testing intervals (e.g. weekly) would be required. Consequently, the cost of pooled milk sampling at this frequency would likely outweigh the benefit of detection only a few days earlier than with passive surveillance methods, as suggested by modelling studies performed by Kompas et al. (2017) based on dairy herds in the Victoria state of Australia. There are however, several applications of the pooled milk surveillance system that could be cost-effective for use in FMD free countries and have been considered potentially valuable for use in the United States (Kompas et al., 2017; Lombard et al., 2017):

(i) Testing of dairy premises in response to the threat of an outbreak in a neighbouring country or region for early preclinical diagnosis;
(ii) Confirmation of a suspect case in a herd of dairy cattle;
(iii) Testing of unpasteurised milk to be moved to a disease-free region;
(iv) Screening of dairy herds after the cessation of clinical signs to signify disease freedom on a farm/region.

A surveillance system designed for these purposes must be highly specific and sensitive to ensure a high degree of confidence in the test results. False results may have costly implications including the unnecessary culling of animals or onward viral transmission (Caporale et al., 2012; Lewerin et al., 2018). Therefore, in order for the milk sampling surveillance system to be implemented for these purposes, further research is required to obtain precise estimates of sensitivity and specificity, from studies performed in settings similar to those of FMD free countries. In the event of a future FMD outbreak

in a normally-free country, bulk milk sampling could be exploited in order for example, to determine the optimal frequency of milk sampling, which has only so far been hypothesised using simulation modelling (Thurmond and Perez, 2006; Garner et al., 2016; Kompas et al., 2017).

7.4 Concluding remarks

In conclusion, this thesis has demonstrated the utility of milk as a diagnostic sample for FMDV detection, and based on the proof-of-concept pilot studies performed, has highlighted the benefits and limitations of its application for FMD surveillance in both FMD endemic and free regions.

Further research and investment are required to inform recommendations on how this simple, cost-effective, risk-based targeted surveillance system that is otherwise expensive and logistically challenging, could be used to contribute to FMD surveillance activities around the globe.

Appendices

Appendix I

Determination of the optimal high-throughput screening method for the detection of FMDV in milk samples

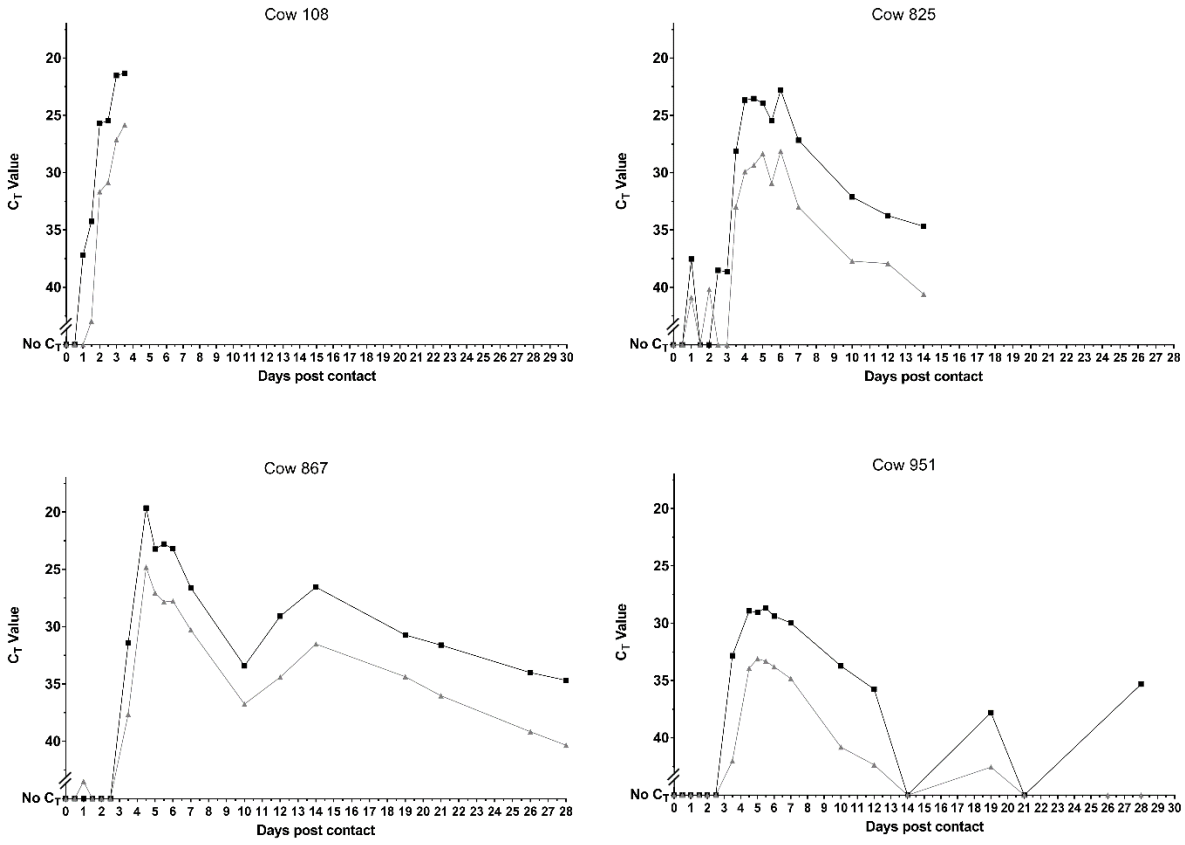

Figure 8.1 Comparison of Methods A and B for the detection of FMDV from whole milk from each cow. C_T values are the mean of two replicates. ▲: Method A, ■: Method B.

Appendix II

FMD detection in milk samples from individual cattle

Table 8.1 List of samples and virus isolates from northern Tanzania used throughout the study. The mean C_T values for the pan-serotypic (3D) rRT-PCR and East Africa (O, A, SAT 1, SAT 2) rRT-PCR typing assays are reported.

Animal ID/ WRLFMD Reference	Collection date	Sample type	Location	3D	O	A	SAT 1	SAT 2
8177	26/05/2012	M	Nyamburi	No C_T	NP	NP	NP	NP
8146	30/07/2012	M	Nyamburi	No C_T	NP	NP	NP	NP
8233	17/08/2012	M	Nyamburi	No C_T	NP	NP	NP	NP
8233	17/08/2012	M	Nyamburi	No C_T	NP	NP	NP	NP
8401	18/08/2012	M	Nyamsingisi	24.93	No C_T	24.10	No C_T	No C_T
8266	17/10/2012	M	Nyamburi	No C_T	NP	NP	NP	NP
8233	18/10/2012	M	Nyamburi	36.46	No C_T	No C_T	No C_T	No C_T
8259	18/10/2012	M	Nyamburi	No C_T	NP	NP	NP	NP
8269	18/10/2012	M	Nyamburi	No C_T	NP	NP	NP	NP
8401	10/11/2012	M	Nyamsingisi	No C_T	NP	NP	NP	NP
8403	10/11/2012	M	Nyamsingisi	No C_T	NP	NP	NP	NP
8406	10/11/2012	M	Nyamsingisi	No C_T	NP	NP	NP	NP
8427	13/11/2012	M	Nyichoka	No C_T	NP	NP	NP	NP
8438	13/11/2012	M	Nyichoka	No C_T	NP	NP	NP	NP
8445	13/11/2012	M	Nyichoka	No C_T	NP	NP	NP	NP
8457	13/11/2012	M	Nyichoka	No C_T	NP	NP	NP	NP
7470	23/11/2012	M	Rwamchanga	No C_T	NP	NP	NP	NP
7476	23/11/2012	M	Rwamchanga	No C_T	NP	NP	NP	NP
7689	23/11/2012	M	N/A	No C_T	NP	NP	NP	NP
7652	24/11/2012	M	Rwamchanga	No C_T	NP	NP	NP	NP
7653	24/11/2012	M	Rwamchanga	No C_T	NP	NP	NP	NP
7655	24/11/2012	M	Rwamchanga	No C_T	NP	NP	NP	NP
7910	20/12/2012	M	Tamau	No C_T	NP	NP	NP	NP
7913	20/12/2012	M	Tamau	No C_T	NP	NP	NP	NP
7930	18/01/2013	M	Mbilikili	31.33	No C_T	32.73	No C_T	No C_T
7941	18/01/2013	M	Mbilikili	No C_T	NP	NP	NP	NP
7945	18/01/2013	M	Mbilikili	35.07	No C_T	35.56	No C_T	No C_T
7950	18/01/2013	M	Mbilikili	34.60	No C_T	No C_T	No C_T	No C_T

7951	18/01/2013	M	Mbilikili	26.99	No C_T	27.17	No C_T	No C_T
7963	18/01/2013	M	Mbilikili	29.58	No C_T	28.75	No C_T	No C_T
7964	18/01/2013	M	Mbilikili	25.93	No C_T	26.76	No C_T	No C_T
8110	04/02/2013	M	Natambiso	No C_T	NP	NP	NP	NP
8225	14/02/2013	M	Motukeri	No C_T	NP	NP	NP	NP
8404	15/02/2013	M	Nyamsingisi	No C_T	NP	NP	NP	NP
8406	15/02/2013	M	Nyamsingisi	No C_T	NP	NP	NP	NP
6605	11/11/2013	M	Nygoti	No C_T	NP	NP	NP	NP
9151	11/11/2013	M	Nygoti	No C_T	NP	NP	NP	NP
9152	11/11/2013	M	Nygoti	No C_T	NP	NP	NP	NP
6778	16/11/2013	M	Nyamburi	No C_T	NP	NP	NP	NP
8261	16/11/2013	M	Nyamburi	No C_T	NP	NP	NP	NP
8562	16/11/2013	M	Nyamburi	36.57	No C_T	No C_T	48.00	No C_T
8806	16/11/2013	M	Nyamburi	No C_T	NP	NP	NP	NP
8808	16/11/2013	M	N/A	No C_T	NP	NP	NP	NP
8809	16/11/2013	M	N/A	36.72	No C_T	No C_T	No C_T	No C_T
8811	16/11/2013	M	N/A	No C_T	NP	NP	NP	NP
8149	18/11/2013	M	Nyamburi	32.39	41.40	No C_T	34.26	No C_T
8413	18/11/2013	M	Nyamburi	No C_T	NP	NP	NP	NP
8194	22/11/2013	M	Motukeri	No C_T	NP	NP	NP	NP
8193	25/11/2013	M	Motukeri	37.96	No C_T	No C_T	No C_T	No C_T
8530	25/11/2013	M	Mbilikili	No C_T	NP	NP	NP	NP
9202	19/01/2014	M	N/A	33.53	No C_T	No C_T	No C_T	No C_T
7024	26/01/2014	M	Nyichoka	No C_T	NP	NP	NP	NP
9232	29/01/2014	M	N/A	33.67	No C_T	No C_T	No C_T	No C_T
6532	05/02/2014	M	Tamau	No C_T	NP	NP	NP	NP
6537	05/02/2014	M	Tamau	No C_T	NP	NP	NP	NP
7700	05/02/2014	M	Tamau	No C_T	NP	NP	NP	NP
8850	05/02/2014	M	N/A	No C_T	NP	NP	NP	NP
6545	06/02/2014	M	Tamau	No C_T	NP	NP	NP	NP
6551	06/02/2014	M	Tamau	No C_T	NP	NP	NP	NP
6582	06/02/2014	M	Tamau	No C_T	NP	NP	NP	NP
7908	06/02/2014	M	Tamau	No C_T	NP	NP	NP	NP
7909	06/02/2014	M	Tamau	35.48	No C_T	No C_T	No C_T	No C_T
7913	06/02/2014	M	Tamau	32.41	38.05	No C_T	35.65	No C_T
7914	06/02/2014	M	Tamau	No C_T	NP	NP	NP	NP
7545	10/02/2014	M	Mbilikili	No C_T	NP	NP	NP	NP
8544	10/02/2014	M	Mbilikili	31.97	34.16	No C_T	36.25	No C_T

8687	10/02/2014	M	N/A	36.97	No C_T	No C_T	40.75	No C_T
7950	12/02/2014	M	Mbilikili	37.04	No C_T	No C_T	38.85	No C_T
7955	12/02/2014	M	Mbilikili	36.18	No C_T	No C_T	36.86	No C_T
7956	12/02/2014	M	Mbilikili	No C_T	NP	NP	NP	NP
7961	12/02/2014	M	Mbilikili	35.44	No C_T	No C_T	40.69	No C_T
7963	12/02/2014	M	Mbilikili	No C_T	NP	NP	NP	NP
8682	12/02/2014	M	N/A	No C_T	NP	NP	NP	NP
8884	12/02/2014	M	N/A	No C_T	NP	NP	NP	NP
8889	12/02/2014	M	N/A	No C_T	NP	NP	NP	NP
8897	12/02/2014	M	N/A	No C_T	NP	NP	NP	NP
8110	18/02/2014	M	Natambiso	36.83	No C_T	No C_T	No C_T	No C_T
8300	19/02/2014	M	Nyamsingisi	No C_T	NP	NP	NP	NP
8401	19/02/2014	M	Nyamsingisi	No C_T	NP	NP	NP	NP
8403	19/02/2014	M	Nyamsingisi	No C_T	NP	NP	NP	NP
8533	19/02/2014	M	Nyamsingisi	39.66	No C_T	No C_T	No C_T	No C_T
8534	19/02/2014	M	Nyamsingisi	No C_T	NP	NP	NP	NP
9200	19/02/2014	M	N/A	34.66	38.01	No C_T	37.20	No C_T
9201	19/02/2014	M	N/A	35.38	No C_T	No C_T	36.38	No C_T
9203	19/02/2014	M	N/A	No C_T	NP	NP	NP	NP
9204	19/02/2014	M	N/A	No C_T	NP	NP	NP	NP
9205	19/02/2014	M	N/A	No C_T	NP	NP	NP	NP
9206	19/02/2014	M	N/A	No C_T	NP	NP	NP	NP
7459	22/02/2014	M	Rwamchanga	37.69	No C_T	No C_T	No C_T	No C_T
7461	22/02/2014	M	Rwamchanga	35.68	No C_T	No C_T	No C_T	No C_T
7472	22/02/2014	M	Rwamchanga	33.69	No C_T	No C_T	No C_T	No C_T
7476	22/02/2014	M	Rwamchanga	No C_T	NP	NP	NP	NP
7479	22/02/2014	M	Rwamchanga	No C_T	NP	NP	NP	NP
7485	22/02/2014	M	Rwamchanga	No C_T	NP	NP	NP	NP
7487	22/02/2014	M	Rwamchanga	No C_T	NP	NP	NP	NP
7494	22/02/2014	M	Rwamchanga	No C_T	NP	NP	NP	NP
7652	22/02/2014	M	Rwamchanga	No C_T	NP	NP	NP	NP
7653	22/02/2014	M	Rwamchanga	No C_T	NP	NP	NP	NP
7665	22/02/2014	M	Rwamchanga	No C_T	NP	NP	NP	NP
7670	22/02/2014	M	Rwamchanga	No C_T	NP	NP	NP	NP
8694	22/02/2014	M	Tamau	No C_T	NP	NP	NP	NP
8698	22/02/2014	M	Rwamchanga	No C_T	NP	NP	NP	NP
7050	24/02/2014	M	Nyichoka	No C_T	NP	NP	NP	NP
9310	24/02/2014	M	N/A	No C_T	NP	NP	NP	NP

9311	24/02/2014	M	Nyichoka	No C_T	NP	NP	NP	NP
9312	24/02/2014	M	N/A	37.41	No C_T	No C_T	No C_T	No C_T
9313	24/02/2014	M	N/A	No C_T	NP	NP	NP	NP
9315	24/02/2014	M	N/A	No C_T	NP	NP	NP	NP
7028	26/02/2014	M	Nyichoka	No C_T	NP	NP	NP	NP
7030	26/02/2014	M	Nyichoka	35.61	40.80	No C_T	No C_T	No C_T
7033	26/02/2014	M	Nyichoka	35.58	No C_T	No C_T	No C_T	No C_T
7040	26/02/2014	M	Nyichoka	37.18	40.35	No C_T	No C_T	No C_T
7043	26/02/2014	M	Nyichoka	No C_T	NP	NP	NP	NP
7048	26/02/2014	M	Nyichoka	No C_T	NP	NP	NP	NP
6625	28/03/2014	M	Natambiso	No C_T	NP	NP	NP	NP
6644	28/03/2014	M	Natambiso	No C_T	NP	NP	NP	NP
6657	28/03/2014	M	Natambiso	No C_T	NP	NP	NP	NP
6585	14/04/2014	M	Nygoti	No C_T	NP	NP	NP	NP
6599	14/04/2014	M	Nygoti	35.06	No C_T	No C_T	39.79	No C_T
6605	14/04/2014	M	Nygoti	No C_T	NP	NP	NP	NP
9151	14/04/2014	M	Nygoti	36.80	37.36	No C_T	35.96	No C_T
9152	14/04/2014	M	Nygoti	36.71	No C_T	No C_T	No C_T	No C_T
6675	19/05/2014	M	Nygoti	No C_T	NP	NP	NP	NP
6700	19/05/2014	M	Nygoti	36.64	No C_T	No C_T	39.71	No C_T
9243	20/05/2014	M	N/A	38.58	No C_T	No C_T	No C_T	No C_T
9247	20/05/2014	M	N/A	No C_T	NP	NP	NP	NP
9248	20/05/2014	M	N/A	No C_T	NP	NP	NP	NP
8257	23/05/2014	M	Nyamburi	No C_T	NP	NP	NP	NP
8565	23/05/2014	M	Nyamburi	35.48	No C_T	No C_T	No C_T	No C_T
8806	23/05/2014	M	Nyamburi	No C_T	NP	NP	NP	NP
8809	23/05/2014	M	N/A	24.54	26.77	31.33	26.52	No C_T
8177	26/05/2014	M	Nyamburi	No C_T	NP	NP	NP	NP
8413	26/05/2014	M	Nyamburi	No C_T	NP	NP	NP	NP
8816	26/05/2014	M	Nyamburi	37.09	No C_T	No C_T	No C_T	No C_T
8829	26/05/2014	M	N/A	No CT	NP	NP	NP	NP
8643	30/05/2014	M	Nygoti	34.60	No C_T	No C_T	No C_T	No C_T
8646	30/05/2014	M	Nygoti	35.53	No C_T	No C_T	39.09	No C_T
9149	30/05/2014	M	Nygoti	No C_T	NP	NP	NP	NP
8225	18/08/2014	M	Motukeri	32.90	37.00	No C_T	37.01	No C_T
9111	18/08/2014	M	Motukeri	36.38	37.25	No C_T	48.18	No C_T
7736	03/09/2014	M	Nyichoka	33.31	No C_T	No C_T	No C_T	No C_T

7601	09/09/2014	M	N/A	23.55	No C_T	No C_T	23.79	No C_T
7602	09/09/2014	M	N/A	31.01	No C_T	No C_T	31.27	No C_T
7608	09/09/2014	M	N/A	23.28	34.92	No C_T	31.76	No C_T
7609	09/09/2014	M	Nyichoka	30.79	33.11	37.07	32.43	No C_T
7716	29/09/2014	M	N/A	No C_T	NP	NP	NP	NP
7730	08/10/2014	M	N/A	No C_T	NP	NP	NP	NP
7743	08/10/2014	M	N/A	No C_T	NP	NP	NP	NP
7805	09/10/2014	M	Bunchugu	27.39	No C_T	No C_T	27.05	No C_T
7808	10/10/2014	M	N/A	No C_T	NP	NP	NP	NP
7815	16/10/2014	M	Rwamchanga	26.30	No C_T	No C_T	24.48	No C_T
7828	16/10/2014	M	N/A	37.75	No C_T	No C_T	No C_T	No C_T
7832	16/10/2014	M	N/A	35.66	No C_T	No C_T	No C_T	No C_T
7834	16/10/2014	M	N/A	No C_T	NP	NP	NP	NP
7848	16/10/2014	M	N/A	32.76	37.76	No C_T	40.70	No C_T
8011	07/11/2014	M	N/A	29.62	No C_T	No C_T	32.62	No C_T
8014	07/11/2014	M	N/A	No C_T	NP	NP	NP	NP
8021	07/11/2014	M	N/A	33.81	No C_T	No C_T	No C_T	No C_T
8032	07/11/2014	M	N/A	34.20	47.61	No C_T	37.21	No C_T
8039	07/11/2014	M	N/A	No C_T	NP	NP	NP	NP
8040	07/11/2014	M	N/A	33.70	37.24	No C_T	36.48	No C_T
8044	07/11/2014	M	N/A	35.13	No C_T	No C_T	No C_T	No C_T
8045	11/11/2014	M	N/A	No C_T	NP	NP	NP	NP
8227	25/11/2014	M	Motukeri	No C_T	NP	NP	NP	NP
7033	27/11/2014	M	Nyichoka	No C_T	NP	NP	NP	NP
9013	27/11/2014	M	Nyichoka	39.58	No C_T	No C_T	No C_T	No C_T
7960	02/12/2014	M	Mbilikili	31.62	35.85	No C_T	33.34	No C_T
7961	02/12/2014	M	Mbilikili	40.31	No C_T	No C_T	No C_T	No C_T
8840	02/12/2014	M	Motukeri	No C_T	NP	NP	NP	NP
8884	02/12/2014	M	N/A	31.78	35.06	No C_T	44.97	No C_T
8502	18/12/2014	M	Mbilikili	37.69	No C_T	No C_T	No C_T	No C_T
8517	18/12/2014	M	Mbilikili	35.43	No C_T	No C_T	No C_T	No C_T
9511	18/12/2014	M	N/A	32.56	36.07	No C_T	33.94	No C_T
8644	26/12/2014	M	Nygoti	No C_T	NP	NP	NP	NP
9205	09/01/2015	M	N/A	36.99	No C_T	No C_T	No C_T	No C_T
9144	28/01/2015	M	Nyichoka	30.14	34.54	37.05	37.40	No C_T
9150	28/01/2015	M	Nygoti	No C_T	NP	NP	NP	NP
9201	28/01/2015	M	N/A	27.37	36.80	No C_T	34.45	No C_T
9202	28/01/2015	M	N/A	36.33	No C_T	No C_T	No C_T	No C_T

6778	29/01/2015	M	Nyamburi	No C_T	NP	NP	NP	NP
6779	29/01/2015	M	Nyamburi	No C_T	NP	NP	NP	NP
8261	29/01/2015	M	Nyamburi	No C_T	NP	NP	NP	NP
8806	29/01/2015	M	Nyamburi	No C_T	NP	NP	NP	NP
8808	29/01/2015	M	N/A	No C_T	NP	NP	NP	NP
8811	29/01/2015	M	N/A	34.58	No C_T	No C_T	No C_T	No C_T
9227	29/01/2015	M	N/A	No C_T	NP	NP	NP	NP
7605	13/03/2015	M	N/A	No C_T	NP	NP	NP	NP
7951	02/12/2015	M	N/A	32.30	35.31	No C_T	No C_T	No C_T
6516	N/A	M	Tamau	32.27	No C_T	No C_T	37.89	No C_T
8297	N/A	M	Nyamsingisi	No C_T	NP	NP	NP	NP
TAN/19/2012 (SAT 2)	28/04/2012	C	Simanjiro					
TAN/39/2012 (O)	31/05/2012	C	Ngorongoro district					
TAN/6/2013 (A)	16/03/2013	C	Nyamburi					
TAN/33/2014 (SAT 1)	16/10/2014	C	Rwamchanga					
TAN/20/2014	03/09/2014	E	Nyichoka					
TAN/22/2014	09/09/2014	E	Nyichoka					
TAN/23/2014	09/09/2014	VF	Nyichoka					
TAN/28/2014	09/10/2014	E	Bunchugu					
TAN/29/2014	09/10/2014	VF	Bunchugu					
TAN/34/2014	16/10/2014	E	Rwamchanga					

N/A – information not available. NP – Not performed. M – Milk. CC – Cell culture isolate. E – Epithelium. VF – Vesicular fluid. No C_T – No C_T value observed (>50).

Appendix III

FMD surveillance in Nakuru County, Kenya

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VETERINARY RESEARCH

RESEARCH ARTICLE Open Access

Knowledge and risk factors for foot-and-mouth disease among small-scale dairy farmers in an endemic setting

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Abstract

Foot-and-mouth disease (FMD) is a highly contagious viral infection of cloven-hoofed animals. In Kenya, the disease is endemic with outbreaks typically occurring throughout the year. A cross-sectional study was undertaken in Nakuru County to investigate farmer knowledge and risk factors for clinical disease. Semi-structured interviews were conducted on 220 smallholder farmers, selected using random spatial sampling. The majority of respondents (207/220 [94.1%]) knew of FMD and 166/207 (80.2%) of them could correctly identify the disease based on their knowledge of the clinical signs. Forty-five out of 220 farmers (20.4%) vaccinated their livestock against FMD in the previous 6 months, although of those who knew of FMD only 96/207 (46.4%) perceived it as a preventive measure undertaken to reduce the risk of disease in their farm. FMD had occurred in 5.9% of the surveyed farms within the previous 6 months (from May to November 2016). Using multivariate analysis, the use of a shared bull (OR = 9.7; $p = 0.014$) and the number of sheep owned (for each additional sheep owned OR = 1.1; $p = 0.066$) were associated with an increased likelihood of a farm experiencing a case of FMD in the previous 6 months, although the evidence for the latter was weak. This study reports risk factors associated with clinical FMD at the farm level in a densely populated smallholder farming area of Kenya. These results can be used to inform the development of risk-based strategic plans for FMD control and as a baseline for evaluating interventions and control strategies.

Introduction

Foot-and-mouth disease (FMD) is a viral disease affecting cloven-hoofed animals. The causative pathogen, FMD virus (FMDV), belongs to the family *Picornaviridae* and genus *Aphthovirus* [1]. The disease causes major economic losses in dairy production [2]. In Kenya where the disease is endemic [3], FMD was ranked second among infectious diseases of livestock with the highest impact on pastoralist livelihoods [4].

Kenya has the largest developed smallholder dairy farming system in sub-Saharan Africa [5] and the sector contributes 70% of all milk produced in the country [6]. Nakuru County is located in the central highlands of Kenya where dairy farming is an important economic activity [7]. Clinical FMD in this area has been regularly reported through field investigations conducted during "real-time" training courses conducted every year by the European Commission for the Control of Foot-and-Mouth Disease (EuFMD).

Putative risk factors for clinical FMD among cattle have been investigated in a variety of endemic settings. Commonly reported risk factors include: the communal sharing of water or feed [8], the type of livestock production system, the number of calves aged up to 6 months present in the holding, and the presence of small ruminants [9–12]. Additional risk factors identified include: the distance of the farm to major roads [12], the frequency of cattle purchased [8], animals residing in an area with history of FMD in the last 12 months [13], and animals owned by livestock traders [13].

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An understanding of these risk factors at country level is an important component of developing a national risk-based control strategy required to progress towards stage 2 of the Progressive Control Pathway for FMD control [14]. Kenya is currently in stage 1, which involves collecting information "to gain an understanding of the epidemiology of FMD in the country and develop a risk-based approach to reduce the impact of FMD" [14]. A control strategy has been developed but has not been fully implemented and is undergoing revision in line with the devolution of veterinary authority to the County level.

FMD vaccination in Kenya is not compulsory; private farmers are entitled to have their animals vaccinated either by hiring private animal health practitioners or through subsidised government vaccination exercises, if sufficient vaccine is available. Although the County government in Nakuru utilises vaccination for FMD control, the scope is limited to a reactive "ring-based" strategy in response to confirmed outbreaks. Despite this, large-scale farms may perform routine vaccination [15]. The currently available vaccines are either monovalent or polyvalent containing a combination of strains from O, A, SAT 1 and SAT 2 serotypes and with at least a 6.0 PD_{50} (50% protective dose). A recent study has found these serotypes to be the most prevalent in Nakuru County for the period from 2010 to 2016 [16].

The Kenyan government's "Vision 2030" recognises that livestock play a very important role in the national economy [17]. In this context, control of infectious diseases of livestock (including FMD) is seen as a pathway to accelerating productivity in the sector with the potential to alleviate poverty [18]. Despite the importance of smallholder dairy farmers to the national milk output and the potential high impact of FMD on productivity, no study has focused on quantifying risk factors for clinical disease in this sector. Knowledge and practices of smallholder dairy farmers in relation to FMD is also poorly quantified. This study aimed to contribute to this knowledge gap by analysing data collected from a cross-sectional survey among smallholder dairy farms within Nakuru County, Kenya.

Materials and methods

Study area

Nakuru County is found in the mid-west area of Kenya with an elevation of approximately 1850 m above sea level, and characterised by an average rainfall of 963 mm per year. The area is home to a national park (Lake Nakuru National Park) and a forest reserve (Mau forest reserve) hosting wildlife. National statistics from the 2009 Kenya Housing and Population Census reported Nakuru County as having a total of 409 836 households [19], 439 994 cattle, 502 035 sheep, 227 037 goats, and 13 894 pigs [20].

This study was performed as part of a larger project investigating the use of milk from pooling facilities for FMDV surveillance. The study area consisted of the catchment areas of five neighbouring milk-pooling facilities located within Molo, Njoro and Rongai sub-counties of Nakuru County, Kenya (Figure 1). All catchment areas either bordered each other or overlapped so a single spatial polygon was created using Google Earth (Google Inc., USA) and exported to QGIS version 2.18.10 (QGIS Development Team, Las Palmas, USA).

Study design

A cross sectional study design was used whereby data regarding farmers' knowledge, occurrence of clinical FMD and putative risk factors were collected and analysed. This represents a cost-effective methodology for generating hypotheses that could be subsequently used as part of larger studies in the area.

The study population was small-scale dairy farmers in Nakuru County, Kenya located within the catchment area of the milk-pooling facilities. Inclusion criteria were: (i) premises with at least one but no more than fifty cattle at the time of the interview, and (ii) having the cattle located in the proximity of the household (i.e. not farmed at another premises). Farms were selected by spatial sampling using QGIS to generate random points within the defined study area. The list of geo-coordinates was uploaded onto GPS units (Garmin eTrex 10, Garmin Corp., UK) which were used to locate the points on the ground. The nearest smallholder farm to the generated random point was selected for the interview. If it did not meet the inclusion criteria, the next closest premises was approached. Coordinates indicating areas where no obvious closest smallholder farm could be identified (e.g. in the centre of a large-scale farm or woodland/plantation) were removed from the study.

Sample size calculation

The sample size was based on the estimated number of farms affected by FMD in the previous 6 months and calculated using the formula for sampling binomial outcomes [21]

$$n = Z_{\alpha}^{2} \times \frac{P(1-P)}{L^{2}}$$

where n is the required sample size, P is the expected proportion of households being affected in the previous 6 months and L is the desired precision at a Z confidence level (corresponding to $\alpha = 95\%$). The estimated prevalence was set as 15% (based on experience of one of the authors [NL] doing surveys in the area as part of EuFMD

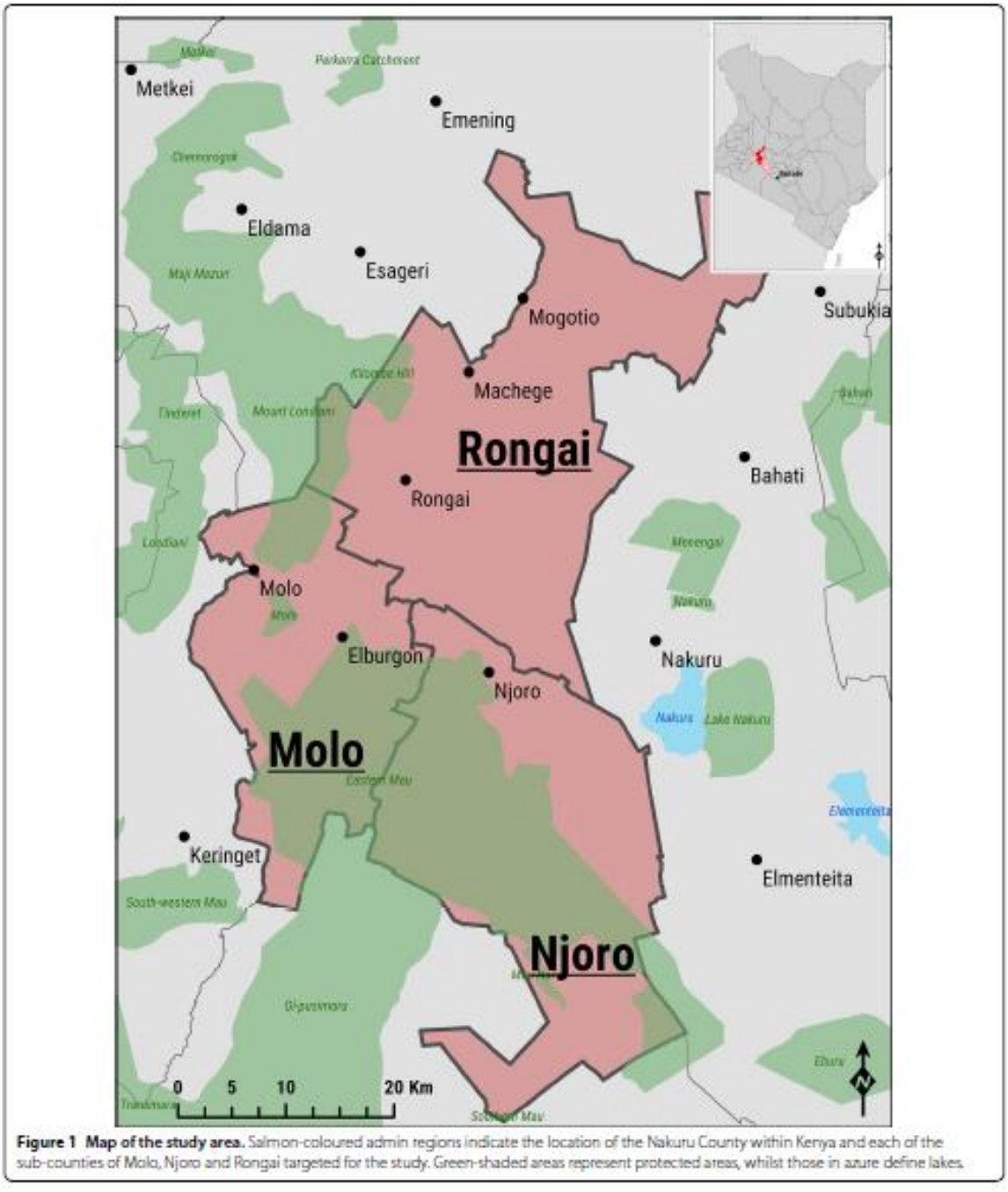

Figure 1 Map of the study area. Salmon-coloured admin regions indicate the location of the Nakuru County within Kenya and each of the sub-counties of Molo, Njoro and Rongai targeted for the study. Green-shaded areas represent protected areas, whilst those in azure define lakes.

training courses) with an absolute precision of 2.5%. This estimated prevalence was used because there was nothing to refer to in the literature or from any other records on the prevalence of clinical disease in the area. This resulted in 197 farms to be interviewed. The sample size was inflated by 20% to account for non-responsiveness

and the potential inaccessibility of some farms, giving a total of 237 GPS coordinates for the study.

Data collection

The survey was conducted between the 16th November and 1st December 2016. Data were collected using a questionnaire developed and uploaded onto the EpiCollect + mobile phone application [22]. The questionnaire included both closed and open-ended questions and was tested in the field with a limited number of smallholder farmers before implementation to make sure the questions were well understood. The main survey was conducted by five investigating teams all comprising a native Swahili speaker and paper questionnaires also available in Swahili. In every case, prior informed consent was obtained verbally from participants before interviews were conducted and after providing an overview of the aims, methodology, and anticipated outcomes of the study. Data were collected on the livestock located at the farm, farm management practices, putative risk factors for FMD, and farmers' knowledge of the disease. An electronic version of the questionnaire is available as an Additional file 1.

To assess if farmers had experienced clinical episodes of FMD in the previous 6 months, they were asked if they had encountered cases of a disease in their livestock showing any one or more of the following clinical signs: lesions in the mouth, tongue, teats, feet, at the coronary band, and interdigital space; lameness; salivation; discharges from the nose and the mouth [23]. A farm was defined as being a case if they reported to the interview team having an animal with two or more of the clinical signs of FMD listed by AU-IBAR in the previous 6 months.

Data analysis

Data collected from the field surveys were exported from EpiCollect + and imported into Stata 13.1 (Stata Corp, College station, Texas, USA) for data cleaning and analysis. Descriptive statistics were first calculated on the data. These included: proportions for categorical variables and means with their 95% confidence intervals (CI), medians with their interquartile range (IQR), or ranges for continuous variables. Cross tabulation was further used to summarise the data.

A spatial Bernoulli model was used to detect clustering of disease events, and estimating the relative risk of a case occurring in the predicted cluster, using SaTScan version 9.4.4 [24]. ArcGIS was used to draw maps of the study area (ESRI 2018. ArcGIS Desktop: Release 10.6 Redlands, CA: Environmental Systems Research Institute). Local spatial autocorrelation of reported FMD events was assessed by estimating the univariable Moran's I correlation coefficient [25].

Univariable logistic regression analysis examined the associations between putative risk factors and having clinical FMD. Variables associated on univariable analysis with a p value less than 0.2 were taken forward into a multivariable logistic regression model. Penalized likelihood ratios were used instead of maximised likelihood ratios in the logistic regression modelling to account for low number of cases [26].

Final multivariable models were constructed using a backward-stepwise approach. Variables were included in the model based on the result of a likelihood ratio test with a p-value less than 0.05. Regression diagnostics were undertaken to evaluate potential multicollinearlity between independent variables by post-estimating the variance inflation factor (VIF), with model fitness assessed using the Wald χ^2 test, Akaike's information criterion (AIC), Bayesian information criterion (BIC), and McFadden's Pseudo R^2 [27]. Linearity between the continuous independent variables and the logit of the dependent variable was assessed by adding an interaction term calculated by multiplying the continuous variable with its logarithm as described before [28] and checking for its significance. Spatial autocorrelation of reported FMD cases was accounted for by including the sub-county as an a priori fixed effect.

Results

Of the 237 GPS coordinates generated, seventeen (17/237 [7.2%]) were located either in inaccessible areas or with no discernible farm. A total of 220 small-scale dairy farmers were interviewed with an average distance between the farm and the randomly generated point of 250.8 meters (IQR 157.7–433.9). The majority of respondents were farm owners (185/220 [84.1%]) while managers and other farm workers represented the remaining 11.4% (25/220) and 4.5% (10/220), respectively. The average age of the respondents was 40.0 years (IQR 30.0–56.5).

The surveyed farmers owned a total of 1205 cattle with the mean number of cattle kept per farmer being 4.0 (IQR 2.0–6.0). On average, more female cattle were kept across all age groups. This difference was most pronounced in cattle aged above 2 years. The majority of bulls were reported being less than 6 months of age, with their number decreasing with increasing age. The age-sex distribution of the animals under study is represented in Figure 2.

A total of 132/220 (60.0%) respondents also owned sheep, whilst 33/220 (15.0%), 22/220 (10.0%) and 1/220 (0.4%) owned goats, donkeys and pigs respectively. Twenty-four out of 220 respondents (10.9%) co-farmed both sheep and goats with cattle.

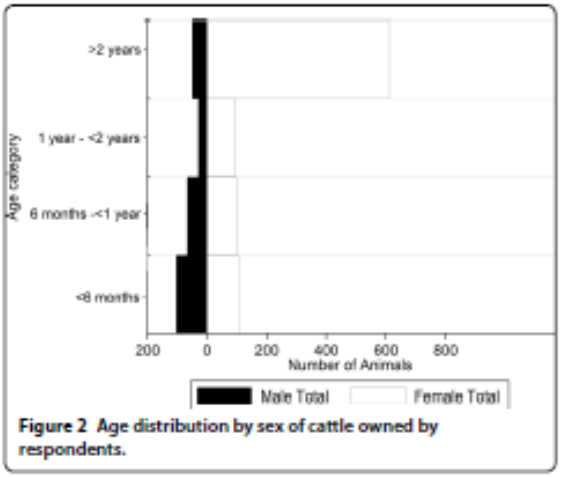

Figure 2 Age distribution by sex of cattle owned by respondents.

Knowledge on FMD

The majority of farmers (207/220 [94.1%]) knew of the existence of FMD. These farmers were asked an open question on what clinical signs were typically seen in cattle affected by FMD (Table 1). The most commonly reported clinical sign was hypersalivation (160/207 [77.3%]) followed by hoof (111/207 [53.6%]) and mouth lesions (109/207 [52.7%]). Only one farmer associated the disease with mortality in adults and no farmers reported mortality in calves. Using the case definition recommended by AU-IBAR [22], 166/207 (80.2%) of the respondents who claimed knowledge of FMD correctly identified the clinical signs. A total of 70% (154/220) mentioned more than 1 clinical sign given in the case definition with 35% (77/220), 7.7% (17/220) and 0.5% (1/220) mentioning 3, 4 and all 5 signs, respectively.

Other vesicular diseases of cattle closely resembling FMD (such as vesicular stomatitis and bovine papular stomatitis) have not been reported in Kenya. The case

Table 1 Farmer knowledge of FMD clinical signs and preventive measures, including vaccination practices, in the study area located within the Nakuru County, Kenya

Knowledge on clinical signs of FMD		Preventive measures for FMD[a]		FMD vaccination practices	
Clinical sign	Response/total[a] (%)	Preventive measure	Response/total (%)	Vaccination practice	Response/total (%)
Hypersalivation	160/207 (77.3)	Vaccination	94/207 (45.4)	Vaccinated ≤ 4 months ago	35/220 (15.9)[b]
Hoof lesions	111/207 (53.6)	Keep cattle within farm compound	76/207 (36.7)	Vaccinated 5–6 months ago	10/220 (4.5)[b]
Mouth lesions	109/207 (52.7)	Avoid other cattle from entering farm compound	15/207 (7.2)	Vaccinated 6–12 months ago	45/220 (20.5)[b]
Lameness	81/207 (39.1)	Keep cattle away from farm compound boundaries	14/207 (6.8)	Vaccinated > 1 year ago	51/220 (31.7)[b]
Lack of appetite	64/207 (30.9)	Do not bring in new cattle	9/207 (4.3)	No vaccination date reported	2/220 (0.9)[b]
Depression	33/207 (15.9)	Avoid use of communal dips	5/207 (2.4)	Vaccinated all cattle	131/143 (91.6)[c]
Drop in milk production	15/207 (7.2)	Do not share equipment with surrounding farms	5/207 (2.4)	Young calves not vaccinated	6/143 (4.2)[c]
Lesions on teats	8/207 (3.9)	Keep visitors away from cattle	4/207 (1.9)	Pregnant Cattle not vaccinated	5/143 (3.5)[c]
Mortality in adult cattle	1/207 (0.5)	Do not do any preventive measure	58/207 (28.0)	Private AHP vaccinates cattle	24/143 (16.8)[c]
				Government AHP vaccinates cattle	118/143 (82.5)[c]
				Non-AHP vaccinates cattle	1/143 (0.7)[c]
				Cattle vaccinated at communal point	84/143 (58.7)[c]
				Cattle vaccinated at farm compound	59/143 (41.3)[c]

AHP, Animal Health Provider (veterinarian or para-veterinarian).

[a] Denominator is all farmers that had heard of FMD.

[b] Denominator is all farms that were surveyed.

[c] Denominator is all farms that had ever vaccinated.

definition also excluded other similar diseases occurring in the area, including Malignant Catarrhal Fever, Mucosal Disease and Bluetongue Disease.

Reported control measures for FMD prevention

The 207 farmers who were aware of FMD were asked about preventive measures undertaken to reduce the risk of disease (Table 1). The most commonly reported preventive measure was vaccination (94/207 [45.4%]) followed by keeping cattle within the farm compound (76/207 [36.7%]). Keeping visitors away from areas on their farm compound where they might come into contact with their cattle was also reported by 4/207 farmers (1.9%). Fifty-eight respondents (28.0%) did not report any preventive measure to reduce the risk of FMD occurring on their farm.

A total of 143/207 respondents (69.1%) reported using FMD vaccine on their cattle at least once since they started farming. Farmers who reported vaccinating their animals in the previous 4 and 6 months were 35/207 (16.9%) and 45/207 (21.7%), respectively.

FMD 6-month occurrence

Of the total 220 smallholder farmers, 13 (5.9% [95% CI 2.8–9.0]) reported having a case of FMD in at least one animal on their farm in the previous 6 months, all correctly identifying the disease according to the AU-IBAR case definition. Of these, 84.6% (11/13) mentioned more than two clinical signs given in the case definition. A total of 53.8% (7/13), 23.1% (3/13) and 7.7% (1/13) mentioned 3, 4 and all 5 clinical signs in the case definition, respectively. When the number of clinical signs reported by respondents was modelled against whether a farm reported a case of FMD, the probability of reporting a clear case of FMD increased by 1.9 ($p=0.027$) when respondents provided an additional clinical feature of the disease.

Of these farms, 60 individual cases of FMD were reported representing an individual level incidence risk of 5.0% (95% CI 3.9–6.4) based on the 1205 cattle owned by surveyed farms at the time of the survey. Based on the estimated numbers of cattle present at the time of the outbreak (mean number per affected farm of 8.2, 95% CI 2.9–13.6) and the numbers affected with FMD (mean number of cases per affected farm of 4.6, 95% CI 0.8–8.5), the mean within-farm incidence risk was 58.0% (95% CI 38.3–77.6).

Spatial clustering of farms reporting FMD cases in the previous 6 months was identified in the northern part of the study area (Northern part of Rongai), observing a significant non-zero positive spatial autocorrelation between cases (Moran's $I=0.508$; $z=7.084$; $p\leq0.001$) (Figure 3). This single disease cluster (with an estimated radius of 5.32 km) was 38.1 times more likely than any other part of the study area to experience FMD ($p<0.001$), with 40.7% of the clinical cases reported within the geographical extent of this cluster.

Retrospective history of FMD occurrence within the surveyed area

Farmers were asked when they last heard of an FMD outbreak in the local area. Of the 220 respondents, 25/220 (11.4%) claimed never to have heard of an FMD case in their local area. For farmers that had heard of FMD in the local area, 34/220 (15.5%) stated FMD was reported in the previous 6 months, 29/220 (13.2%) between 6 months and 1 year, 33/220 (15.0%) between 1 and 2 years, and 45/220 (20.5%) more than 2 years ago. A total of 54/220 (24.6%) farmers did not respond to the question although the reasons for non-response were not included.

Reports of wildlife in the area

The majority of farmers (130/220 [59.0%]) had not seen or heard of reports of FMD susceptible wildlife in the surrounding areas and outside of the parks. A total of 21 out of 220 (9.5%) heard of reports of antelopes, 1/220 (0.5%) of gazelles and 1/220 (0.5%) of wild pigs. Other wildlife not naturally susceptible to FMD reported by these respondents included aardvarks, cheetahs, hyena, hares, leopards, monkeys, porcupines, squirrels, and wild dogs.

Farm level risk factors for FMD occurrence

Putative risk factors for FMD among smallholder farmers in the study area are shown in Table 1. Most farmers used artificial insemination (AI) (115/220 [52.3%]) to breed their cattle. This consisted of 96/220 (43.6%) who used AI solely, 2/220 (0.9%) who used AI together with their own bull, and 17/220 (7.7%) who used AI together with a shared bull. A total of 67/220 (30.5%) reported using a shared bull, subdivided further into 46/220 (20.9%) who used a shared bull only and 4/220 (1.8%) using both shared and their own bull.

A total of 89/220 (40.5%) had acquired new cattle in the previous 12 months. Of these 89 respondents, 54 (60.7%) had acquired only one replacement animal in the previous year, with the rest acquiring two or more. For sourcing new cattle, 96/220 (43.6%) used surrounding farms while 43/220 (19.6%) used livestock markets.

Less than half of farmers (97/220 [44.1%]) used communal grazing either as the sole source of pasture (34/220 [15.5%]) or in addition to that available within the farm compound (63/220 [28.6%]). For the 97 respondents that used communal grazing, roadsides were the most commonly used (68/97 [70.1%]), while 58/97 (59.8%) used other non-questionnaire listed communal places (e.g.

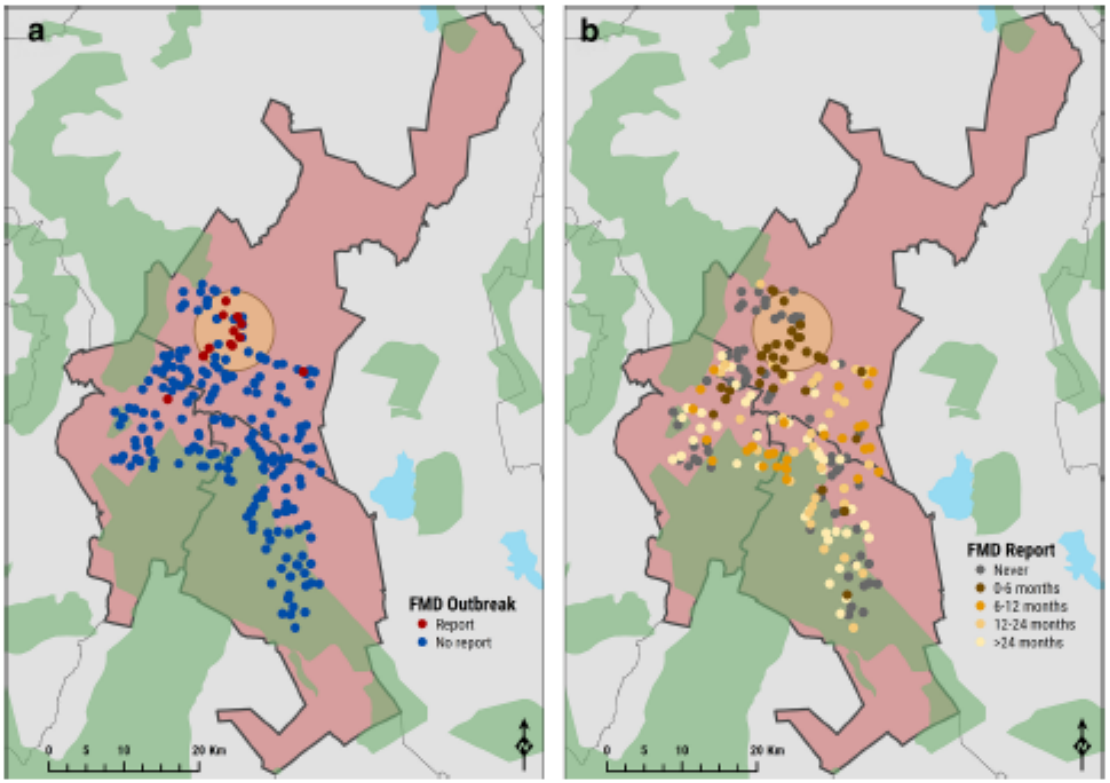

Figure 3 Map of occurrence of FMD cases. Farmer reported FMD outbreak locations within the study area are represented in red in **A**, whilst the distribution of farmer reported FMD occurrence reported during the survey is reported in **B**. The geographical cluster of cases is also shown (light orange colour).

harvested fields), 10/97 (10.3%) forests and 9/97 (9.3%) used fields within towns. Communal watering points for cattle were used by 64/220 (29.1%) of the farmers interviewed, whilst communal acaricide dips were used by 14/220 (6.4%).

Several factors were associated with an increased risk of clinical FMD in the previous 6 months based on univariable analysis, including: use of a shared bull; the number of additional cattle sourced from outside the farm in the previous 12 months; buying cattle from livestock markets; grazing sheep both within towns and the farm compound; grazing cattle within towns; use of a communal dip; and the number of sheep present on farm. The odds of disease being reported was significantly lower in farms that had used vaccination at some point in their past (OR = 0.2, 95% CI 0.07–0.7; $p = 0.013$). The results of the univariable analysis for all putative risk factors are shown in Additional file 2.

The final multivariable model contained an a priori term (subcounty) to correct for spatial autocorrelation (Table 2). Based on this final multivariable model, the use of a shared bull was significantly associated with the FMD status of a farm (OR = 9.7, 95% CI 1.6–59.1; $p = 0.014$) when compared to those not using this breeding method. A number of farmers (49/220) did not respond to the question on breeding method. These were included as a separate category and there was no evidence of an association with reporting FMD (OR = 3.4 (95% CI 0.4–25.1; $p = 0.238$). Due to collinearity with the variable representing the use of a shared bull, the use of AI was dropped as a separate variable in the model. The odds of FMD increased 1.1 times for each additional sheep owned (95% CI 1.0–1.2; $p = 0.066$). The interaction term for the number of sheep and its logarithm in the final multivariable model was not significant ($p = 0.251$) indicating that linearity with the logit of the dependent variable was not violated.

Table 2 Odds ratios from logistic regression indicating associations between exposure variables and the odds of having FMD in the previous 6 months

Univariable analysis				Multivariable analysis	
Variable	Type of variable	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Use of a shared bull	Categorical				
Did not use a shared bull		Base category		Base category	
Used a shared bull		12.7 (0.4–396.4)	0.147	9.7 (1.6–59.1)	0.014
Did not respond to the question		15.4 (2.1–112.5)	0.007	3.4 (0.4–25.1)	0.238
Number of additional cattle sourced from outside the farm in the previous 12 months	Continuous	1.2 (1.0–1.5)	0.043	1.1 (1.0–1.3)	0.207
Buying cattle from livestock markets in the previous 12 months	Categorical	3.9 (1.3–12.4)	0.019		
Grazing sheep within towns	Categorical	34.5 (3.5–337.8)	0.002		
Grazing cattle within towns	Categorical	8.3 (1.6–43.4)	0.012		
Use of communal dips	Categorical	8.6 (2.3–32.8)	0.002		
Number of sheep	Continuous	1.1 (1.0–1.2)	0.025	1.1 (1.0–1.2)	0.066
Ever vaccinated cattle for FMD	Categorical	0.2 (0.07–0.7)	0.013		
Subcounty	Categorical				
Njoro		Base category		Base category	
Molo		5.3 (0.2–133.5)	0.307	4.5 (0.2–113.6)	0.365
Rongai		37.4 (2.2–643.2)	0.013	30.0 (1.7–528.9)	0.020

From the univariable analysis, only variables with a p-value <0.2 are included (a list of all examined variables is reported in Additional file 1) which were taken forward into the final multivariable model using a backward-stepwise approach. Subcounty of the interviewed farm was included a priori as a fixed effect to account for potential spatial autocorrelation. Variables were retained in the final model if the likelihood ratio test had a p-value less than 0.2. The final multivariable model had a Wald Chi square of 20.0 with 6 degrees of freedom giving a p value of 0.0027. The model had an AIC of 64.5, a BIC of 95.0 and a McFadden's R^2 of 0.443.

Discussion

Foot-and-mouth disease has major economic implications to dairy farming systems in Kenya and other developing countries within the African continent [2]. Despite various studies ranking FMD among the most important animal diseases among cattle keepers in Kenya [4, 29–32], no other study has aimed to determine the knowledge, attitudes and practices towards it among small-holder dairy farmers communities.

The number of cattle kept on the farms surveyed in this study were similar to other smallholder studies from the region [33–35] and, in addition to keeping cattle, the small-scale farmers interviewed also kept sheep, goats and pigs which are susceptible to FMD. This diversification of livestock was also reported by Njarui et al. [34] in a study conducted in the highland counties of Kenya. The same finding was also reported by Kosgey et al. [36] in an earlier study in Nakuru, Nandi and Nyeri Counties of Kenya. In Kenya and other African countries small stock are kept as a quick source of liquidity in the face of family needs such as school fees and payment of dowry [37]. The age-sex distribution in the study population was consistent with the dominance of dairy production systems in this region, with high numbers of adult females and a gradual decrease in numbers of males with increasing age indicating likely retention for breeding purposes.

By using random spatial sampling, it was possible that farms in high density areas might have had a lower probability of being selected than those in low density areas. The authors accept that this bias potentially exists although in the absence of a sampling frame and with a population census several years out of date, this was considered the optimal approach with the resources available. It was assumed that small scale dairy farmers were evenly distributed in the study area. However, the geographical extent of the study area was not large and the author's knowledge of the study area would suggest that this is a reasonable assumption and the potential for bias was limited.

The survey results revealed that the majority of farmers in the study population had knowledge of FMD. The most commonly reported clinical sign was hypersalivation, followed by hoof and mouth lesions. Based on the FMD case definition recommended by the AU-IBAR [23], the majority of respondents correctly identified the disease providing some internal validity to the study results. Only one respondent reported observing mortality due to FMD, which was in adult cattle. No farmer reported mortality among calves. This is consistent with a SAT 2 outbreak on a large-scale farm in the study area in 2012 that reported a mortality rate of 0.44%, reflecting a single adult death related to FMD [15]. FMD is often associated with deaths among young stock from myocardial

infection [38]. In neighbouring Ethiopia, a study reported the highest mortality among cattle less than 2 years of age at 2.8% [39]. Reasons for the low mortality in the present study may be attributable to farmers associating sudden deaths with other diseases, different pathogenicity of the circulating strains, or the presence of maternal immunity associated with previous exposure and vaccination.

Vaccination was the most frequently reported preventive measure against FMD, followed by restricting contact with other cattle by keeping them within the farm compound. Nevertheless, nearly a third of respondents reported doing nothing to prevent FMD from occurring in their livestock, although it is unclear if this is due to a lack of knowledge, a perceived low risk of disease, or difficulties achieving recommended preventive measures. Follow-up studies are required to explore this observation and may indicate a requirement for public awareness and education programmes on FMD prevention among farmers in this region.

The only FMD vaccine available at the time of the study was an aqueous-adjuvanted, inactivated vaccine with a recommended vaccination interval of 6 months [40]. Despite vaccination being the most reported preventive measure (45%), the estimated vaccination coverage for the last 6 months was lower (21.7%). This may indicate either a lack of knowledge over the necessary vaccination schedules or poor vaccine availability. However, the percentage of farms that had ever vaccinated was markedly higher (69.1%) than those stating vaccination was used to prevent FMD. This disparity may indicate that some farmers were unaware of the purpose of vaccination. Quantifying and deploying effective vaccination coverage at a population level is an essential component of any FMD control programme in an endemic setting. Uncertainty in vaccination coverage estimates could be addressed through improved record keeping including the use of vaccination record cards as recommended in the FAO-OIE Post Vaccination Monitoring Guidelines [41]. Some farmers reported not vaccinating young and pregnant cattle. Young calves are often not included in vaccination campaigns due to the presence of maternal antibodies that can interfere with the immune response. There may also be a perception that the impact of disease is lower among this group leading to reluctance to pay for vaccination. The lower vaccination among pregnant cattle may be due to an association with pre-term calving or abortion. Further studies and subsequent public awareness programs would be useful in educating farmers on recommended vaccination practices.

Spatial clustering of FMD affected farms was identified within the Rongai sub-county, which may indicate a geographical structure of FMD circulation. Identifying clinical disease clusters is useful for informing a risk-based control strategy by targeting control measures to these areas. The clustering observed in this study is likely to be attributable to a transit route for pastoralists in this area [39]. Although pastoralist routes within the study area have not been mapped, many farmers suggested that the occurrence of FMD coincided with the arrival of Maasai pastoralists to utilise available grazing.

The proportion of farmers that reported having heard of FMD in the study area 6 months prior to the survey was 15.5% compared to 5.9% that reported having disease. This means that more farmers had heard of outbreaks in their area than those that actually experienced a case in their farm, perhaps because they had not received information on an outbreak being reported in the area. Although data were not collected on how disease information was conveyed, this finding suggests that communication of outbreaks could be improved so that farmers could initiate preventative measures. This could be achieved through public awareness campaigns, mobile phone messaging or social media. The prevalence estimate in the present study was less than the expected prevalence used for sampling size calculation, and the results from a previous serological survey of the area [3]. The expected prevalence was based on a limited number of respondents usually interviewed during EuFMD training activities in the county, thus potentially not providing enough power and also bias as these studies were performed in areas of known FMD virus circulation. It was indeed lower than that estimated by serology and this difference can be explained by the fact that seroprevalence reports levels of lifetime exposure to the virus. In addition, the present study investigated the presence of clinical disease which may not correlate with seropositivity.

The present study used clinical signs for the case definition with no laboratory confirmation. There are limitations to this approach although there was some validation through comparing reported clinical signs to the AU-IBAR case definition [23]. However, because of the imperfectness of our case definition methodology (specificity and sensitivity is unlikely to be close to 1), both the FMD occurrence and FMD odds here reported are likely to be biased estimators of the true FMD status in the area 6 months prior to the study [42]. In a cross sectional study in Cambodia, Bellet et al. [43] compared participatory epidemiology tools (including farmer description of clinical signs of FMD) with serological tests. The authors found participatory methods as characterised by high sensitivity and low specificity in the identification of FMD cases. To overcome this, serosurveys could be useful. However, serosurveys used to estimate the burden of infection can be time-consuming and expensive. Moreover it is difficult to estimate the timing of infection as antibody levels can persist for years post-infection [44]

and previous vaccination complicates interpretation particularly if not using vaccines that have been specifically purified of non-structural proteins [13]. Surveys for clinical disease offer a low-cost alternative that is likely to be more achievable in resource poor settings, although these do not replace the need for serosurveys in understanding the epidemiology of FMD.

The majority of farmers used AI for breeding their livestock (52.3%). This is higher than that reported by Baltenweck et al. [45] who found only 18.6% of the smallholder dairy farmers in Kirinyaga, Nakuru and Kisumu Counties in Kenya using this breeding method. This high figure may be due to an increased accessibility to AI services. Temporal changes in management practices may be related to a dynamic risk of FMD exposure and affect the impact of risk-based control measures.

Using communal resources for grazing and water was commonly reported in this study and consistent with other studies in Kenya [39, 40]. Farmers often resort to communal sources during the dry seasons when grazing and water are scarce, increasing the potential for transmission of infectious pathogens like FMDV. Despite many farms using communal grazing and water, neither was associated with the occurrence of FMD in this study. This may be related to the timing of the study (November, with the main dry season running from January through to March) since farmers were asked if clinical FMD had occurred in the previous 6 months. This may indicate that using communal resources are relatively lower risk outside this dry season although further studies are required to investigate this hypothesis. Communal acaricide dips are another potential cause of livestock contact and are used all year around. Despite their use being associated with clinical FMD on univariable analysis, this variable was dropped from the multivariable model. Relatively few farmers (6.4%) used communal dips for tick control so the study is likely to be underpowered to show an association if present.

Contact with FMDV susceptible wild animals is a potential risk factor for disease [46]. Farmers reported the presence of antelopes and wild pigs in the surrounding areas although the presence of wildlife was not a significant risk factor in this study. This result is not surprising since the majority of small scale farmers in Kenya do not graze animals in protected areas where they might interact with wildlife [47]. In addition, Lake Nakuru National Park is fenced so likely reducing the probability of contact [48], confirmed by the minimal sightings of wildlife in the study area.

Several risk factors for FMD were identified by univariable analyses at the farm level while only vaccination was associated with a lower risk of disease. This is in agreement with studies conducted elsewhere on similar and differing settings [10–12, 49]. Vaccination was not associated with a reduced disease risk in the multivariable model so it is likely that there was confounding with the univariable association. However, this study was not designed to evaluate vaccine effectiveness, therefore no reliable assessment of vaccine performance can be made.

Multivariable analysis indicated that the use of a shared bull was related to FMD occurrence on the farm in the previous 6 months. Shared bulls present a high risk for moving between farms and having contact with potentially infected animals. Forty-nine (49/220, 22.3%) farmers did not report any breeding method. There was no evidence that these farmers were at greater or lesser risk of having reported clinical disease in the multivariable model. It is possible that the reason for these farmers not reporting a breeding method was that they did not breed their cattle, although this information was not recorded. For every sheep owned by a farm the odds of introducing FMD increased by 10%. This finding agrees with Mergesa et al. [9] who identified co-farming cattle with small ruminants as a risk factor for FMD in pastoralist systems in Ethiopia, although they did not investigate the effect of the number of small ruminants. In a study by Anderson et al. [50] on the role of sheep and goats in FMD epidemiology in Kenya, a high seropositivity level was reported thus indicating likely exposure in small ruminants. Observations from the study area indicated that mixed cattle-small ruminant farms were often managed differently to farms that only kept cattle. This may include factors that increase the risk of exposure to FMD virus in small ruminants (e.g. communal grazing over wider areas and for longer periods), which could be transmitted to cattle where disease is more apparent. Small ruminants are commonly excluded from vaccination strategies (including Kenya) though their inclusion could be beneficial by reducing interspecies transmission. Although challenge studies have indicated a limited role for sheep in FMD transmission to cattle [51], further evidence derived from field conditions are required to support their inclusion in vaccination strategies.

In conclusion, FMD is regularly reported among smallholder dairy farmers in Nakuru County, Kenya, which in this study affected 1 in 17 farms over a six-month period. Farmers had knowledge of FMD and the associated clinical signs, but the disease control by vaccination and its coverage reported in this area was low. There is a need to educate farmers on the risk of FMD and associated control measures including vaccination, enhancing their access. Improved understanding of FMD epidemiology can help identify risk-based control measures that can be implemented to reduce disease impact. Use of shared bulls and co-farming sheep with cattle were identified as risk factors for disease in this study. Although

semi-structured questionnaire-based surveys have limitations, the current study shows that useful information on the burden of disease can be easily extracted from rural farming communities in low resource settings.

Additional files

Additional file 1. Questionnaire for dairy farmers. This file contains the paper form of the questionnaire tha was used in the data collection for the study.

Additional file 2. Table showing the results of univariable analysis of all putative risk factors against each investigated variable. This file contains the results of univariable logistic regression carried out on the relevant study variables against an outcome of whether or not a farm experienced a case of FMD.

Abbreviations

AIC: akaike information criterion; AI: artificial insemination; AU-IBAR: African Union Intergovernmental Bureau of Animal Resources; BIC: Bayesian Information Criterion; EUFMD: European Union Commission for the control of Foot and Mouth Disease; FMD: foot and mouth disease; FMDV: foot and mouth disease virus; PCP: progressive control pathway; PD_{50}: protective dose 50; SAT 1: Southern African Territories 1; SAT 2: Southern African Territories 2; VIF: variance inflation factor.

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Authors' contributions

All authors have directly participated in the planning, execution & analysis of this study. DM participated in data collection, analysis and writing up of the final manuscript. ADN, BA and BS-B participated in data collection and editing of the manuscript. PMK assisted in analysis of results and editing of the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The dataset supporting the conclusions of this article is available in the Harvard Dataserve repository: https://doi.org/10.7910/DVN/KQMKPZ.

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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References

1. Domingo E, Baranowski E, Escarmis C, Sobrino F (2002) Foot-and-mouth disease virus. Comp Immunol Microbiol Infect Dis 25:297–308
2. Knight-Jones TJD, Rushton J (2013) The economic impacts of foot and mouth disease—what are they, how big are they and where do they occur? Prev Vet Med 112:161–173
3. Kibore B, Gitao CG, Sangula A, Kitala P (2013) Foot and mouth disease sero-prevalence in cattle in Kenya. J Vet Med Anim Heal 5:262–268
4. Onono JO, Wieland B, Rushton J (2013) Constraints to cattle production in a semiarid pastoral system in Kenya. Trop Anim Health Prod 45:1415–1422
5. Omore AO, Muriuki H, Kenyanjui M, Owango M, Staal S (1999) The Kenya dairy sub-sector: a rapid appraisal. ILRI, Nairobi
6. Government of Kenya (2008) National Livestock Policy—Session Paper No. 2. Nairobi
7. Foeken DWJ, Owuor SO (2008) Farming as a livelihood source for the urban poor of Nakuru, Kenya. Geoforum 39:1978–1990
8. Cleland PC, Baldock FC, Chamnanpood P, Gleeson LJ (1996) Village level risk factors for foot-and-mouth disease in Northern Thailand. Prev Vet Med 26:253–261
9. Megersa B, Beyene B, Abunna F, Regassa A, Amenu K, Rufael T (2009) Risk factors for foot and mouth disease seroprevalence in indigenous cattle in Southern Ethiopia: the effect of production system. Trop Anim Health Prod 41:891–898
10. Elnekave E, Zamir L, Hamd F, Even Tov B, Klement E (2015) Risk factors for foot and mouth disease outbreaks in grazing beef cattle herds. Prev Vet Med 120:236–240
11. Bronsvoort BMC, Nfon C, Hamman SM, Tanya VN, Kitching RP, Morgan KL (2004) Risk factors for herdsman-reported foot-and-mouth disease in the Adamawa Province of Cameroon. Prev Vet Med 66:127–139
12. Hamoonga R, Stevenson MA, Allepuz A, Carpenter TE, Sinkala Y (2014) Risk factors for foot-and-mouth disease in Zambia, 1981–2012. Prev Vet Med 114:64–71
13. Emami J, Rasouli N, McLaws M, Bartels CJM (2015) Risk factors for infection with Foot-and-Mouth Disease virus in a cattle population vaccinated with a non-purified vaccine in Iran. Prev Vet Med 119:114–122
14. FAO (2012) The progressive control pathway for FMD control (PCP-FMD): Principles, Stage Descriptions and Standards, Rome
15. Lyons NA, Stark KDC, van Maanen C, Thomas SL, Chepkwony EC, Sangula AK, Dulu TD, Fine PEM (2015) Epidemiological analysis of an outbreak of foot-and-mouth disease (serotype SAT2) on a large dairy farm in Kenya using regular vaccination. Acta Trop 143:103–111
16. Machira DN, Kitala P (2017) Epidemiological analysis of passive surveillance data on foot and mouth disease occurrence in Nakuru County, Kenya. J Dairy Vet Anim Res 6:298–300
17. Kenya National Planning Commission (2013) National development plan vision 2030. Nairobi
18. Perry BD, Rich KM (2007) Poverty impacts of foot-and-mouth disease and the poverty reduction implications of its control. Vet Rec 160:238–241
19. Kenya National Bureau of Statistics (2013) County Statistics. https://www.knbs.or.ke/publications. Accessed 30 May 2018

20. Kenya National Bureau of Statistics (2009) Kenya—2009 Kenya Population and Housing Census (10 Per Cent sample, every 10th household). Population and Housing Census, Nairobi
21. Dohoo IR, Martin SW, Stryhn H (2009) Veterinary Epidemiologic Research. University of Prince Edward Island, Charlottetown, P.E.I
22. Aanensen DM, Huntley DM, Feil EJ, Al-Own F, Spratt BG (2009) EpiCollect: linking smartphones to web applications for epidemiology, ecology and community data collection. PLoS ONE 4:e6968
23. AU-IBAR (2014) Standard Methods and Procedures (SMPs) for Control of Foot and Mouth Disease in the Greater Horn of Africa. African Union—Inter-African Bureau for Animal Resources, Nairobi
24. Kulldorff M (1997) A spatial scan statistic. Commun Stat Theory Methods 26:1481–1496
25. Anselin L (2018) Local indicators of spatial association—LISA. Geogr Anal 27:93–115
26. Firth D (1993) Bias reduction of maximum likelihood estimates. Biometrika 80:27–38
27. McFadden D (1974) Conditional logit analysis of qualitative choice behavior. In: Zarembka P (ed) Frontiers in econometrics. Academic Press, New York, pp 105–142
28. Box GEP, Tidwell PW (1962) Transformation of the independent variables. Technometrics 4:531–550
29. Jost CC, Mariner JC, Roeder PL, Sawitri E, Macgregor-Skinner GJ (2007) Participatory epidemiology in disease surveillance and research. Rev Sci Tech Int Des Epizoot 26:537
30. Jost CC, Nzietchueng S, Kihu S, Bett B, Njogu G, Swai ES, Mariner JC (2010) Epidemiological assessment of the Rift Valley fever outbreak in Kenya and Tanzania in 2006 and 2007. Am J Trop Med Hyg 83:65–72
31. Bedelian C, Nkedianye D, Herrero M (2007) Maasai perception of the impact and incidence of malignant catarrhal fever (MCF) in southern Kenya. Prev Vet Med 78:296–316
32. Mwacharo JM, Drucker AG (2005) Production objectives and management strategies of livestock keepers in South-East Kenya: implications for a breeding programme. Trop Anim Health Prod 37:635–652
33. Bebe BO, Udo HMJ, Rowlands GJ, Thorpe W (2003) Smallholder dairy systems in the Kenya highlands: cattle population dynamics under increasing intensification. Livest Prod Sci 82:211–221
34. Njarui DMG, Gichangi EM, Gatheru M, Nyambati EM, Ondiko CN, Njunie MN, Ndungu-Magiroi KW, Kitya WW, Kute CAO, Ayako W (2016) A comparative analysis of livestock farming in smallholder mixed crop-livestock systems in Kenya: 1. Livestock inventory and management. Livest Res Rural Dev 28:66
35. Phiri BJ, Benschop J, French NP (2010) Systematic review of causes and factors associated with morbidity and mortality on smallholder dairy farms in eastern and southern Africa. Prev Vet Med 94:1–8
36. Kosgey IS, Rowlands GJ, Van Arendonk JAM, Baker RL (2008) Small ruminant production in smallholder and pastoral/extensive farming systems in Kenya. Small Rumin Res 77:11–24
37. Arasio RL (2003) Participatory assesment and analysis of livestock markets, off take and marketing constraints in Loima division, Turkana district. University of Nairobi, Kenya
38. Kitching RP (2002) Clinical variation in foot and mouth disease: cattle. Rev Sci Tech 21:499–504
39. Rufael T, Catley A, Bogale A, Sahle M, Shiferaw Y (2008) Foot and mouth disease in the Borana pastoral system, southern Ethiopia and implications for livelihoods and international trade. Trop Anim Health Prod 40:29–38
40. KEVEVAPI (2018) Kenya Veterinary Vaccine Production Institute—FOOTIVAX Vaccine. https://kevevapi.org/index.php/products/item/15-footivax-tm. Accessed 3 Apr 2018
41. Ferrari G, Paton D, Duffy S, Bartels C, Knight-Jones T (2016) Foot and mouth disease vaccination and post-vaccination monitoring Guidelines. The Food and Agriculture Organization of the United Nations and the World Organisation for Animal Health, Rome
42. Diggle P (2011) Estimating prevalence using an imperfect test. Epidemiol Res Int 2011:1–5
43. Bellet C, Vergne T, Grosbois V, Holl D, Roger F, Goutard F (2012) Evaluating the efficiency of participatory epidemiology to estimate the incidence and impacts of foot-and-mouth disease among livestock owners in Cambodia. Acta Trop 123:31–38
44. Paton DJ, Fussel A-E, Vosloo W, Dekker A, De Clercq K (2014) The use of serosurveys following emergency vaccination, to recover the status of "foot-and-mouth disease free where vaccination is not practised". Vaccine 32:7050–7056
45. Baltenweck I, Ouma R, Anunda F, Okeyo AM, Romney D (2004) Artificial or natural insemination: the demand for breeding services by smallholders. In: Ninth KARI (Kenya Agricultural Research Institute) annual scientific conference and agricultural research forum. ILRI, Nairobi
46. Miguel E, Grosbois V, Caron A, Boulinier T, Fritz H, Cornélis D, Foggin C, Makaya Pious V, Tshabalala Priscilla T, de Garine-Wichatitsky M (2013) Contacts and foot and mouth disease transmission from wild to domestic bovines in Africa. Ecosphere 4:art51
47. Bebe B (2003) Herd dynamics of smallholder dairy in the Kenya highlands. Wageningen Universiteit, Wageningen
48. Kenya Wildlife Service (2009) Kenya wildlife service annual report
49. Hassig M, Meier AB, Braun U, Urech Hassig B, Schmidt R, Lewis F (2015) Cattle movement as a risk factor for epidemics. Schweiz Arch Tierheilkd 157:441–448
50. Anderson EC, Doughty WJ, Anderson J (1976) The role of sheep and goats in the epizootiology of foot-and-mouth disease in Kenya. J Hyg (Lond) 76:395–402
51. Bravo de Rueda C, de Jong MCM, Eblé PL, Dekker A (2014) Estimation of the transmission of foot-and-mouth disease virus from infected sheep to cattle. Vet Res 45:58

Publisher's Note

Figure 8.2 Description of the study area and Survey 1 (Chapter 5) are described in the above research article, published in *Veterinary Research.*

Farm ID: _____ **Date: ____/____/_____**

Interviewer: _____

Questionnaire: FQv1.2

Use of milk for foot-and-mouth disease surveillance: Field Validation in Endemic Settings

Follow-up questionnaire for interim surveys

1. Position at the farm: (please circle the correct choice)
    a. Owner
    b. Employee
    c. Milker
    d. Herdsman
    e. Manager
    f. Family member of main owner
    g. Other _____
2. GPS coordinates: longitude_____ latitude_____
3. How many years have the owners been in cattle farming? _____years
4. How many cattle are currently on the farm? _____

    Please complete the following grid to give the sex and age categories for cattle on the farm

Age of cow	Male	Female
< 6 months		
6-12 months		
1-2 years		
>2 years		

5. What other species are found in your farm (state number):
    a. Goats _____
    b. Sheep_____
    c. Pigs_____
    d. Donkeys_____

Farm ID: _____ **Date: ____/____/_____**

Interviewer: _____

Risk factors

6. What grazing methods do you use for your animals?
    a. Zero grazing
    b. Within farm grazing
    c. Outside farm grazing
    d. Don't know
7. For within farm grazing, do animals graze close to the boundary of the farm?
    a. Yes (Always)
    b. Yes (Sometimes)
    c. Yes (Rarely)
    d. Never
    e. Don't know
8. For grazing outside the farm, do you use communal grazing fields to feed your animals?
    a. Yes (Always)
    b. Yes (Sometimes)
    c. Yes (Rarely)
    d. Never
    e. Don't know

Please complete table showing how grazing varies for each species:

Species	**Grazing method** **1 = Zero; 2 = within farm grazing;** **3 = outside farm grazing**	**Communal grazing?** **Y=Yes; N=No**
Cattle		
Sheep		
Goat		
Pigs		

9. How is water provided for the animals?
    a. Stored rain water
    b. Piped water on farm
    c. Bore-hole on farm
    d. Private access to river
    e. Communal access to river
    f. Access to other communal access point (e.g. dam)
    g. Other (please state) _____

Farm ID: _____ **Date: ____/____/_____**

Interviewer: _____

10. Which of the following communal grazing areas are used:
    a. Forests_____
    b. By roadsides_____
    c. Fields post-harvest _____
    d. Other communal place _____
11. Do you employ any workers on the farm?
    a. Yes
    b. No
    c. Don't know
12. Do you share workers these worker(s) with other farms?
    a. Yes
    b. No
    c. Don't know
13. Do the worker(s) live on the farm?
    a. Yes
    b. No
    c. Don't know
14. Do the workers own any cattle/sheep/goats/pigs on other farms?
    a. Yes
    b. No
    c. Don't know
15. Do you share equipment with surrounding farms?
    a. Yes
    b. No
    c. Don't Know
16. How do you get replacement animals?
    a. Buying from markets
    b. Buying from other farms
    c. From my own animals
    d. Other (please state)
17. How do you breed your cattle?
    a. AI
    b. Own bull
    c. Shared bull
18. Do you use a dip or spray for tick control?
    a. Dip
    b. Spray
    c. Both
    d. Other (please state) _____
    e. Don't know

Farm ID: _____ **Date: ____/____/_____**

Interviewer: _____

19. How frequently do you apply tick control? _____

Disease

20. Have you had any sick animals in the last four months?
    a. Yes
    b. No (Go to question 38)
    c. Don't know (Go to question 38)
21. What species have you seen illness in?
    a. Cattle (see question 21)
    b. Sheep (see question 26)
    c. Goats (see question 30)
    d. Pigs (see question 34)
    e. Other (please state) _____
22. What disease(s) have you seen in cattle? Ask as an open question. State how many months ago.
    a. FMD _____months
    b. LSD _____months
    c. CBPP _____months
    d. ECF _____months
    e. Bluetongue _____months
    f. Anaplasmosis _____months
    g. Mastitis _____months
    h. Pneumonia _____months
    i. Anthrax _____months
    j. RVF _____months
    k. Worms _____months
    l. Not sure (go to Q22)
    m. Other (please state) _____ _____months
23. What signs did the animals show?
    a. Salivation
    b. Lameness
    c. Fever
    d. Inappetence
    e. Sudden death
    f. Diarrhoea/Gastrointestinal disease
    g. Respiratory disease
    h. Abortion
    i. Drop in milk yield
    j. Other (please state) _____

Farm ID: _____ **Date: ____/____/_____**

Interviewer: _____

24. How many cattle were sick? _____
25. Of the animals affected how many were:

< 6 months	
6-12 months	
1-2 years	
>2 years	

26. What signs have you seen in **Sheep**? Ask as an open question. State how many months ago.
    a. PPR _____months
    b. FMD _____months
    c. Bluetongue _____months
    d. Sheep pox _____months
    e. Mastitis _____months
    f. Respiratory disease _____months
    g. Worms _____months
    h. Abortion _____months
    i. Sudden death _____months
    j. Other (please state) _____ _____months
27. What signs did the animals show?
    a. Salivation
    b. Lameness
    c. Fever
    d. Inappetence
    e. Sudden death
    f. Diarrhoea/Gastrointestinal disease
    g. Respiratory disease
    h. Abortion
    i. Skin lesions
    j. Other (please state) _____
28. How many sheep were sick? _____
29. Of the animals affected how many were:

	Sick
< 12 months	
1-2 years	
>2 years	

Farm ID: _____ **Date:** ____/____/_____

Interviewer: _____

30. What signs have you seen in **Goats**? Ask as an open question. State how many months ago.
    a. PPR _____months
    b. FMD _____months
    c. CCPP _____months
    d. Goatpox _____months
    e. Bluetongue _____months
    f. Mastitis _____months
    g. Respiratory disease _____months
    h. Worms _____months
    i. Abortion _____months
    j. Sudden death _____months
    k. Other (please state) _____
31. What signs did the animals show?
    a. Salivation
    b. Lameness
    c. Fever
    d. Inappetence
    e. Sudden death
    f. Diarrhoea/Gastrointestinal disease
    g. Respiratory disease
    h. Abortion
    i. Other (please state) _____
32. How many goats were sick? _____
33. Of the animals affected how many were:

	Sick
< 12 months	
1-2 years	
>2 years	

34. What signs have you seen in **pigs**? Ask as an open question. State how many months ago.
    a. FMD _____months
    b. Respiratory disease _____months
    c. Worms _____months
    d. Abortion _____months
    e. Sudden death _____months
    f. Other (please state) _____ _____months

Farm ID: _____ **Date: ____/____/_____**

Interviewer: _____

35. What signs did the animals show?
    a. Salivation
    b. Lameness
    c. Fever
    d. Inappetence
    e. Sudden death
    f. Diarrhoea/Gastrointestinal disease
    g. Respiratory disease
    h. Abortion
    i. Other (please state) _____
36. How many pigs were sick? _____
37. Of the animals affected how many were:

	Sick
< 12 months	
1-2 years	
>2 years	

38. Did you call a vet or animal health assistant when you animals were sick?
    a. Yes (state disease event)
    b. No
    c. Don't know
39. Was this a private or government employee?
    a. Government
    b. Private
    c. Don't know

Farm ID: _____ **Date: ____/____/_____**

Interviewer: _____

Local disease reports and vaccination

40. Have you heard reports of any other diseases in the local area in the last four months?
    a. Yes
    b. No (Go to 42)
    c. Don't know (Go to 42)
41. Which diseases did you hear about (state how many months ago)?
    a. FMD _____months
    b. LSD _____months
    c. PPR _____months
    d. ECF _____months
    e. Anaplasmosis _____months
    f. Mastitis_____months
    g. Pneumonia _____months
    h. Anthrax _____months
    i. Bluetongue _____months
    j. Other (please state) _____
    k. Don't know
42. Has there been any vaccination on the farm in the last four months?
    a. Yes
    b. No
    c. Don't know
43. Which disease(s) have been vaccinated for (state how many months ago)?
    a. Foot-and-mouth disease _____months
    b. Lumpy Skin disease _____months
    c. PPR _____months
    d. Anthrax _____months
    e. ECF _____ _____months
    f. Other (please state) _____ _____months
    g. Don't know
44. Who did the vaccination?
    a. Private animal health assistant/veterinarian
    b. Government animal health assistant/veterinarian
    c. Government
    d. Other (please state) _____

Farm ID: _____ **Date:** ____/____/_____

Interviewer: _____

Milk Production and Delivery

45. How much milk did your farm produce yesterday? _____ litres
46. How much of this did you sell? _____ litres
47. How many cows were lactating? _____cows
48. To whom do you sell your milk?
    a. Neighbours
    b. Local hotels
    c. Hawkers
    d. Direct to co-operative or other pooling facility
    e. Other (please state) _____
49. What is the name of the co-operative/pooling facility? _____
50. What does the hawker do with the milk?
    a. Sells to private places
    b. Sells to milk pooling facility
    c. Other (please state) _____
    d. Don't know
51. If the farmer had FMD in the last 4 months, what did they do with the milk while the animals were sick? Sell it to the cooperative society
    a. Consume it at home
    b. Boil and consume at home
    c. Sell it to others
    d. Dispose of it
    e. Give to other animals (e.g. dogs)
    f. Give it to calves
    g. Give it to calves (after boiling)
    h. Other (please specify) _____

Figure 8.3 The paper form of the questionnaire used when interviewing small-holder dairy farmers during surveys 2 and 3.

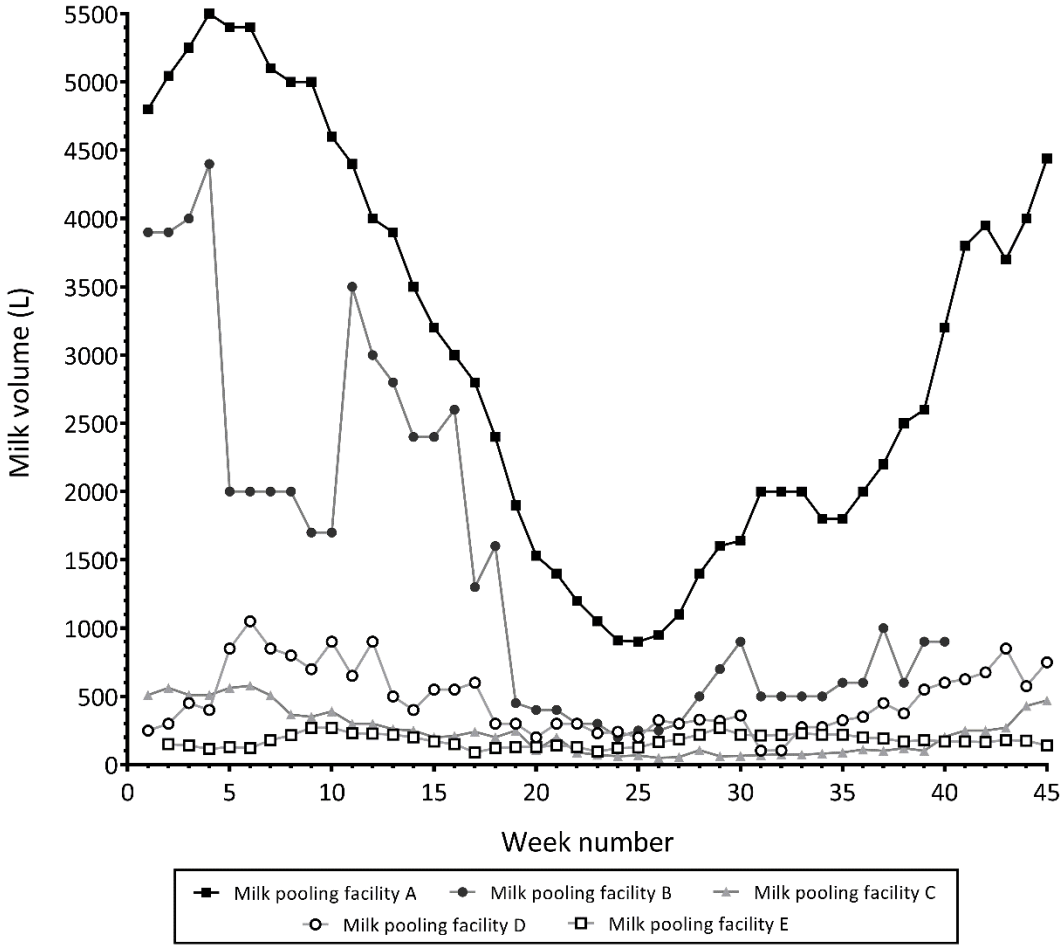

Figure 8.4 Graph showing the temporal trend of the total volume of milk supplied to each milk pooling facility on the sampling dates (week 1 –16/11/2016, week 45 – 20/09/2017).

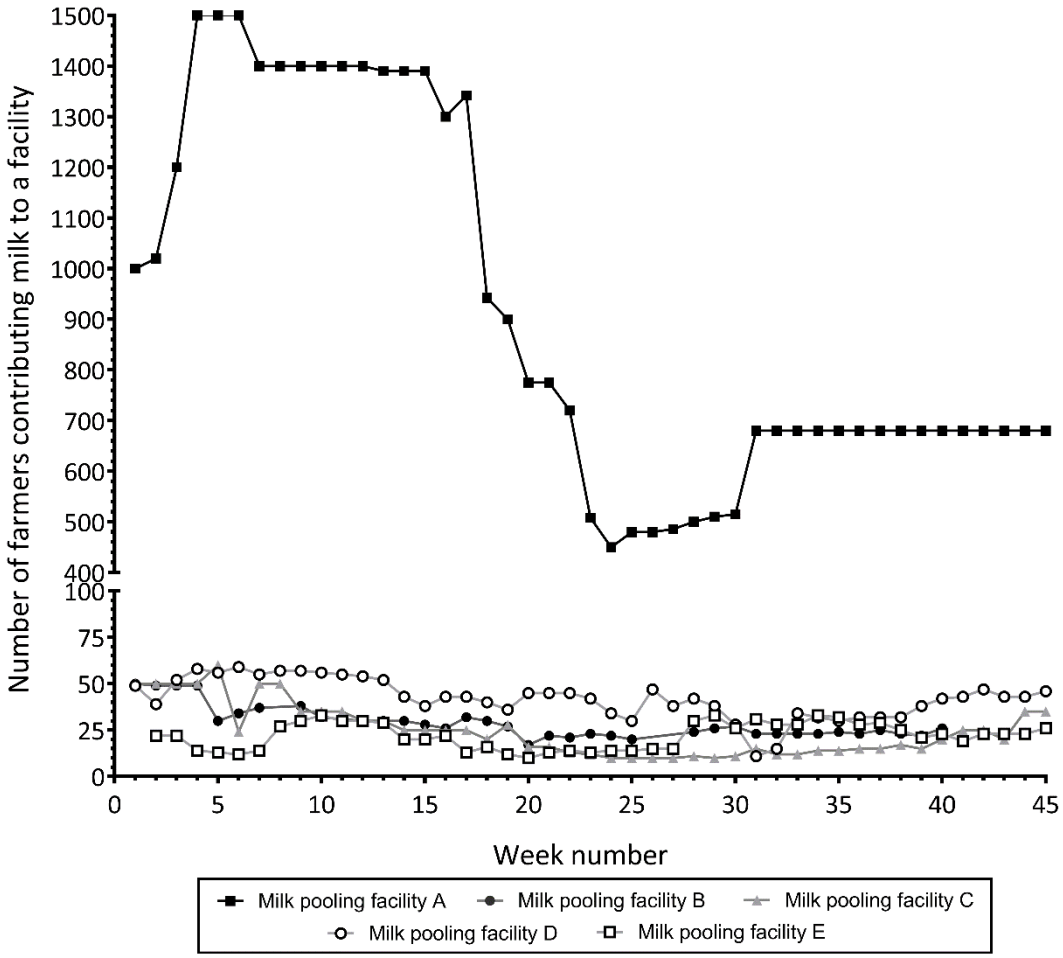

Figure 8.5 Graph showing the temporal trend of the total number of farmers supplying to each milk pooling facility on the sampling dates (week 1 – 16/11/2016, week 45 – 20/09/2017).

Appendix IV

FMD surveillance on a large scale dairy farm in Saudi Arabia

		New clinical cases observed		
		Positive	Negative	Total
Pan-serotypic rRT-PCR	Positive	6	36	42
	Negative	8	682	690
	Total	14	718	732

Se = 42.9%, Sp = 95.0%, A_{obs} = 0.94, K = 0.19

Figure 8.6 Comparison of pan-serotypic rRT-PCR assay results for pooled milk with the number of new clinical cases observed on the milk sample collection day for all houses.

		'Predicted' rRT-PCR		
		Positive	Negative	Total
'Observed' rRT-PCR	Positive	9	19	28
	Negative	17	657	674
	Total	26	676	702

Se = 34.6%, Sp = 97.2%, A_{obs} = 0.95, K = 0.31

*Houses 17 and 18 were not included in the analysis due to incomplete epidemiological data.

Figure 8.7 Comparison of actual rRT-PCR assay results for pooled milk with the predicted results for all houses* for '1/10' virus excretion with a cut-off C_T value of 40.

References

Aanensen, D.M., D.M. Huntley, E.J. Feil, F. Al-Own, and B.G. Spratt, 2009: EpiCollect: Linking smartphones to web applications for epidemiology, ecology and community data collection. *PLoS One* **4**, DOI: 10.1371/journal.pone.0006968.

Abrahão, J.S., T.M.L. Oliveira, R.K. Campos, M.C. Madureira, E.G. Kroon, and Z.I.P. Lobato, 2009: Bovine vaccinia outbreaks: detection and isolation of vaccinia virus in milk samples. *Foodborne Pathog. Dis.* **6**, 1141–1146, DOI: 10.1089/fpd.2009.0324.

Acharya, R., E. Fry, D. Stuart, G. Fox, D. Rowlands, and F. Brown, 1989: The three-dimensional structure of foot-and-mouth disease virus at 2.9 Å resolution. *Nature* **337**, 709–716.

Ahmed, H.A., S.A.H. Salem, A.R. Habashi, A.A. Arafa, M.G.A. Aggour, G.H. Salem, A.S. Gaber, O. Selem, S.H. Abdelkader, N.J. Knowles, M. Madi, B. Valdazo-Gonzalez, J. Wadsworth, G.H. Hutchings, V. Mioulet, J.M. Hammond, and D.P. King, 2012: Emergence of Foot-and-Mouth Disease Virus SAT 2 in Egypt During 2012. *Transbound. Emerg. Dis.* **59**, 476–481, DOI: 10.1111/tbed.12015.

Ahmed, H.I., U. Farooq, A. Bin Zahur, K. Naeem, A. Latif, and H. Irshad, 2017: Evidence of foot-and-mouth disease virus excretion in the milk of apparently healthy vaccinated buffaloes in Islamabad, Pakistan. *Turkish J. Vet. Anim. Sci.* **41**, 431–434, DOI: 10.3906/vet-1512-96.

Ahmed, S.S.U., 2015: Comparing pooling schemes for infectious disease surveillance. In: *ISVEE 14, Novemb. 2015, Merida, Yucatan, Mex.*

Alexandersen, S., M. Quan, C. Murphy, J. Knight, and Z. Zhang, 2003: Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentally infected with foot-and-mouth disease virus. *J. Comp. Pathol.* **129**, 268–282, DOI: 10.1016/S0021-9975(03)00045-8.

Alexandersen, S, Z. Zhang, A.. Donaldson, and A.J.. Garland, 2003: The Pathogenesis and Diagnosis of Foot-and-Mouth Disease. *J. Comp. Pathol.* **129**, 1–36, DOI: 10.1016/S0021-9975(03)00041-0.

Alexandersen, S., M.B. Oleksiewicz, and A.I. Donaldson, 2001: The early pathogenesis of foot-and-mouth disease in pigs infected by contact: a quantitative time-course study using TaqMan RT–PCR. *J. Gen. Virol.* **82**, 747–755, DOI: 10.1099/0022-1317-82-4-747.

Ali, W., M. Habib, R.S.A. Khan, M.A. Zia, I.U. Khan, U. Saliha, M. Farooq, M.S. Shah, and H.M. Muzammil, 2017: Reverse transcription-polymerase chain reaction (RT-PCR) based detection and economic impact of foot-and-mouth disease in district Faisalabad, Pakistan during the year 2015. *Iraqi J. Vet. Sci.* **31**, 1–6.

Aly, S.A., and A.S. Gaber, 2007: Inactivation of foot and mouth disease virus in milk and milk products. *Milchwissenschaft* **62**, 3–5.

Amaral-Doel, C.M.F., N.E. Owen, N.P. Ferris, R.P. Kitching, and T.R. Doel, 1993: Detection of foot-and-mouth disease viral sequences in clinical specimens and ethyleneimine-inactivated preparations by the polymerase chain reaction. *Vaccine* **11**, 415–421.

Ansari-Lari, M., M. Mohebbi-Fani, N.A. Lyons, and N. Azizi, 2017: Impact of FMD outbreak on milk production and heifers' growth on a dairy herd in southern Iran. *Prev. Vet. Med.* **144**, 117–122, DOI: 10.1016/j.prevetmed.2017.05.022.

APHIS, 2008: Prevalence of Contagious Mastitis Pathogens on U.S. Dairy Operations, 2007. *Vet. Serv. Centers Epidemiol. Anim. Heal.*

Armson, B., V. Mioulet, C. Doel, M. Madi, S. Parida, K.A. Lemire, D.J. Holder, A. Das, M.T. McIntosh, and D.P. King, 2018: Detection of foot-and-mouth disease virus in milk samples by real-time reverse transcription polymerase chain reaction: optimisation and evaluation of a high-throughput screening method with potential for disease surveillance. *Vet. Microbiol.* **223**, 189–194, DOI: 10.1016/j.vetmic.2018.07.024.

Armson, B., J. Wadsworth, T. Kibona, D. Mshanga, V.L. Fowler, N.J. Knowles, V. Mioulet, R. Reeve, D.P. King, K. Bachanek-Bankowska, and T. Lembo, 2019: Opportunities for enhanced surveillance of foot-and-mouth disease in endemic settings using milk samples. *Transbound. Emerg. Dis.* **66**, 1405–1410, DOI: 10.1111/tbed.13146.

Armstrong, R., and E. Mathew, 2001: Predicting herd protection against foot-and-mouth disease by testing individual and bulk tank milk samples. *J. Virol. Methods* **97**, 87–99, DOI: S0166093401003421 [pii].

Armstrong, R.M., 1997: Development of tests for antibodies against foot-and-mouth disease virus in cattle milk. *J. Virol. Methods* **63**, 175–180, DOI: 10.1016/S0166-0934(96)02126-X.

Armstrong, R.M., E.S. Mathew, and D.K.J. Mackay, 2000: Validation of the specific isotype assay to detect antibodies against foot-and-mouth disease virus in bovine milk. *J. Virol. Methods* **85**, 193–201, DOI: 10.1016/S0166-0934(99)00171-8.

Asghar, A.H., A.M. Mohamed, S. Arabia, S. Arabia, and S. Arabia, 2016: The impact of importation of live ruminants on the epizootiology of foot and mouth disease in Saudi Arabia. *Rev. sci. tech. Off. int. Epiz* **35**, 1–22.

AU-IBAR, 2014: Standard Methods and Procedures (SMPs) for Control of Foot and Mouth Disease in the Greater Horn of Africa.

Azad, T., and S. Ahmed, 2016: Common milk adulteration and their detection techniques. *Int. J. Food Contam.* **3**, DOI: 10.1186/s40550-016-0045-3.

Bachanek-Bankowska, K., H.R. Mero, J. Wadsworth, V. Mioulet, R. Sallu, G.J. Belsham, C.J. Kasanga, N.J. Knowles, and D.P. King, 2016: Development and evaluation of tailored specific real-time RT-PCR assays for detection of foot-and-mouth disease virus serotypes circulating in East Africa. *J. Virol. Methods* **237**, 114–120, DOI: 10.1016/j.jviromet.2016.08.002.

Bachanek-Bankowska, K., A. Di Nardo, J. Wadsworth, E.K.M. Henry, Ü. Parlak, A. Timina, A. Mischenko, I.A. Qasim, D. Abdollahi, M. Sultana, M.A. Hossain, D.P. King, N.J. Knowles, E.K.M. Henry, D.P. King, and N.J. Knowles, 2018: Foot-and-mouth disease in the Middle East caused by an A/ASIA/G-VII virus lineage, 2015-2016. *Emerg. Infect. Dis.* **24**, 1073–1078, DOI: 10.3201/eid2406.170715.

Bachrach, H.L., S.S. Breese, J.J. Callis, W.R. Hess, and R.E. Patty, 1957: Inactivation of foot- and-mouth disease virus by pH and temperature changes and by formaldehyde. *Exp. Biol. ad Med.* **95**.

Bao, H.-F., D. Li, J.-H. Guo, Z.-J. Lu, Y.-L. Chen, Z.-X. Liu, X.-T. Liu, and Q.-G. Xie, 2008: A highly sensitive and specific multiplex RT-PCR to detect foot-and-mouth disease virus in tissue and food samples. *Arch. Virol.* **153**, 205–209, DOI: 10.1007/s00705-007-1082-y.

Barasa, M., A. Catley, D. MacHuchu, H. Laqua, E. Puot, D.T. Kot, and D. Ikiror, 2008: Foot-and-mouth disease vaccination in South Sudan: Benefit-cost analysis and livelihoods impact. *Transbound. Emerg. Dis.* **55**, 339–351, DOI: 10.1111/j.1865-1682.2008.01042.x.

Barnard, S.E., 1977: Collecting and Handling Milk Samples. *J. Dairy Sci.* **60**, 791–794, DOI: 10.4315/0022-2747-38.2.108.

Bastola, U., Lembo, T. Cleaveland, S., Casey, M., Auty, H., Kibona, T., Mshanga, D., Marsh, T.L., 2015: Economic consequences of Foot-and-Mouth Disease on traditional livestock-dependent communities on northern Tanzania. In: *ISVEE 14, Novemb. 2015, Merida, Yucatan, Mex.*

Bates, T.W., M.C. Thurmond, S.K. Hietala, K.S. Venkateswaran, T.M. Wilson, B.W. Colston Jr, J.E. Trebes, and F.P. Milanovich, 2003: Surveillance for detection of foot-and-mouth disease. *J. Am. Vet. Med. Assoc.* **223**, 609–614.

Bauer, A.E., S. Olivas, M. Cooper, H. Hornstra, P. Keim, T. Pearson, and A.J. Johnson, 2015: Estimated herd prevalence and sequence types of Coxiella burnetii in bulk tank milk samples from commercial dairies in Indiana. *BMC Vet. Res.* **11**, 186, DOI: 10.1186/s12917-015-0517-3.

Baxt, B., D.O. Morgan, B.H. Robertson, and C. A. Timpone, 1984: Epitopes on foot-and-mouth disease virus outer capsid protein VP1 involved in neutralization and cell attachment. *J. Virol.* **51**, 298–305.

Bayissa, B., G. Ayelet, M. Kyule, Y. Jibril, and E. Gelaye, 2011: Study on seroprevalence, risk factors, and economic impact of foot-and-mouth disease in Borena pastoral and agro-pastoral system, southern Ethiopia. *Trop. Anim. Health Prod.* **43**, 759–766, DOI: 10.1007/s11250-010-9728-6.

Bertram, M.R., A. Delgado, S.J. Pauszek, G.R. Smoliga, B. Brito, C. Stenfeldt, E.J. Hartwig,

S.D. Jumbo, M. Abdoulmoumini, A.A. Oliva Marie, R. Salhine, L.L. Rodriguez, R. Garabed, and J. Arzt, 2018: Effect of vaccination on cattle subclinically infected with foot-and-mouth disease virus in Cameroon. *Prev. Vet. Med.* **155**, 1–10, DOI: 10.1016/j.prevetmed.2018.04.003.

Bickley, J., J.K. Short, D.G. McDowell, and H.C. Parkes, 1996: Polymerase chain reaction (PCR) detection of Listeria monocytogenes in diluted milk and reversal of PCR inhibition caused by calcium ions. *Lett. Appl. Microbiol.* **22**, 153–8.

Blackwell, J.H., and J.L. Hyde, 1976: Effect of heat on foot-and-mouth disease virus (FMDV) in the components of milk from FMDV-infected cows. *J. Hyg. (Lond).* **77**, 77–83.

Blackwell, J.H., and P.D. McKercher, 1982: Concentration of Foot-and-Mouth Disease Virus in Milk of Cows Infected Under Simulated Field Conditions. *J. Dairy Sci.* **65**, 1624–1631, DOI: 10.3168/jds.S0022-0302(82)82388-6.

Brehm, K.E., N.P. Ferris, M. Lenk, R. Riebe, and B. Haas, 2009: Highly sensitive fetal goat tongue cell line for detection and isolation of foot-and-mouth disease virus. *J. Clin. Microbiol.* **47**, 3156–3160, DOI: 10.1128/JCM.00510-09.

Brito, B.P., L.L. Rodriguez, J.M. Hammond, J. Pinto, and A.M. Perez, 2015: Review of the Global Distribution of Foot-and-Mouth Disease Virus from 2007 to 2014. *Transbound. Emerg. Dis.*n/a-n/a, DOI: 10.1111/tbed.12373.

Brock, K. V., D.L. Grooms, J. Ridpath, and S.R. Bolin, 1998: Changes in levels of viremia in cattle persistently infected with bovine viral diarrhea virus. *J. Vet. Diagnostic Investig.* **10**, 22–26, DOI: 10.1177/104063879801000105.

Burrows, R., 1968: Excretion of foot-and-mouth disease virus prior to the development of lesions. *Vet. Rec.* **82**, 387–388.

Burrows, R., J.A. Mann, A. Greig, W.G. Chapman, and Goodridg.D, 1971: Growth and Persistence of Foot-and-Mouth Disease Virus in Bovine Mammary Gland. *J. Hyg.* **69**, 307-.

BVDFree, 2019: BVDFree England [Online] Available at https://bvdfree.org.uk/.

Callahan, J., F. Brown, F. Osorio, J. Sur, E. Kramer, G. Long, J. Lubroth, S. Ellis, K. Shoulars, K. Gaffney, D. Rock, and W.M. Nelson, 2002: Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *J. Am. Vet. Med. Assoc.* **220**, 1636–1642.

Callens, M., and K. De Clercq, 1997: Differentiation of the seven serotypes of foot-and-mouth disease virus by reverse transcriptase polymerase chain reaction. *J. Virol. Methods* **67**, 35–44, DOI: 10.1016/S0166-0934(97)00074-8.

Cao, Y., Z. Lu, and Z. Liu, 2016: Foot-and-mouth disease vaccines: progress and problems. *Expert Rev. Vaccines* **15**, 783–789, DOI: 10.1586/14760584.2016.1140042.

Caporale, V., A. Giovannini, and C. Zepeda, 2012: Surveillance strategies for foot and mouth disease to prove absence of disease and absence of viral circulation. *Rev. Sci. Tech.* **31**, 747–59, DOI: 10.20506/rst.31.3.2156.

Caraguel, C.G.B., H. Stryhn, N. Gagné, I.R. Dohoo, and K.L. Hammell, 2011: Selection of a cutoff value for real-time polymerase chain reaction results to fit a diagnostic purpose: Analytical and epidemiologic approaches. *J. Vet. Diagnostic Investig.* **23**, 2–15, DOI: 10.1177/104063871102300102.

Casey-Bryars, M., R. Reeve, U. Bastole, N.J. Knowles, H. Auty, K. Bachanek-Bankowska, V.L. Fowler, R. Fyumagwa, R. Kazwala, T. Kibona, A. King, D.P. King, F. Lankester, A.B. Ludi, A. Lugelo, F.F. Maree, D. Mshanga, G. Ndhlovu, K. Parekh, D.J. Paton, B. Perry, J. Wadsworth, S. Parida, D.T. Haydon, T.L. Marsh, S. Cleaveland, and T. Lembo, 2018: Waves of endemic foot-and-mouth disease in eastern Africa suggest feasibility of proactive vaccination approaches. *Nat. Ecol. Evol.*DOI: 10.1038/s41559-018-0636-x.

Casey, M.B., T. Lembo, N.J. Knowles, R. Fyumagwa, F. Kivaria, H. Maliti, C. Kasanga, R. Sallu, R. Reeve, S. Parida, D.P. King, and S. Cleaveland, 2013: Patterns of Foot-and-Mouth Disease Virus Distribution in Africa: The Role of Livestock and Wildlife in Virus Emergence.*Role Anim. Emerg. Viral Dis.* Elsevier.

CDC, 1999: Biosafety in Microbiological and Biomedical Laboratories *HHS Publ. no. 93-*

8395.

Chand, P., B.S. Rajpurohit, A.K. Malhotra, and J.S. Poonia, 2005: Comparison of milk-ELISA and serum-ELISA for the diagnosis of Brucella melitensis infection in sheep. *Vet. Microbiol.* **108**, 305–311, DOI: 10.1016/j.vetmic.2005.04.006.

Charleston, B., B.M. Bankowski, S. Gubbins, M.E. Chase-Topping, D. Schley, R. Howey, P. V. Barnett, D. Gibson, N.D. Juleff, and M.E.J. Woolhouse, 2011: Relationship Between Clinical Signs and Transmission of an Infectious Disease and the Implications for Control. *Science (80).* **332**, 726–729, DOI: 10.1126/science.1199884.

Chaters, G., J. Rushton, T.D. Dulu, and N.A. Lyons, 2018: Impact of foot-and-mouth disease on fertility performance in a large dairy herd in Kenya. *Prev. Vet. Med.* **159**, 57–64, DOI: 10.1016/j.prevetmed.2018.08.006.

Chen, H., J. Zhang, Y. Liu, and X. Liu, 2011: Rapid typing of foot-and-mouth disease serotype Asia 1 by reverse transcription loop-mediated isothermal amplification. *Virol. J.* **8**, 489, DOI: 10.1186/1743-422X-8-489.

Chowdhury, S.M.Z.H., M.F. Rahman, M.B. Rahman, and M.M. Rahman, 1993: Foot-and-mouth disease and its effects on morbidity, mortality, milk yield and draft power in Bangladesh. *Asian-Australasian J. Anim. Sci.* **6**, 423–426.

Christensen, J., and I. a Gardner, 2000: Herd-level interpretation of test results for epidemiologic studies of animal diseases. *Prev. Vet. Med.* **45**, 83–106, DOI: 10.1016/S0167-5877(00)00118-5.

Christiansson, A., J. Bertilsson, and B. Svensson, 1999: Bacillus cereus spores in raw milk: factors affecting the contamination of milk during the grazing period. *J. Dairy Sci.* **82**, 305–314, DOI: 10.3168/jds.S0022-0302(99)75237-9.

Clarke, T., S.P. Andrews, P.J. Moate, C.A. Pollino, and W.L. Schmidt, 1997: Evaluation of low cost in-line milk samplers for estimating individual cow somatic cell counts. *J. Dairy Res.* **64**, 13–22.

Condy, J.B., R.S. Hedger, C. Hamblin, and I.T.R. Barnett, 1985: The duration of the foot-

and-mouth disease virus carrier state in African buffalo (i) in the individual animal and (ii) in a free-living herd. *Comp. Immunol. Microbiol. Infect. Dis.* **8**, 259–265, DOI: https://doi.org/10.1016/0147-9571(85)90004-9.

Cottam, E.M., J. Wadsworth, A.E. Shaw, R.J. Rowlands, L. Goatley, S. Maan, N.S. Maan, P.P.C. Mertens, K. Ebert, Y. Li, E.D. Ryan, N. Juleff, N.P. Ferris, J.W. Wilesmith, D.T. Haydon, D.P. King, D.J. Paton, and N.J. Knowles, 2008: Transmission Pathways of Foot-and-Mouth Disease Virus in the United Kingdom in 2007. *PLoS Pathog.* **4**, e1000050, DOI: 10.1371/journal.ppat.1000050.

Daly, J.M., B. King, R.A. Tarlinton, K.C. Gough, B.C. Maddison, and R. Blowey, 2015: Comparison of Schmallenberg virus antibody levels detected in milk and serum from individual cows. *BMC Vet. Res.* **11**, 56, DOI: 10.1186/s12917-015-0365-1.

DEFRA, 2015a: EXPLANATORY MEMORANDUM TO THE BRUCELLOSIS (ENGLAND) ORDER 2015 [Online] Available at http://www.legislation.gov.uk/uksi/2015/364/pdfs/uksiem_20150364_en.pdf.

DEFRA, 2015b: Consultation on a proposal to change the Brucellosis bulk milk tank sampling regime for producer-retailers. Summary of responses.

Department of Environment Food and Rural Affairs, 2010: Maps of livestock populations in 2000 and 2010 across England Vol. 44.

Dhikusooka, M.T., C. Ayebazibwe, A. Namatovu, G.J. Belsham, H.R. Siegismund, S.N. Wekesa, S.N. Balinda, V.B. Muwanika, and K. Tjørnehøj, 2016: Unrecognized circulation of SAT 1 foot-and-mouth disease virus in cattle herds around Queen Elizabeth National Park in Uganda. *BMC Vet. Res.* **12**, 5, DOI: 10.1186/s12917-015-0616-1.

Di Nardo, a, N.J. Knowles, and D.J. Paton, 2011: Combining livestock trade patterns with phylogenetics to help understand the spread of foot and mouth disease in sub-Saharan Africa, the Middle East and Southeast Asia. *Rev. Sci. Tech.* **30**, 63–85.

Didenko, V. V., 2001: DNA Probes Using Fluoresence Resonance Energy Transfer (FRET): Designs and Applications. *Biotechniques* **31**, 1106–1121, DOI: 10.2144/01315rv02.

Doel, T.R., 1996: Natural and vaccine-induced immunity to foot and mouth disease: the prospects for improved vaccines. *Rev. Sci. Tech.* **15**, 883–911, DOI: 10.20506/rst.15.3.955.

Domingo, E., C. Escarmís, E. Baranowski, C.M. Ruiz-Jarabo, E. Carrillo, J.I. Núñez, and F. Sobrino, 2003: Evolution of foot-and-mouth disease virus. *Virus Res.* **91**, 47–63, DOI: 10.1016/S0168-1702(02)00259-9.

Donaldson, A.I., and R.P. Kitching, 1989: Transmission of foot-and-mouth disease by vaccinated cattle following natural challenge. *Res. Vet. Sci.* **46**, 9–14.

Drew, T.W., F. Yapp, and D.J. Paton, 1999: The detection of bovine viral diarrhoea virus in bulk milk samples by the use of a single-tube RT-PCR. *Vet. Microbiol.* **64**, 145–154, DOI: 10.1016/S0378-1135(98)00266-1.

Drewe, J.A., L.J. Hoinville, A.J.C. Cook, T. Floyd, and K.D.C. Stark, 2011: Evaluation of animal and public health surveillance systems: a systematic review. *Epidemiol. Infect.* **140**, 575–590, DOI: 10.1017/s0950268811002160.

Dubovi, E.J., and V. Section, 1995: Bulk Milk Tank Testing For the Detection of BVDV Persistently Infected Lactating Cows. 14853.

Ehizibolo, D.O., A.M. Perez, C. Carrillo, S. Pauszek, M. Alkhamis, I. Ajogi, J.U. Umoh, H.M. Kazeem, P.O. Ehizibolo, A. Fabian, M. Berninger, K. Moran, L.L. Rodriguez, and S.A. Metwally, 2014: Epidemiological analysis, serological prevalence and genotypic analysis of Foot-and-mouth disease in Nigeria 2008-2009. *Transbound. Emerg. Dis.* **61**, 500–510, DOI: 10.1111/tbed.12054.

Elnekave, E., H. Shilo, B. Gelman, and E. Klement, 2015: The longevity of anti NSP antibodies and the sensitivity of a 3ABC ELISA - A 3 years follow up of repeatedly vaccinated dairy cattle infected by foot and mouth disease virus. *Vet. Microbiol.* **178**, 14–18, DOI: 10.1016/j.vetmic.2015.04.003.

Elvander, M., S. Edwards, K. Naslund, and N. Linde, 1995: Evaluation and application of an indirect ELISA for the detection of antibodies to bovine respiratory syncytial virus in milk, bulk milk, and serum. *J Vet Diagn Invest* **7**, 177–182, DOI: 10.1177/104063879500700202.

Falzon, L.C., L. Alumasa, F. Amanya, E. Kang'ethe, S. Kariuki, K. Momanyi, P. Muinde, M.K. Murungi, S.M. Njoroge, A. Ogendo, J. Ogola, J. Rushton, M.E.J. Woolhouse, and E.M. Fèvre, 2019: One Health in Action: Operational Aspects of an Integrated Surveillance System for Zoonoses in Western Kenya. *Front. Vet. Sci.* **6**, 1–13, DOI: 10.3389/fvets.2019.00252.

Farooq, U., Z. Ahmed, K. Naeem, M. Bertram, B. Brito, C. Stenfeldt, S.J. Pauszek, M. Larocco, L. Rodriguez, and J. Arzt, 2018: Characterization of naturally occurring , new and persistent subclinical foot-and-mouth disease virus infection in vaccinated Asian buffalo in Islamabad Capital Territory , Pakistan. *Transbound. Emerg. Dis.*1836–1850, DOI: 10.1111/tbed.12963.

Fayed, A.A., M. Abdel-Halim, N. Degheidy, M. EL-Bayoumy, and H. Fakry, 2013: Value of Individual and Bulk Milk Serology for Surveillance and Evaluation of Vaccination Programs Used in Dairy Farms in Egypt to Control FMD Virus Infection. *Int. J. Vet. Med. Res. Reports* **2013**, 1–11, DOI: 10.5171/2013.730973.

Felkai, V., F. Solyom, M. Szent-Ivanyi, and A. Wagner, 1970: Heat resistance of foot-and-mouth disease virus in milk. *Magy. Allatorvosok Lapja* **25**, 378–384.

Ferrari, G., L. Tasciotti, E. Khan, and A. Kiani, 2014: Foot-and-Mouth Disease and Its Effect on Milk Yield: An Economic Analysis on Livestock Holders in Pakistan. *Transbound. Emerg. Dis.* **61**, e52–e59, DOI: 10.1111/tbed.12072.

Ferris, N. P., D.P. King, S.M. Reid, G.H. Hutchings, A.E. Shaw, D.J. Paton, N. Goris, B. Haas, B. Hoffmann, E. Brocchi, M. Bugnetti, A. Dekker, and K. De Clercq, 2006: Foot-and-mouth disease virus: A first inter-laboratory comparison trial to evaluate virus isolation and RT-PCR detection methods. *Vet. Microbiol.* **117**, 130–140, DOI: 10.1016/j.vetmic.2006.06.001.

Ferris, N. P., J.M. Oxtoby, and J.F. Hughes, 1995: Multiple-infected diagnostic specimens from foot and mouth disease endemic regions. *Rev Sci Tech* **14**, 557–65.

Ferris, N.P., and M. Dawson, 1988: Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. *Vet. Microbiol.* **16**, 201–209, DOI:

10.1016/0378-1135(88)90024-7.

Ferris, N.P., D.P. King, S.M. Reid, A.E. Shaw, and G. Hutchings, 2006: Comparisons of original laboratory results and retrospective analysis by real-time reverse transcriptase-PCR of virological samples collected from confirmed cases of foot-and-mouth disease in the UK in 2001. *Vet. Rec.* **159**, 373–378.

Ferris, N.P., A. Nordengrahn, G.H. Hutchings, S.M. Reid, D.P. King, K. Ebert, D.J. Paton, T. Kristersson, E. Brocchi, S. Grazioli, and M. Merza, 2009: Development and laboratory validation of a lateral flow device for the detection of foot-and-mouth disease virus in clinical samples. *J. Virol. Methods* **155**, 10–7, DOI: 10.1016/j.jviromet.2008.09.009.

Fishbourne, E., A.B. Ludi, G. Wilsden, P. Hamblin, B. Statham, A. Bin-Tarif, E. Brocchi, S. Grazioli, A. Dekker, P. Eblé, and D.P. King, 2017: Efficacy of a high potency O1Manisa foot-and-mouth disease vaccine in cattle against heterologous challenge with a field virus from the O/ME-SA/Ind-2001 lineage collected in North Africa. *Vaccine* **35**, 2761–2765, DOI: 10.1016/j.vaccine.2017.02.047.

Food and Agriculture Organization (FAO), 2011: The Progressive Control Pathway for FMD control Principles , Stage Descriptions and Standards. .

Food and Agriculture Organization (FAO), 2018: The Progressive Control Pathway for Foot and Mouth Disease control (PCP-FMD) Principles, Stage Descriptions and Standards.

Fowler, V.L., B.M. Bankowski, B. Armson, A. Di Nardo, B. Valdazo-Gonzalez, S.M. Reid, P. V Barnett, J. Wadsworth, N.P. Ferris, V. Mioulet, and D.P. King, 2014: Recovery of Viral RNA and Infectious Foot-and-Mouth Disease Virus from Positive Lateral-Flow Devices. *PLoS One* **9**, e109322, DOI: 10.1371/journal.pone.0109322.

Garner, M.G., I.J. East, T. Kompas, P. V. Ha, S.E. Roche, and H.T.M. Nguyen, 2016: Comparison of alternatives to passive surveillance to detect foot and mouth disease incursions in Victoria, Australia. *Prev. Vet. Med.* **128**, 78–86, DOI: 10.1016/j.prevetmed.2016.04.009.

Gelberg, H.B., and R.M. Lewis, 1982: The Pathogenesis of Vesicular Exanthema of Swine

Virus and San Miguel Sea Lion Virus in Swine. *Vet. Pathol.* **19**, 424–443, DOI: 10.1177/030098588201900408.

Gilchrist, C.A., S.D. Turner, M.F. Riley, W.A. Petri, and E.L. Hewlett, 2015: Whole-Genome Sequencing in Outbreak Analysis. *Clin. Microbiol. Rev.* **28**, 541–563, DOI: 10.1128/CMR.00075-13.

Giridharan, P., D. Hemadri, C. Tosh, A. Sanyal, and S.K. Bandyopadhyay, 2005: Development and evaluation of a multiplex PCR for differentiation of foot-and-mouth disease virus strains native to India. *J. Virol. Methods* **126**, 1–11, DOI: 10.1016/j.jviromet.2005.01.015.

Goller, K. V., V. Dill, M. Madi, P. Martin, Y. Van der Stede, V. Vandenberge, B. Haas, S. Van Borm, F. Koenen, C.J. Kasanga, N. Ndusilo, M. Beer, L. Liu, V. Mioulet, B. Armson, D.P. King, and V.L. Fowler, 2018: Rapid and simple detection of foot-and-mouth disease virus: Evaluation of a cartridge-based molecular detection system for use in basic laboratories. *Transbound. Emerg. Dis.* **65**, 578–584, DOI: 10.1111/tbed.12744.

Gomaa Hemida, M., W. Rizk El-Ghareeb, F. Al-Hizab, and A. Ibrahim, 2018: Foot-and-mouth disease virus O/ME-SA/Ind 2001 lineage outbreak in vaccinated Holstein Friesian cattle in Saudi Arabia in 2016. *Vet. Q.* **38**, 88–98, DOI: 10.1080/01652176.2018.1539568.

González-Warleta, M., J.A. Castro-Hermida, C. Carro-Corral, and M. Mezo, 2011: Anti-Neospora caninum antibodies in milk in relation to production losses in dairy cattle. *Prev. Vet. Med.* **101**, 58–64, DOI: 10.1016/j.prevetmed.2011.04.019.

Goris, N., F. Vandenbussche, C. Herr, J. Villers, Y. Van der Stede, and K. De Clercq, 2009: Validation of two real-time RT-PCR methods for foot-and-mouth disease diagnosis: RNA-extraction, matrix effect, uncertainty of measurement and precision. *J. Virol. Methods* **160**, 157–162, DOI: 10.1016/j.jviromet.2009.05.005.

Grubman, M.J., and B. Baxt, 2004: Foot-and-mouth disease. *Clin. Microbiol. Rev.* **17**, 465–93, DOI: 10.1128/CMR.17.2.465.

Guatelli, J.C., T.R. Gingeras, and D.D. Richman, 1989: Nucleic acid amplification in vitro:

Detection of sequences with low copy numbers and application to diagnosis of human immunodeficiency virus type 1 infection. *Clin. Microbiol. Rev.* **2**, 217–226, DOI: 10.1128/CMR.2.2.217.

Hadorn, D., and K. Stark, 2008: Evaluation and optimization of surveillance systems for rare and emerging infectious diseases. *Vet. Res.* **39**, DOI: 10.1051/vetres.

Hafez, S.M., M.A. Farag, K.S. Mazloum, and A.M. al-Bokmy, 1994: Serological survey of foot and mouth disease in Saudi Arabia. *Rev Sci Tech* **13**, 711–719.

Hall, M.D., N.J. Knowles, J. Wadsworth, A. Rambaut, and M.E.J. Woolhouse, 2013: Reconstructing geographical movements and host species transitions of foot-and-mouth disease virus serotype SAT 2. *MBio* **4**, e00591-13, DOI: 10.1128/mBio.00591-13.

Hall, T.A., 1999: BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**, 95–98.

Hamblin, C., I.T.R. Barnett, and R.S. Hedger, 1986: A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus I. Development and method of ELISA. *J. Immunol. Methods* **93**, 115–121, DOI: 10.1016/0022-1759(86)90441-2.

Hamdy, M.E.R., and A.S. Amin, 2002: Detection of Brucella species in the milk of infected cattle, sheep, goats and camels by PCR. *Vet. J.* **163**, 299–305, DOI: 10.1053/tvjl.2001.0681.

Hansen, S., V. Dill, M.A. Shalaby, M. Eschbaumer, S. Böhlken-Fascher, B. Hoffmann, C.P. Czerny, and A. Abd El Wahed, 2019: Serotyping of foot-and-mouth disease virus using oxford nanopore sequencing. *J. Virol. Methods* **263**, 50–53, DOI: 10.1016/j.jviromet.2018.10.020.

Hayer, S.S., R. Ranjan, J.K. Biswal, S. Subramaniam, J.K. Mohapatra, G.K. Sharma, M. Rout, B.B. Dash, B. Das, B.R. Prusty, A.K. Sharma, C. Stenfeldt, A. Perez, L.L. Rodriguez, B. Pattnaik, K. VanderWaal, and J. Arzt, 2018: Quantitative characteristics of the foot-and-mouth disease carrier state under natural conditions in India. *Transbound. Emerg. Dis.* **65**, 253–260, DOI: 10.1111/tbed.12627.

Hayer, S.S., K. VanderWaal, R. Ranjan, J.K. Biswal, S. Subramaniam, J.K. Mohapatra, G.K. Sharma, M. Rout, B.B. Dash, B. Das, B.R. Prusty, A.K. Sharma, C. Stenfeldt, A. Perez, A.H. Delgado, M.K. Sharma, L.L. Rodriguez, B. Pattnaik, and J. Arzt, 2018: Foot-and-mouth disease virus transmission dynamics and persistence in a herd of vaccinated dairy cattle in India. *Transbound. Emerg. Dis.* **65**, e404–e415, DOI: 10.1111/tbed.12774.

Hedger, R., and P. Dawson, 1970: Foot-and-mouth disease virus in milk: an epidemiological study. *Vet. Rec.* **Aug 15**, 186–8.

Heid, C.A., J. Stevens, K.J. Livak, and P.M. Williams, 1996: Real Time Quantitative PCR. *Genome Res.* **6**, 986–994.

Hill, F.I., M.P. Reichel, and D.J. Tisdall, 2010: Use of molecular and milk production information for the cost-effective diagnosis of bovine viral diarrhoea infection in New Zealand dairy cattle. *Vet. Microbiol.* **142**, 87–89, DOI: 10.1016/j.vetmic.2009.09.047.

Hoinville, L.J., L. Alban, J.A. Drewe, J.C. Gibbens, L. Gustafson, B. Häsler, C. Saegerman, M. Salman, and K.D.C. Stärk, 2013: Proposed terms and concepts for describing and evaluating animal-health surveillance systems. *Prev. Vet. Med.* **112**, 1–12, DOI: 10.1016/j.prevetmed.2013.06.006.

Holland, P.M., R.D. Abramson, R. Watson, and D.H. Gelfand, 1991: Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 7276–80.

House of Commons, 2002: The 2001 Outbreak of Foot and Mouth Disease. *Natl. Audit Off.*1–138.

Howson, E.L.A., B. Armson, N.A. Lyons, E. Chepkwony, C.J. Kasanga, S. Kandusi, N. Ndusilo, W. Yamazaki, D. Gizaw, S. Cleaveland, T. Lembo, R. Rauh, W.M. Nelson, B.A. Wood, V. Mioulet, D.P. King, and V.L. Fowler, 2018: Direct detection and characterization of foot-and-mouth disease virus in East Africa using a field-ready real-time PCR platform. *Transbound. Emerg. Dis.* **65**, 221–231, DOI: 10.1111/tbed.12684.

Howson, E.L.A., B. Armson, M. Madi, C.J. Kasanga, S. Kandusi, R. Sallu, E. Chepkwony, A. Siddle, P. Martin, J. Wood, V. Mioulet, D.P. King, T. Lembo, S. Cleaveland, and V.L. Fowler, 2017: Evaluation of Two Lyophilized Molecular Assays to Rapidly Detect Foot-and-Mouth Disease Virus Directly from Clinical Samples in Field Settings. *Transbound. Emerg. Dis.* **64**, 861–871, DOI: 10.1111/tbed.12451.

Hutber, A.M., R.P. Kitching, and D.A. Conway, 1999: Predicting the level of herd infection for outbreaks of foot-and-mouth disease in vaccinated herds. *Epidemiol. Infect.* **122**, 539–44, DOI: 10.1017/S0950268899002356.

IATA, 2019: Infectious Substances Shipping Guidelines, 60th Edition.

Jamal, S.M., and G.J. Belsham, 2013: Foot-and-mouth disease: Past, present and future. *Vet. Res.* **44**, 1–14, DOI: 10.1186/1297-9716-44-116.

Jamal, S.M., and G.J. Belsham, 2015: Development and Characterization of Probe-Based Real Time Quantitative RT-PCR Assays for Detection and Serotyping of Foot-And-Mouth Disease Viruses Circulating in West Eurasia. *PLoS One* **10**, e0135559, DOI: 10.1371/journal.pone.0135559.

James, A.D., and J. Rushton, 2002: The economics of foot and mouth disease. *Rev. Sci. Tech.* **21**, 637–644.

Jemberu, W.T., M.C.M. Mourits, T. Woldehanna, and H. Hogeveen, 2014: Economic impact of foot and mouth disease outbreaks on smallholder farmers in Ethiopia. *Prev. Vet. Med.* **116**, 26–36, DOI: 10.1016/j.prevetmed.2014.06.004.

Johansson, M.K., 2006: Choosing reporter-quencher pairs for efficient quenching through formation of intramolecular dimers. *Methods Mol. Biol.* **335**, 17–29, DOI: 10.1385/1-59745-069-3:17.

Kamel, M., A. El-Sayed, and H. Castañeda Vazquez, 2019: Foot-and-mouth disease vaccines: recent updates and future perspectives. *Arch. Virol.* **164**, 1501–1513, DOI: 10.1007/s00705-019-04216-x.

Karanja, A.M., 2003: The Dairy Industry in Kenya: The Post-Liberalization Agenda. In: Dairy industry stakeholders workshop. *Dairy Ind. stakeholders Work. Tegemeo*

Inst. Agric. Policy Dev. Egert. Univ. Nairobi, Kenya.

Karber, G., 1931: 50% end-point calculation. *Arch. EXp. Pathol. Pharmak* **162**, 480–483.

Kasambula, L., G.J. Belsham, H.R. Siegismund, V.B. Muwanika, A.R. Ademun-Okurut, and C. Masembe, 2012: Serotype Identification and VP1 Coding Sequence Analysis of Foot-and-Mouth Disease Viruses from Outbreaks in Eastern and Northern Uganda in 2008/9. *Transbound. Emerg. Dis.* **59**, 323–330, DOI: 10.1111/j.1865-1682.2011.01276.x.

Kasanga, C.J., J. Wadsworth, C. a. R. Mpelumbe-Ngeleja, R. Sallu, F. Kivaria, P.N. Wambura, M.G.S. Yongolo, M.M. Rweyemamu, N.J. Knowles, and D.P. King, 2015: Molecular Characterization of Foot-and-Mouth Disease Viruses Collected in Tanzania Between 1967 and 2009. *Transbound. Emerg. Dis.* **62**, e19–e29, DOI: 10.1111/tbed.12200.

Kasanga, C.J., R. Sallu, F. Kivaria, M. Mkama, J. Masambu, M. Yongolo, S. Das, C. Mpelumbe-Ngeleja, P.N. Wambura, D.P. King, and M.M. Rweyemamu, 2012: Foot-and-mouth disease virus serotypes detected in Tanzania from 2003 to 2010: Conjectured status and future prospects. *Onderstepoort J. Vet. Res.* **79**, DOI: 10.4102/ojvr.v79i2.462.

Kasza, L., J.A. Shadduck, and G.J. Christofinis, 1972: Establishment, Viral Susceptibility and Biological Characteristics of a Swine Kidney Cell Line SK-6. *Res. Vet. Sci.* **13**, 46–53.

Kazimi, S.E., and S.K. Shah, 1980: Effect on production performance in cattle due to foot-and-mouth disease. *Bull. l'Office Int. des Epizoot.* **92**, 159–166.

Kerfua, S., G. Shirima, L. Kusiluka, C. Ayebazibwe, R. Mwebe, S. Cleaveland, and D. Haydon, 2018: Spatial and temporal distribution of foot-and-mouth disease in four districts situated along the Uganda – Tanzania border: Implications for cross-border efforts in disease control. *Onderstepoort J. Vet. Res.* **85**, 1–8.

Kibore, B., C.G. Gitao, A. Sangula, and P. Kitala, 2013: Foot and mouth disease sero-prevalence in cattle in Kenya. *J. Vet. Med. Anim. Heal.* **5**, 262–268, DOI: 10.5897/JVMAH2013.0220.

Kim, S.G., E.H. Kim, C.J. Lafferty, and E. Dubovi, 2005: Coxiella burnetii in bulk tank milk samples, United States. *Emerg. Infect. Dis.* **11**, 619–621, DOI: 10.3201/eid1104.041036.

Kimani, T.M., J.W. Mwirigi, and R.M. Murithi, 2005: Financial Impact Assessment of Foot and Mouth Disease in Large Scale Farms in Nakuru District, Kenya. *Kenya Vet.* **29**, 7–9.

Kimura, M., 1980: A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111–120.

King, D.J., G.L. Freimanis, R.J. Orton, R.A. Waters, D.T. Haydon, and D.P. King, 2016: Investigating intra-host and intra-herd sequence diversity of foot-and-mouth disease virus. *Infect. Genet. Evol.* **44**, 286–292, DOI: 10.1016/j.meegid.2016.07.010.

King, D.P., N.P. Ferris, A.E. Shaw, S.M. Reid, G.H. Hutchings, A.C. Giuffre, J.M. Robida, J.D. Callahan, W.M. Nelson, and T.R. Beckham, 2006: Detection of foot-and-mouth disease virus: comparative diagnostic sensitivity of two independent real-time reverse transcription-polymerase chain reaction assays. *J. Vet. Diagn. Invest.* **18**, 93–97, DOI: 10.1177/104063870601800114.

King, D.P., M. Madi, V. Mioulet, J. Wadsworth, C.F. Wright, B. Valdazo-González, N.P. Ferris, N.J. Knowles, and J. Hammond, 2012: New technologies to diagnose and monitor infectious diseases of livestock: Challenges for sub-Saharan Africa. *Onderstepoort J. Vet. Res.* **79**, 1–6, DOI: 10.4102/ojvr.v79i2.456.

Kitching, R.P., 2002: Clinical variation in foot and mouth disease: cattle. *Rev. Sci. Tech.* **21**, 499–504.

Kitching, R.P., and G.J. Hughes, 2002: Clinical variation in foot and mouth disease: sheep and goats. *Rev. Sci. Tech.* **21**, 505–512.

Kivaria, F.M., 2003: Foot and mouth disease in Tanzania: An overview of its national status. *Vet. Q.* **25**, 72–78, DOI: 10.1080/01652176.2003.9695147.

Knight-Jones, T.J.D., A.N. Bulut, S. Gubbins, K.D.C. Stärk, D.U. Pfeiffer, K.J. Sumption, and D.J. Paton, 2014: Retrospective evaluation of foot-and-mouth disease vaccine effectiveness in Turkey. *Vaccine* **32**, 1848–1855, DOI: 10.1016/j.vaccine.2014.01.071.

Knight-Jones, T.J.D., M. McLaws, and J. Rushton, 2016: Foot-and-Mouth Disease Impact on Smallholders - What Do We Know, What Don't We Know and How Can We Find Out More? *Transbound. Emerg. Dis.*1–15, DOI: 10.1111/tbed.12507.

Knight-Jones, T.J.D., and J. Rushton, 2013: The economic impacts of foot and mouth disease – What are they, how big are they and where do they occur? *Prev. Vet. Med.* **112**, 161–173, DOI: 10.1016/j.prevetmed.2013.07.013.

Knowles, N. J., K. Bachanek-Bankowska, J. Wadsworth, V. Mioulet, B. Valdazo-González, I.M. Eldaghayes, A.S. Dayhum, A.M. Kammon, M.A. Sharif, S. Waight, A.M. Shamia, S. Tenzin, U. Wernery, S. Grazioli, E. Brocchi, S. Subramaniam, B. Pattnaik, and D.P. King, 2015: Outbreaks of Foot-and-Mouth Disease in Libya and Saudi Arabia During 2013 Due to an Exotic O/ME-SA/Ind-2001 Lineage Virus. *Transbound. Emerg. Dis.* **2**, 1–5, DOI: 10.1111/tbed.12299.

Knowles, N.J., J. Wadsworth, K. Bachanek-Bankowska, and D.P. King, 2016: VP1 sequencing protocol for foot and mouth disease virus molecular epidemiology. *Rev. Sci. Tech. l'OIE* **35**, 741–755, DOI: 10.20506/rst.35.3.2565.

Knowles, N.J, and a. . Samuel, 2003: Molecular epidemiology of foot-and-mouth disease virus. *Virus Res.* **91**, 65–80, DOI: 10.1016/S0168-1702(02)00260-5.

Knowles, Nick J., J. Wadsworth, K. Bachanek-Bankowska, and D.P. King, 2016: VP1 sequencing protocol for foot-and-mouth disease virus molecular epidemiology. *Rev. Sci. Tech. l'OIE* **35**, 741–755, DOI: 10.20506/rst.35.3.2565.

Kompas, T., P. Van Ha, H.T.M. Nguyen, I. East, S. Roche, and G. Garner, 2017: Optimal surveillance against foot-and-mouth disease: the case of bulk milk testing in Australia. *Aust. J. Agric. Resour. Econ.* **61**, 515–538, DOI: 10.1111/1467-8489.12224.

Kuijk, H., P. Franken, M.H. Mars, W. Bij De Weg, and B. Makoschey, 2008: Monitoring of

BVDV in a vaccinated herd by testing milk for antibodies to NS3 protein. *Vet. Rec.* **163**, 482–484, DOI: 10.1136/vr.163.16.482.

Kumar, S., G. Stecher, and K. Tamura, 2016: MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **33**, 1870–4, DOI: 10.1093/molbev/msw054.

Kurwijila, L.R., A. Omore, and D. Grace, 2012: Tanzania Dairy Industry Overview 2012.

Landis, J.R., and G.G. Koch, 1977: The measurement of observer agreement for categorical data. *Biometrics* **33**, 159–174.

LaRocco, M., P.W. Krug, E. Kramer, Z. Ahmed, J.M. Pacheco, H. Duque, B. Baxt, and L.L. Rodriguez, 2013: A continuous bovine kidney cell line constitutively expressing bovine αVβ6 integrin has increased susceptibility to foot-and-mouth disease virus. *J. Clin. Microbiol.* **51**, 1714–1720, DOI: 10.1128/JCM.03370-12.

Lebailly, C., 1920: La virulence du lait dans la fievre aphteuse. *Compte rendu l'Academie des Sci. Paris* **171**.

Lee, F., M.H. Jong, and D.W. Yang, 2006: Presence of antibodies to non-structural proteins of foot-and-mouth disease virus in repeatedly vaccinated cattle. *Vet. Microbiol.* **115**, 14–20, DOI: 10.1016/j.vetmic.2005.12.017.

Leeuw, P.W. de, J.G. van Bekkum, and J.W.A. Tiessink, 1978: Excretion of foot-and-mouth disease virus in oesophageal-pharyngeal fluid and milk of cattle after intranasal infection. *J. Hyg. (Lond).* **81**, 415.

Legesse, Y., Y. Asfaw, M. Sahle, G. Ayelet, S. Jenberie, and H. Negussie, 2013: First confirmation of foot and mouth disease virus serotype SAT-1 in cattle and small ruminants in Ethiopia in 2007/08. *Trop. Anim. Health Prod.* **45**, 1265–1267, DOI: 10.1007/s11250-012-0339-2.

Lewerin, S.S., C. Wolff, C. Masembe, K. Ståhl, S. Boqvist, and M.A. Franko, 2018: Methodological aspects of serosurveillance in resource-poor settings. *Vet. Rec. Open* **5**, 1–7, DOI: 10.1136/vetreco-2017-000273.

Liu, Y., Y. Ding, J. Dai, B. Ma, J. He, W. Ma, J. Lv, X. Ma, Y. Ou, J. Wang, Y. Liu, H. Chang, Y.

Wang, and Q. Zhang, 2018: Development of a new RT-PCR with multiple primers for detecting Southern African Territories foot-and-mouth disease viruses. **2004**, DOI: 10.2478/jvetres-2018-0064.

Lloyd-Jones, K., M. Mahapatra, S. Upadhyaya, D.J. Paton, A. Babu, G. Hutchings, and S. Parida, 2017: Genetic and antigenic characterization of serotype O FMD viruses from East Africa for the selection of suitable vaccine strain. *Vaccine* **35**, 6842–6849, DOI: 10.1016/j.vaccine.2017.10.040.

Logan, G., G.L. Freimanis, D.J. King, B. Valdazo-González, K. Bachanek-Bankowska, N.D. Sanderson, N.J. Knowles, D.P. King, and E.M. Cottam, 2014: A universal protocol to generate consensus level genome sequences for foot-and-mouth disease virus and other positive-sense polyadenylated RNA viruses using the Illumina MiSeq. *BMC Genomics* **15**, 828, DOI: 10.1186/1471-2164-15-828.

Lohse, L., J. Arzt, G.J. Belsham, A. Bøtner, and C. Stenfeldt, 2018: Transmission of Foot-and-Mouth Disease from Persistently Infected Carrier Cattle to Naive Cattle via Transfer of Oropharyngeal Fluid. *mSphere* **3**, 1–12, DOI: 10.1128/msphere.00365-18.

Lombard, J., M. Remmenga, and K. Moreno-Torres, 2017: Potential uses of a rRT-PCR assay for FMD in bulk-tank milk in the United States.

Lyons, N.A., N. Alexander, K.D.C. Stärk, T.D. Dulu, K.J. Sumption, A.D. James, J. Rushton, and P.E.M. Fine, 2015: Impact of foot-and-mouth disease on milk production on a large-scale dairy farm in Kenya. *Prev. Vet. Med.* **120**, 177–86, DOI: 10.1016/j.prevetmed.2015.04.004.

Lyons, N.A., N. Alexander, K.D. Stärk, T.D. Dulu, J. Rushton, and P.E. Fine, 2015: Impact of foot-and-mouth disease on mastitis and culling on a large-scale dairy farm in Kenya. *Vet. Res.* **46:41**, DOI: 10.1186/s13567-015-0173-4.

Lyons, N.A., A.B. Ludi, G. Wilsden, P. Hamblin, I.A. Qasim, S. Gubbins, and D.P. King, 2017: Evaluation of a polyvalent foot-and-mouth disease virus vaccine containing A Saudi-95 against field challenge on large-scale dairy farms in Saudi Arabia with the emerging A/ASIA/G-VII viral lineage. *Vaccine* **35**, 6850–6857, DOI:

10.1016/J.VACCINE.2017.10.029.

Lyons, N.A., K.D.C. Stärk, K. van Maanen, S.L. Thomas, E.C. Chepkwony, A.K. Sangula, T.D. Dulu, and P.E.M. Fine, 2015: Epidemiological analysis of an outbreak of foot-and-mouth disease (serotype SAT2) on a large dairy farm in Kenya using regular vaccination. *Acta Trop.* **143**, 103–111, DOI: 10.1016/j.actatropica.2014.09.010.

Machira, D., and P. Kitala, 2017: Epidemiological Analysis of Passive Surveillance Data on Foot and Mouth Disease Occurrence in Nakuru County, Kenya. *J. Dairy, Vet. Anim. Res.* **6**, 3–6, DOI: 10.15406/jdvar.2017.06.00178.

Madi, M., A. Hamilton, D. Squirrell, V. Mioulet, P. Evans, M. Lee, and D.P. King, 2012: Rapid detection of foot-and-mouth disease virus using a field-portable nucleic acid extraction and real-time PCR amplification platform. *Vet. J.* **193**, 67–72, DOI: 10.1016/j.tvjl.2011.10.017.

Maree, F., L.M. de Klerk-Lorist, S. Gubbins, F. Zhang, J. Seago, E. Pérez-Martín, L. Reid, K. Scott, L. van Schalkwyk, R. Bengis, B. Charleston, and N. Juleff, 2016: Differential persistence of foot-and-mouth disease virus in African buffalo is related to virus virulence. *J. Virol.* **90**, JVI.00166-16, DOI: 10.1128/JVI.00166-16.

Marquardt, O., and K.-H. Adam, 1990: Foot-and-mouth disease virus subtyping by sequencing VP1 genes. *Vet. Microbiol.* **23**, 175–183, DOI: 10.1016/0378-1135(90)90147-N.

Marquardt, O., O.C. Straub, R. Ahl, and B. Haas, 1995: Detection of foot-and-mouth disease virus in nasal swabs of asymptomatic cattle by RT-PCR within 24 hours. *J. Virol. Methods* **53**, 255–261, DOI: 10.1016/0166-0934(95)00015-M.

Marston, D. A., L.M. McElhinney, R.J. Ellis, D.L. Horton, E.L. Wise, S.L. Leech, D. David, X. De Lamballerie, and A.R. Fooks, 2013: Next generation sequencing of viral RNA genomes. *BMC Genomics* **14**, 444, DOI: 10.1186/1471-2164-14-444.

Mazengia, H., M. Taye, H. Negussie, S. Alemu, and A. Tassew, 2010: Incidence of foot and mouth disease and its effect on milk yield in dairy cattle at Andassa dairy farm, Northwest Ethiopia. *Agric. Biol. J. North Am.* **1**, 969–973, DOI: 10.5251/abjna.2010.1.5.969.973.

Morgan, K.L., I.G. Handel, V.N. Tanya, S.M. Hamman, C. Nfon, I.E. Bergman, V. Malirat, K.J. Sorensen, and B.M. d. C. Bronsvoort, 2014: Accuracy of herdsmen reporting versus serologic testing for estimating foot-and-mouth disease prevalence. *Emerg. Infect. Dis.* **20**, 2048–2054, DOI: 10.3201/eid2012.140931.

Mulei, C.M., J.K. Wabacha, and P.M. Mbithi, 2001: Short-term economic impact of Foot and Mouth disease outbreak in a large Diary Farm in Kiambu District, Kenya. *Kenya Vet.* **22**, 76–78.

Muriuki, H., 2011: Dairy development in Kenya *Dairy Reports*.

Musallam, I., J. McGiven, and A. Akakpo, 2017: Using ELISA for the detection of Brucella antibodies in milk.

Nakazawa, M., 2017: Package "fmsb." .

Namatovu, A., S.N. Wekesa, K. Tjørnehøj, M.T. Dhikusooka, V.B. Muwanika, H.R. Siegsmund, and C. Ayebazibwe, 2013: Laboratory capacity for diagnosis of foot-and-mouth disease in Eastern Africa: implications for the progressive control pathway. *BMC Vet. Res.* **9**, 19, DOI: 10.1186/1746-6148-9-19.

Nardelli, L., E. Lodetti, G.L. Gualandi, R. Burrows, D. Goodridge, F. Brown, and B. Cartwright, 1968: A foot and mouth disease syndrome in pigs caused by an enterovirus. *Nature* **219**, 1275–6.

Nelson, N., D.J. Paton, S. Gubbins, C. Colenutt, E. Brown, S. Hodgson, and J.L. Gonzales, 2017: Preclinical diagnosis: predicting its ability to improve control of farm-to-farm foot-and-mouth disease transmission in cattle. *J. Clin. Microbiol.*JCM.00179-17, DOI: 10.1128/JCM.00179-17.

Niedbalski, W., 2016: Recent progress in the diagnosis of foot-and-mouth disease: Rapid field-based assays. *Med. Weter.* **72**, 339–344, DOI: 10.21521/mw.5524.

Nielsen, P.K., M.B. Petersen, L.R. Nielsen, T. Halasa, and N. Toft, 2015: Latent class analysis of bulk tank milk PCR and ELISA testing for herd level diagnosis of Mycoplasma bovis. *Prev. Vet. Med.* **121**, 338–342, DOI: 10.1016/j.prevetmed.2015.08.009.

Nishi, T., T. Kanno, N. Shimada, K. Morioka, M. Yamakawa, and K. Fukai, 2019: Reverse transcription-PCR using a primer set targeting the 3D region detects foot-and-mouth disease virus with high sensitivity. *Transbound. Emerg. Dis.*DOI: 10.1111/tbed.13202.

Niskanen, R., S. Alenius, B. Larsson, and S.-O. Jacobsson, 1991: Determination of level of antibodies to bovine virus diarrhoea virus (BVDV) in bulk tank milk as a tool in the diagnosis and prophylaxis of BVDV infections in dairy herds, pp. 245–251. In: Liess B., Moennig V., Pohlenz J., Trautwein G. (eds) Ruminant Pestivirus Infections. Archives of Virology (Supplementum 3), vol3.Springer, Vienna.

Nthiwa, D., S. Alonso, D. Odongo, E. Kenya, and B. Bett, 2019: A participatory epidemiological study of major cattle diseases amongst Maasai pastoralists living in wildlife-livestock interfaces in Maasai Mara , Kenya. *Trop. Anim. Health Prod.*DOI: https://doi.org/10.1007/s11250-018-01790-1.

Nyaguthii, D.M., B. Armson, P.M. Kitala, B. Sanz-Bernardo, A. Di Nardo, and N.A. Lyons, 2019: Knowledge and risk factors for foot-and-mouth disease among small-scale dairy farmers in an endemic setting. *Vet. Res.* **50**, 33, DOI: 10.1186/s13567-019-0652-0.

OIE, 2017a: Foot and mouth disease (infection with foot and mouth disease virus). *Man. Diagnostic Tests Vaccines Terr. Anim.* **2.1.8**.

OIE, 2017b: The 12th Annual Meeting of the OIE/FAO FMD Reference Laboratories Network.

OIE, 2018: Foot and mouth disease (infection with foot and mouth disease virus)*OIE Terr. Man.*

OIE, 2019a: Chapter 8.8. Infection With Foot and Mouth Disease. *Terr. Anim. Heal. Code*1–24.

OIE, 2019b: List of FMD free Members. Resolution No.15. 87th General Session of World Assembly.

Omore, A., H. Muriuki, M. Kenyanjui, M. Owango, and S. Staal, 1999: THE KENYAN

DAIRY SUB-SECTOR A Rapid Appraisal - Smallholder Dairy (Research & Development) Project Report. 57.

Onono, J.O., B. Wieland, and J. Rushton, 2013: Constraints to cattle production in a semiarid pastoral system in Kenya. *Trop. Anim. Health Prod.* **45**, 1415–1422, DOI: 10.1007/s11250-013-0379-2.

Orsel, K., M.C.M. de Jong, A. Bouma, J.A. Stegeman, and A. Dekker, 2007: The effect of vaccination on foot and mouth disease virus transmission among dairy cows. *Vaccine* **25**, 327–335, DOI: 10.1016/j.vaccine.2006.07.030.

Orton, R.J., C.F. Wright, M.J. Morelli, N. Juleff, G. Thébaud, N.J. Knowles, B. Valdazo-González, D.J. Paton, D.P. King, and D.T. Haydon, 2013: Observing micro-evolutionary processes of viral populations at multiple scales. *Philos. Trans. R. Soc. Biol. Sci.* **368**, 20120203, DOI: 10.1098/rstb.2012.0203.

Palladino, R.A., F. Buckley, R. Prendiville, J.J. Murphy, J. Callan, and D.A. Kenny, 2010: A comparison between Holstein-Friesian and Jersey dairy cows and their F1 hybrid on milk fatty acid composition under grazing conditions. *J. Dairy Sci.* **93**, 2176–2184, DOI: 10.3168/jds.2009-2453.

Parida, S., 2009: Vaccination against foot-and-mouth disease virus: strategies and effectiveness. *Expert Rev. Vaccines* **8**, 347–365, DOI: 10.1586/14760584.8.3.347.

Parthiban, A.B.R., M. Mahapatra, S. Gubbins, and S. Parida, 2015: Virus excretion from foot-and-mouth disease virus carrier cattle and their potential role in causing new outbreaks. *PLoS One* **10**, 1–13, DOI: 10.1371/journal.pone.0128815.

Paton, D. J., K.J. Sumption, and B. Charleston, 2009: Options for control of foot-and-mouth disease: knowledge, capability and policy. *Philos. Trans. R. Soc. B Biol. Sci.* **364**, 2657–2667, DOI: 10.1098/rstb.2009.0100.

Paton, D J, K.H. Christiansen, S. Alenius, M.P. Cranwell, G.C. Pritchard, and T.W. Drew, 1998: Prevalence of antibodies to bovine virus diarrhoea virus and other viruses in bulk tank milk in England and Wales. *Vet Rec.* **142**, 385–391, DOI: 10.1136/vr.142.15.385.

Paton, D.J., K. de Clercq, M. Greiner, A. Dekker, E. Brocchi, I. Bergmann, D.J. Sammin, S. Gubbins, and S. Parida, 2006: Application of non-structural protein antibody tests in substantiating freedom from foot-and-mouth disease virus infection after emergency vaccination of cattle. *Vaccine* **24**, 6503–6512, DOI: 10.1016/j.vaccine.2006.06.032.

Picado, A., N. Speybroeck, F. Kivaria, R.M. Mosha, R.D. Sumaye, J. Casal, and D. Berkvens, 2011: Foot-and-Mouth Disease in Tanzania from 2001 to 2006. *Transbound. Emerg. Dis.* **58**, 44–52, DOI: 10.1111/j.1865-1682.2010.01180.x.

Poonsuk, K., L. Giménez-Lirola, and J.J. Zimmerman, 2018: A review of foot-and-mouth disease virus (fmdv) testing in livestock with an emphasis on the use of alternative diagnostic specimens. *Anim. Heal. Res. Rev.* **19**, 100–112, DOI: 10.1017/S1466252318000063.

Power, A.P., and S.A. Harris, 1973: A Cost-Benefit Evaluation of Alternative Control Policies for Foor-and-Mouth Disease in Great Britain. *J. Agric. Econ.* **24**, 573–600.

R Core Team, 2019: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/. .

Rademaker, I., R. Koech, A. Jansen, and J. van der Lee, 2016: Smallholder Dairy Value Chain Interventions. The Kenya Market-led Dairy Programme (KMDP) – Status Report. .

Radström, P., and W.A. Al-soud, 2001: Purification and Characterization of PCR-Inhibitory Components in Blood Cells. *J. Clin. Microbiol.* **39**, 485–493, DOI: 10.1128/JCM.39.2.485.

Ranjan, R., J.K. Biswal, G.K. Sharma, A.K. Sharma, K.P. Singh, J. Misri, and B. Pattnaik, 2016: Use of nucleic acid recognition methods (m-PCR and RT-LAMP) for the detection of foot-and-mouth disease virus excreted in cow milk. *Indian J. Anim. Sci.* **86**, 865–868.

Recheis, D., 2019: The Renewable Energy and Energy Efficiency Partnership, Sustainable Milk Cooling – Entry points in the Kenyan and Indian dairy sectors

[Online] Available at https://www.reeep.org/sustainable-milk-cooling-–-entry-points-kenyan-and-indian-dairy-sectors.

Reichel, M.P., S.R. Lanyon, and F.I. Hill, 2016: Moving past serology: Diagnostic options without serum. *Vet. J.*DOI: 10.1016/j.tvjl.2016.04.010.

Reid, S.M., K. Ebert, K. Bachanek-Bankowska, C. Batten, a. Sanders, C. Wright, a. E. Shaw, E.D. Ryan, G.H. Hutchings, N.P. Ferris, D.J. Paton, and D.P. King, 2009: Performance of Real-Time Reverse Transcription Polymerase Chain Reaction for the Detection of Foot-and-Mouth Disease Virus during Field Outbreaks in the United Kingdom in 2007. *J. Vet. Diagnostic Investig.* **21**, 321–330, DOI: 10.1177/104063870902100303.

Reid, S.M, M.. Forsyth, G.. Hutchings, and N.. Ferris, 1998: Comparison of reverse transcription polymerase chain reaction, enzyme linked immunosorbent assay and virus isolation for the routine diagnosis of foot-and-mouth disease. *J. Virol. Methods* **70**, 213–217, DOI: 10.1016/S0166-0934(97)00181-X.

Reid, S.M, N.P. Ferris, G.H. Hutchings, K. De Clercq, B.J. Newman, N.J. Knowle, and a R. Samuel, 2001: Diagnosis of foot-and-mouth disease by RT-PCR: use of phylogenetic data to evaluate primers for the typing of viral RNA in clinical samples. *Arch. Virol.* **146**, 2421–34, DOI: 10.1007/s007050170012.

Reid, S.M., N.P. Ferris, G.H. Hutchings, Z. Zhang, G.J. Belsham, and S. Alexandersen, 2002: Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. *J. Virol. Methods* **105**, 67–80, DOI: 10.1016/S0166-0934(02)00081-2.

Reid, S.M., V. Mioulet, N.J. Knowles, N. Shirazi, G.J. Belsham, and D.P. King, 2014: Development of tailored real-time RT-PCR assays for the detection and differentiation of serotype O, A and Asia-1 foot-and-mouth disease virus lineages circulating in the Middle East. *J. Virol. Methods* **207**, 146–153, DOI: 10.1016/j.jviromet.2014.07.002.

Reid, S.M., S. Parida, D.P. King, G.H. Hutchings, A.E. Shaw, N.P. Ferris, Z. Zhang, J.E. Hillerton, and D.J. Paton, 2006: Utility of automated real-time RT-PCR for the

detection of foot-and-mouth disease virus excreted in milk. *Vet. Res.* **37**, 121–132, DOI: 10.1051/vetres:2005040.

Reid, S.M, S.S. Grierson, N.P. Ferris, G.H. Hutchings, and S. Alexandersen, 2003: Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *J. Virol. Methods* **107**, 129–139, DOI: 10.1016/S0166-0934(02)00210-0.

Renshaw, R.W., R. Ray, and E.J. Dubovi, 2000a: Comparison of virus isolation and reverse transcription polymerase chain reaction assay for detection of bovine viral diarrhea virus in bulk milk tank samples. *J Vet Diagn Invest* **12**, 184–186, DOI: 10.1177/104063870001200219.

Renshaw, R.W., R. Ray, and E.J. Dubovi, 2000b: Comparison of virus isolation and reverse transcription polymerase chain reaction assay for detection of bovine viral diarrhea virus in bulk milk tank samples. *J Vet Diagn Invest* **12**, 184–186, DOI: 10.1177/104063870001200219.

Roberts, L.C., and G.T. Fosgate, 2018: Stakeholder perceptions of foot-and-mouth disease control in South Africa. *Prev. Vet. Med.* **156**, 38–48, DOI: 10.1016/j.prevetmed.2018.05.001.

Robinson, T.P., P.. Thornton, G. Franceschini, R.L. Kruska, F. Chiozza, A. Notenbaert, G. Cecchi, M. Herrero, M. Epprecht, S. Fritz, L. You, G. Conchedda, and L. See, 2011: Global livestock production systems, p. 152. In: Rome, Food and Agriculture Organization of the United Nations (FAO) and International Livestock Research Institute (ILRI).

Robinson, T.P., G.R.W. Wint, G. Conchedda, T.P. Van Boeckel, V. Ercoli, E. Palamara, G. Cinardi, L. D'Aietti, S.I. Hay, and M. Gilbert, 2014: Mapping the Global Distribution of Livestock. *PLoS One* **9**, e96084, DOI: https://doi.org/10.1371/journal.pone.0096084.

Robson, K.J., T.J. Harris, and F. Brown, 1977: An assessment by competition hybridization of the sequence homology between the RNAs of the seven serotypes of FMDV. *J Gen Virol* **37**, 271–276, DOI: 10.1099/0022-1317-37-2-271.

Rodriguez, L.L., and C.G. Gay, 2011: Development of vaccines toward the global control and eradication of foot-and-mouth disease. *Expert Rev. Vaccines* **10**, 377–387.

Rodriquez, L.L., and S.T. Nichol, 1999: Vesicular Stomatitis Viruses (Rhabdoviridae), pp. 1910–1919. In: Encyclopedia of Virology (Second Edition).

Rolfe, D.C., and W.E. Sykes, 1987: Monitoring of dairy herds for Brucella abortus infection when prevalence is low. *Aust. Vet. J.* **64**, 97–100.

Romey, A., A. Relmy, K. Gorna, E. Laloy, S. Zientara, S. Blaise-Boisseau, and L. Bakkali Kassimi, 2018: Safe and cost-effective protocol for shipment of samples from Foot-and-Mouth Disease suspected cases for laboratory diagnostic. *Transbound. Emerg. Dis.* **65**, 197–204, DOI: 10.1111/tbed.12648.

Rossen, L., P. Norskov, K. Holmstrom, and O.F. Rasmussen, 1992: Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *Int. J. Food Microbiol.* **17**, 37–45.

RStudio Team, 2019: RStudio: Integrated Development for R. RStudio Inc., Boston, MA. URL http://www.rstudio.com/. .

Rufael, T., A. Catley, A. Bogale, M. Sahle, and Y. Shiferaw, 2008: Foot and mouth disease in the Borana pastoral system, southern Ethiopia and implications for livelihoods and international trade. *Trop. Anim. Health Prod.* **40**, 29–38, DOI: 10.1007/s11250-007-9049-6.

Rushton, J., T. Knight-Jones, A. Donaldson, P. de Leeuw, G. Ferrari, and J. Domenech, 2012: The impact of foot and mouth disease. FAO/OIE Global conference on foot and mouth disease control. p. Bangkok, Thailand. .

Rweyemamu, M.M., 1984: Antigenic variation in foot-and-mouth disease: studies based on the virus neutralization reaction. *J. Biol. Stand.* **13**, 323–337, DOI: 10.1016/S0092-1157(84)80013-X.

Ryan, E., J. Gloster, S.M. Reid, Y. Li, N.P. Ferris, R. Waters, N. Juleff, B. Charleston, B. Bankowski, S. Gubbins, J.W. Wilesmith, D.P. King, and D.J. Paton, 2008: Clinical and laboratory investigations of the outbreaks of foot-and-mouth disease in southern

England in 2007. *Vet. Rec.* **163**, 139–147, DOI: 10.1136/vr.163.5.139.

Saduakassova, M.A., A.A. Sultanov, L.B. Kutumbetov, J. Wadsworth, B.A. Wood, N.J. Knowles, D.P. King, and K. Bachanek-Bankowska, 2017: Development and evaluation of a novel real-time RT-PCR to detect foot-and-mouth disease viruses from the emerging A/ASIA/G-VII lineage. *J. Virol. Methods* **252**, 37–41, DOI: 10.1016/j.jviromet.2017.10.023.

Saeed, A., Q.M. Khan, U. Waheed, M. Arshad, M. Asif, and M. Farooq, 2011: RT-PCR evaluation for identification and sequence analysis of foot-and-mouth disease serotype O from 2006 to 2007 in Punjab, Pakistan. *Comp. Immunol. Microbiol. Infect. Dis.* **34**, 95–101, DOI: 10.1016/j.cimid.2009.10.004.

Saitou, N., and M. Nei, 1987: The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–25, DOI: 10.1093/oxfordjournals.molbev.a040454.

Salazar-Gonzalez, J.F., M.G. Salazar, G.H. Learn, G.G. Fouda, H.H. Kang, T. Mahlokozera, A.B. Wilks, R. V Lovingood, A. Stacey, L. Kalilani, S.R. Meshnick, P. Borrow, D.C. Montefiori, T.N. Denny, N.L. Letvin, G.M. Shaw, B.H. Hahn, and S.R. Permar, 2011: Origin and evolution of HIV-1 in breast milk determined by single-genome amplification and sequencing. *J. Virol.* **85**, 2751–63, DOI: 10.1128/JVI.02316-10.

Samuel, A.R., and N.J. Knowles, 2001: Foot-and-mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). *J. Gen. Virol.* **82**, 609–21.

Sanger, F., S. Nicklen, and A.R. Coulson, 1977: DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–7, DOI: 10.1073/pnas.74.12.5463.

Sangula, A. K., H.R. Siegismund, G.J. Belsham, S.N. Balinda, C. Masembe, and V.B. Muwanika, 2011: Low diversity of foot-and-mouth disease serotype C virus in Kenya: evidence for probable vaccine strain re-introductions in the field. *Epidemiol. Infect.* **139**, 189–196, DOI: 10.1017/s0950268810000580.

Sangula, A.K., G.J. Belsham, V.B. Muwanika, R. Heller, S.N. Balinda, C. Masembe, and H.R. Siegismund, 2010: Evolutionary analysis of foot-and-mouth disease virus

serotype SAT 1 isolates from east Africa suggests two independent introductions from southern Africa. *BMC Evol. Biol.* **10**, 371, DOI: 10.1186/1471-2148-10-371.

Saxena, R., 1994: Economic Value of Milk Loss Caused by Foot-and-Mouth Disease in India.

Schrader, C., A. Schielke, L. Ellerbroek, and R. Johne, 2012: PCR inhibitors - occurrence, properties and removal. *J. Appl. Microbiol.* **113**, 1014–1026, DOI: 10.1111/j.1365-2672.2012.05384.x.

Sekiya, M., A. Zintl, and M.L. Doherty, 2013: Bulk milk ELISA and the diagnosis of parasite infections in dairy herds: a review. *Ir. Vet. J.* **66**, 14, DOI: 10.1186/2046-0481-66-14.

Sellers, R.F., 1969: Inactivation of foot-and-mouth disease virus in milk. *Br. Vet. J.* **125**, 163–168.

Senturk, B., and C. Yalcin, 2005: Financial impact of foot-and-mouth disease in Turkey: acquisition of required data via Delphi expert opinion survey. *Vet. Med. (Praha).* **50**, 451–460.

Sergeant, E., 2019: Epitools epidemiological calculators. Ausvet Pty Ltd. Available at: http://epitools.ausvet.com.au [Online].

Sharma, N., 2010: Foot and mouth disease-mastitis cascade in dairy cattle: A field study. *Int. J. Zool. Res.* **6**, 356–359, DOI: 10.3923/ijzr.2008.64.67.

Shaw, A.E, S.M. Reid, D.P. King, G.H. Hutchings, and N.P. Ferris, 2004: Enhanced laboratory diagnosis of foot and mouth disease by real-time polymerase chain reaction. *Rev. Sci. Tech.* **23**, 1003–1009, DOI: 10.20506/rst.23.3.1544.

Shaw, A.E., S.M. Reid, K. Ebert, G.H. Hutchings, N.P. Ferris, and D.P. King, 2007: Implementation of a one-step real-time RT-PCR protocol for diagnosis of foot-and-mouth disease. *J. Virol. Methods* **143**, 81–85, DOI: 10.1016/j.jviromet.2007.02.009.

Shen, F., P.D. Chen, A.M. Walfield, J. Ye, J. House, F. Brown, and C.Y. Wang, 1999: Differentiation of convalescent animals from those vaccinated against foot-and-mouth disease by a peptide ELISA. *Vaccine* **17**, 3039–3049, DOI: 10.1016/S0264-

410X(99)00148-6.

Simms, D., P.E. Cizdziel, and P. Chomczynski, 1993: Trizol ™- a New Reagent for Optimal Single-Step Isolation of RNA. *Focus (Madison).* **15**, 99–102.

Singh, K., S. Corner, S.G. Clark, G. Scherba, and R. Fredrickson, 2012: Seneca Valley Virus and Vesicular Lesions in a Pig with Idiopathic Vesicular Disease. *J. Vet. Sci. Technol.* **03**, DOI: 10.4172/2157-7579.1000123.

Smith, M.T., A.M. Bennett, M.J. Grubman, and B.C. Bundy, 2014: Foot-and-mouth disease: Technical and political challenges to eradication. *Vaccine* **32**, 3902–3908, DOI: 10.1016/j.vaccine.2014.04.038.

Snowdon, W.A., 1966: Growth of FMDV in monolayer cultures of calf thyroid cells. *Nature* **210**, 1079–1080.

Sørensen, K.J., K.G. Madsen, E.S. Madsen, J.S. Salt, J. Nqindi, and D.K. Mackay, 1998: Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. *Arch. Virol.* **143**, 1461–76, DOI: 10.1007/s007050050390.

Souley Kouato, B., F.M. Elliot, D.P. King, J. Hyera, N.J. Knowles, A.B. Ludi, V. Mioulet, G. Matlho, K. De Clercq, E. Thys, H. Marichatou, S. Issa, and C. Saegerman, 2018: Outbreak investigations and molecular characterization of foot-and-mouth disease viruses circulating in south-west Niger. *Transbound. Emerg. Dis.* **65**, 146–157, DOI: 10.1111/tbed.12642.

Spickler, A.R., and J.A. Roth, 2012: Inactivation of foot-and-mouth disease virus in milk products. *Vet. Microbiol. Prev. Med. Reports*.

Stenfeldt, C., M. Bertram, and J. Arzt, 2018: The complexities of subclinical foot-and-mouth disease. *Glob. Foot-and-Mouth Dis. Res. Alliance Newsl.*

Stenfeldt, C., M. Eschbaumer, J.M. Pacheco, S.I. Rekant, L.L. Rodriguez, and J. Arzt, 2015: Pathogenesis of primary foot-and-mouth disease virus infection in the nasopharynx of vaccinated and non-vaccinated cattle. *PLoS One* **10**, DOI:

10.1371/journal.pone.0143666.

Stenfeldt, C., M. Eschbaumer, S.I. Rekant, J.M. Pacheco, G.R. Smoliga, E.J. Hartwig, L.L. Rodriguez, and J. Arzt, 2016: The foot-and-mouth disease carrier state divergence in cattle. *J. Virol.* **90**, JVI.00388-16-, DOI: 10.1128/JVI.00388-16.

Stenfeldt, C., E.J. Hartwig, G.R. Smoliga, R. Palinski, E.B. Silva, M.R. Bertram, I.H. Fish, S.J. Pauszek, and J. Arzt, 2018: Contact Challenge of Cattle with Foot-and-Mouth Disease Virus Validates the Role of the Nasopharyngeal Epithelium as the Site of Primary and Persistent Infection. *mSphere* **3**, 1–18, DOI: 10.1128/msphere.00493-18.

Stenfeldt, C., L. Lohse, and G.J. Belsham, 2013: The comparative utility of oral swabs and probang samples for detection of foot-and-mouth disease virus infection in cattle and pigs. *Vet. Microbiol.* **162**, 330–337, DOI: 10.1016/j.vetmic.2012.09.008.

Stone, S.S., and P.D. DeLay, 1960: Serum and Colostral Antibody Levels in Cattle Convalescent from Foot-and-Mouth Disease: Tests in Calves and Fetal Tissue. *J. Immunol.* **84**, 458 LP – 462.

Sumption, K., M. Rweyemamu, and W. Wint, 2008: Incidence and distribution of foot-and-mouth disease in Asia, Africa and South America; Combining expert opinion, official disease information and livestock populations to assist risk assessment. *Transbound. Emerg. Dis.* **55**, 5–13, DOI: 10.1111/j.1865-1682.2007.01017.x.

Sutmoller, P., and O.R. Casas, 2002: Unapparent foot and mouth disease infection (sub-clinical infections and carriers): implications for control. *Rev. Sci. Tech.* **21**, 519–29.

TechnoServe Kenya, 2008: The Dairy Value Chain in Kenya.

Terbruggen, F., 1932: Survival of foot-and-mouth disease virus in milk and milk products. *Dtsch. Tierarztl. Wochenschr.* **40**, 129–134.

Thurmond, M.C., and A.M. Perez, 2006: Modeled detection time for surveillance for foot-and-mouth disease virus in bulk tank milk. *Am. J. Vet. Res.* **67**, 2017–2024, DOI: 10.2460/ajvr.67.12.2017.

Thwiny, H.T., 2016: Diagnosis of Carrier State of Foot and Mouth Disease Virus in Vaccinated and Unvaccinated Cattle By RT-PCR. *Basrah J. Vet. Res.* **15**, 30–38.

Tomasula, P.M., and R.P. Konstance, 2004: The Survival of Foot-and-Mouth Disease Virus in Raw and Pasteurized Milk and Milk Products. *J. Dairy Sci.* **87**, 1115–1121, DOI: 10.3168/jds.S0022-0302(04)73258-0.

Tomasula, P.M., M.F. Kozempel, R.P. Konstance, D. Gregg, S. Boettcher, B. Baxt, and L.L. Rodriguez, 2007: Thermal Inactivation of Foot-and-Mouth Disease Virus in Milk Using High-Temperature, Short-Time Pasteurization1. *J. Dairy Sci.* **90**, 3202–3211, DOI: 10.3168/jds.2006-525.

TPI, 2019: VAC-METH-10. Heat inactivation of serum. .

Uttenthal, A., S. Parida, T.B. Rasmussen, D.J. Paton, B. Haas, and W.G. Dundon, 2010: Strategies for differentiating infection in vaccinated animals (DIVA) for foot-and-mouth disease, classical swine fever and avian influenza. *Expert Rev. Vaccines* **9**, 73–87, DOI: 10.1586/erv.09.130.

Valdazo-González, B., N.J. Knowles, and D.P. King, 2014: Genome Sequences of Foot-and-Mouth Disease Virus O/ME-SA/Ind-2001 Lineage from Outbreaks in Libya, Saudi Arabia, and Bhutan during 2013. *Genome Announc.* **2**, 2013–2014, DOI: 10.1128/genomeA.00242-14.

Vanzini, V.R., N. Aguirre, C.I. Lugaresi, S.T. De Echaide, V.G. De Canavesio, A.A. Guglielmone, M.D. Marchesino, and K. Nielsen, 1998: Evaluation of an indirect ELISA for the diagnosis of bovine brucellosis in milk and serum samples in dairy cattle in Argentina. *Prev. Vet. Med.* **36**, 211–217, DOI: 10.1016/S0167-5877(98)00080-4.

Vosloo, W., A.D.S. Bastos, O. Sangare, S.K. Hargreaves, and G.R. Thomson, 2002: Review of the status and control of foot and mouth disease in sub-Saharan Africa. *Rev. Sci. Tech.* **21**, 437–449, DOI: http://web.oie.int/boutique/extrait/11vosloo.pdf.

Vosloo, W., A.D. Bastos, E. Kirkbride, J.J. Esterhuysen, D. Janse Van Rensburg, R.G. Bengis, D.W. Keet, and G.R. Thomson, 1996: Persistent infection of African buffalo (Syncerus caffer) with SAT-type foot-and-mouth disease viruses: Rate of fixation

of mutations, antigenic change and interspecies transmission. *J. Gen. Virol.* **77**, 1457–1467, DOI: 10.1099/0022-1317-77-7-1457.

Vosloo, W., L.M. de Klerk, C.I. Boshoff, B. Botha, R.M. Dwarka, D. Keet, and D.T. Haydon, 2007: Characterisation of a SAT-1 outbreak of foot-and-mouth disease in captive African buffalo (Syncerus caffer): Clinical symptoms, genetic characterisation and phylogenetic comparison of outbreak isolates. *Vet. Microbiol.* **120**, 226–240, DOI: 10.1016/j.vetmic.2006.11.002.

Walker, T.M., C.L.C. Ip, R.H. Harrell, J.T. Evans, G. Kapatai, M.J. Dedicoat, D.W. Eyre, D.J. Wilson, P.M. Hawkey, D.W. Crook, J. Parkhill, D. Harris, A.S. Walker, R. Bowden, P. Monk, E.G. Smith, and T.E.A. Peto, 2013: Whole-genome sequencing to delineate Mycobacterium tuberculosis outbreaks: A retrospective observational study. *Lancet Infect. Dis.* **13**, 137–146, DOI: 10.1016/S1473-3099(12)70277-3.

Waters, R., A.B. Ludi, V.L. Fowler, G. Wilsden, C. Browning, S. Gubbins, B. Statham, A. Bin-Tarif, V. Mioulet, D.J. King, C. Colenutt, E. Brown, P. Hudelet, and D.P. King, 2018: Efficacy of a high-potency multivalent foot-and-mouth disease virus vaccine in cattle against heterologous challenge with a field virus from the emerging A/ASIA/G-VII lineage. *Vaccine* **36**, 1901–1907, DOI: 10.1016/j.vaccine.2018.02.016.

Wekesa, S.N., A.K. Sangula, G.J. Belsham, K. Tjornehoj, V.B. Muwanika, F. Gakuya, D. Mijele, and H.R. Siegismund, 2015: Characterisation of recent foot-and-mouth disease viruses from African buffalo (Syncerus caffer) and cattle in Kenya is consistent with independent virus populations. *BMC Vet. Res.* **11**, 1–15, DOI: 10.1186/s12917-015-0333-9.

Wilson, I.G., 1997: Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* **63**, 3741–3751, DOI: 0099-2240/97/$04.00?0.

Wittwer, C.T., and S.L. City, 1989: Nucleic Acids Research. **17**, 4353–4357, DOI: 10.1093/nar/16.5.2269.

Woodbury, E.L., A.R. Samuel, N.J. Knowles, S.M. Hafez, and R.P. Kitching, 1994: Analysis of mixed foot–and–mouth disease virus infections in Saudi Arabia: Prolonged

circulation of an exotic serotype. *Epidemiol. Infect.* **112**, 201–212, DOI: 10.1017/S0950268800057575.

World Food Programme Kenya, 2017: Drought Situation Report #01. 1–2.

World Organisation for Animal Health (OIE), 2019: Recognition of the Foot and Mouth Disease Status of Members*Resolut. No. 15 (87th Gen. Sess. World Assem. May 2019)*.

Wright, C.F., M.J. Morelli, G. Thébaud, N.J. Knowles, P. Herzyk, D.J. Paton, D.T. Haydon, and D.P. King, 2011: Beyond the consensus: dissecting within-host viral population diversity of foot-and-mouth disease virus by using next-generation genome sequencing. *J. Virol.* **85**, 2266–2275, DOI: 10.1128/JVI.01396-10.

WRLFMD, 2015: Genotyping Rep. FMDV type SAT1. Ctry. Tanzania. FAO World Ref. Lab. Foot-and-Mouth Dis. (WRLFMD), Pirbright Institute, Surrey, United Kingdom. http//www.wrlfmd.org/sites/world/files/quick_media/WRLFMD-2015-00008-TAN-GTR-O-SAT1.

WRLFMD, 2017: Genotyping Report. FMDV type O. Country: Kenya. FAO World Ref. Lab. Foot-and-Mouth Dis. (WRLFMD), Pirbright Institute, Surrey, United Kingdom. http://www.wrlfmd.org/sites/world/files/WRLFMD-2017-00031-KEN-GTR-O-O_001.pdf. .

WRLFMD, 2018a: WRLFMD Quarterly Report January to March 2018.

WRLFMD, 2018b: WRLFMD Quarterly Report April to June 2018.

WRLFMD, 2018c: WRLFMD Quarterly Report July to September 2018.

WRLFMD, 2018d: WRLFMD Quarterly Report October to December 2018.

WRLFMD, 2019: WRLFMD Quarterly Report April to June 2019.

Yadav, V., A. Sharma, and R. Sharma, 2007: Detection of FMD virus type specific IgG1, IgG2 and IgA antibodies in milk and serum of buffaloes vaccinated with oil adjuvanted polyvalent FMD vaccine. *Ital. J. Anim. Sci.* **6**, 869–871, DOI: 10.4081/ijas.2007.s2.869.

Young, J.R., S. Suon, C.J. Andrews, L.A. Henry, and P.A. Windsor, 2013: Assessment of

Financial Impact of Foot and Mouth Disease on Smallholder Cattle Farmers in Southern Cambodia. *Transbound. Emerg. Dis.* **60**, 166–174, DOI: 10.1111/j.1865-1682.2012.01330.x.

Zinsstag, J., M.F. Akabar, M. Ibrahim, R. Tschopp, L. Crump, B. Bonfoh, and E. Schelling, 2016: Cost-effective control strategies for animal and zoonotic diseases in pastoralist populations. *Rev. Sci. Tech. l'OIE* **35**, 673–681, DOI: 10.20506/rst.35.2.2548.