The development and application of molecular tools for the diagnosis of foot-and-mouth disease in field and low-resource laboratory settings

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Abstract

The requirements for prompt diagnosis of foot-and-mouth disease (FMD) during outbreaks, and the need to establish robust laboratory testing capacity within FMD-endemic countries, have motivated the development of point-of-care tests (POCTs) to support current diagnostic strategies. Despite numerous publications detailing the design of platforms and assays for this purpose, the majority have only been evaluated in laboratory settings, using protocols incompatible for use in challenging environments. To address this gap, this thesis describes the development of an end-to-end molecular toolbox for the detection and characterisation of FMD virus (FMDV) RNA in decentralised settings. A critical review and multiway comparison of seven assay formats and 11 sample preparation methods revealed that reverse transcription loop-mediated isothermal amplification (RT-LAMP) and real-time reverse transcription PCR (rRT-PCR) POCT-formats exhibited comparable analytical and diagnostic sensitivity to their laboratory-based equivalents. Additionally, reagent lyophilisation provided a solution for cold chain and storage considerations, whilst not compromising assay performance. Both assays were compatible with simple sample preparation methods, removing the requirement for nucleic acid extraction. For example, dilution of samples in nuclease-free water enabled FMDV RNA to be detected in multiple sample types (epithelial tissue suspensions, serum, oesophageal-pharyngeal fluid and lesion swabs), from as early as one day post infection. Notably, when the robust field-ready protocols were deployed into challenging low-resource laboratory and field-settings within East Africa, POCT results (rRT-PCR = 144; RT-LAMP = 145) were consistent with clinical observations and a reference rRT-PCR, with FMDV detected from acutely infected as well as convalescent cattle. Furthermore, transitioning of East Africa-specific FMDV-typing rRT-PCR assays (for serotypes O, A, Southern African Territories [SAT] 1 and SAT 2) into a multiplex POCT-format enabled rapid identification of FMDV serotype *in situ*, confirming active outbreaks of both O and A. This thesis also describes the development of GoPrime, a novel real-time PCR (rPCR) primer/probe validation tool. By parameterising GoPrime with experimental data, collected to investigate the effects of primer/probe-template mismatches on cycle threshold and limit of detection, it was possible to quantitatively predict the performance of rPCR assays *in silico*. The work of this thesis supports the deployment of molecular POCTs into non-specialised, resource-limited and challenging settings for simple, highly sensitive and rapid detection and/or characterisation of FMDV.

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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.

Emma Howson

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Abbreviations

AMV	Avian Myeloblastosis Virus
Ag	Antigen
Ag-LFD	Antigen-Detection Lateral-Flow Device
B3	Backward External Primer
BFS	British Field Strain
BHK	Baby Hamster Kidney
BLoop	Backward Loop Primer
BIP	Backward Internal Primer
Btn	Biotin
BTY	Bovine Thyroid
cDNA	Complementary Deoxyribonucleic Acid
CFT	Complement Fixation Test
CIDLID	Combating Infectious Diseases of Livestock for International Development
C_T	Cycle Threshold
CPE	Cytopathic Effects
DFO	Dragonfly Orange™
DNA	Deoxyribonucleic Acid
DPC	Days Post Challenge
dsDNA	Double-Stranded Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPs	External Primers
EARLN	Eastern Africa Regional Laboratory Network
EuFMD	European Commission for the Control of Foot-and-Mouth Disease
F3	Forward External Primer
FAM	6-Fluorescein Amidite
FAO	Food and Agriculture Organization of the United Nations
FERA	The Food and Environment Research Agency
Flc	Fluorescein
FLoop	Forward Loop Primer
FIP	Forward Internal Primer
FMD(V)	Foot-and-Mouth Disease Virus
HDA	Helicase Dependent Amplification
IC	Internal Control
iiPCR	Insulated Isothermal PCR
IPs	Internal Primers
LAMP	Loop-mediated isothermal AMPlification
LATE-PCR	Linear-After-The-Exponential PCR
LFD	Lateral-Flow Device
LMIC	Low to Middle Income Countries
LOD	Limit of Detection

Log_x	Logarithm to the power X
Loops	Loop primers
MAb	Monoclonal Antibody
NASBA	Nucleic Acid Sequence Based Amplification
NFW	Nuclease-Free Water
NSP	Non-Structural Protein
OIE	World Organisation for Animal Health
OP	Oesophageal-Pharyngeal
OS	Original Sample
OF	Original Fluid
PCP-FMD	Progressive Control Pathway for FMD
PCR	Polymerase Chain Reaction
POC	Point-Of-Care
POCT	Point-Of-Care Test
RMSD	Root-Mean-Square Deviation
RNA	Ribonucleic Acid
rRT	Real-Time Reverse Transcription
RS	Renal Swine
RT	Reverse Transcription
RPA	Recombinase Polymerase Amplification
SAT	Southern African Territories
SD	Standard Deviation
SP	Structural protein
SVD(V)	Swine Vesicular Disease (Virus)
SVV	Seneca Valley Virus 1
TPI	The Pirbright Institute
TVLA	Tanzania Veterinary Laboratory Agency
TxR	Texas Red®
UTR	Untranslated Region
VES	Vesicular Exanthema of Swine
VI	Virus Isolation
VP1	Viral Protein 1
VP2	Viral Protein 2
VP3	Viral Protein 3
VP4	Viral Protein 4
VPg	Viral genome-linked protein
VS	Vesicular Stomatitis
VSIV	Vesicular Stomatitis Indiana Virus
VSNJV	Vesicular Stomatitis New Jersey Virus
WHO	World Health Organization
WRLFMD	World Reference Laboratory for Foot-and-Mouth Disease

Glossary of Terms

Carrier	Animals in which foot-and-mouth disease virus persists in the oropharynx for more than 28 days after infection, and serves as a potential source of infection. In order to confirm persistence in an animal, virus isolation needs to be performed to test for the presence of live virus (OIE, 2012).
Convalescent	An animal recovering from disease.
Decentralised settings	Low-resource laboratory or field settings.
Delayed clearance	Animals in which the virus persists in the oropharynx after recovery from clinical signs.
Pool	A closed geographic region that maintains unique and discrete foot-and-mouth disease virus (FMDV) lineages. Seven major pools of infection are defined (Paton *et al.*, 2009). Sometimes FMDV lineages are transmitted between these pools.
Reservoir	An ecological system in which the pathogen can be permanently maintained, and from which infection can be transmitted to the target population (Haydon *et al.*, 2002).
Serotype	A group of viruses distinguished by their specific shared antigens, as determined by serologic testing. Recovery from infection, or vaccination, with one serotype will not protect against subsequent infection with another (Alexandersen *et al.*, 2003a).
Sensitivity (analytical)	The smallest detectable amount of analyte that can be measured with a defined certainty (OIE, 2012). Synonymous with “limit of detection”.
Sensitivity (diagnostic)	Proportion of known positive samples that test positive in an assay (OIE, 2012). Known positive samples which test negative are considered false-negative results.
Specificity (analytical)	Degree to which an assay distinguishes between the target analyte and other components in the sample matrix (OIE, 2012).
Specificity (diagnostic)	Proportion of known negative samples that test negative in an assay (OIE, 2012). Known negative samples which test positive are considered false-positive results.
Topotype	Geographically clustered viruses from a single genetic lineage which generally share >85% (O, A, C, and Asia 1) or >80% (SAT 1, SAT 2, and SAT 3) nucleotide sequence identity in the VP1-coding region (Samuel and Knowles 2001a; 2001b; Ayelet *et al.* 2009).

CHAPTER 1

Foot-and-mouth disease: a review of diagnostic strategies for a transboundary disease of global importance

Elements of this chapter have been published in the following peer-reviewed article:

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1.1 Summary

Sustainable food production, to support a growing human population, is a key global priority and is encompassed within the Global Goals for Sustainable Development. Livestock infectious diseases pose important threats to the achievements of these global goals, with viral diseases such as foot-and-mouth disease (FMD) amongst the most important. Consequently, management of these threats is key to sustainable agricultural development. Accurate and rapid diagnostic tests are an essential component of contingency and surveillance plans to diagnose, control and eradicate FMD. Diagnosis involves a pipeline that normally starts with clinical suspicion, followed by collection of samples, transport of specimens to a centralised laboratory setting (e.g. national / international reference laboratories), analysis of samples using a range of diagnostic tests and reporting of results. However, transport of specimens from the field to the laboratory can be a lengthy process and logistically challenging in remote locations. These issues can delay critical decision-making and reduce the quality of samples. Furthermore, many diagnostic tests require well-equipped laboratories, often problematic for endemic countries which lack infrastructure and financial resources for disease surveillance and diagnostics. These important limitations of centralised diagnostic testing have motivated the development of tools for rapid, simple, detection of FMD virus (FMDV), which are compatible with use in low-resource settings. Recent advances in the development of such technologies for human medicine have provided a range of prototype diagnostic solutions, which could be applied to a wide selection of diseases of livestock. Based on a critical review of current and emerging diagnostic technologies, this chapter will highlight the most promising candidates for revolutionising FMD diagnostics, with a particular focus on tests that can be utilised in decentralised settings.

1.2 Foot-and-mouth disease

1.2.1 Disease overview

Foot-and-mouth disease (FMD) is a highly infectious trans-boundary disease, affecting both domesticated and wild cloven-hooved animals. Endemic across most of Asia, Africa and parts of South America (FAO, 2016), FMD is characterised by acute fever, coupled with the development of lesions primarily on the epithelial surfaces of the feet, mouth, nasal region and mammary glands/teats (Alexandersen *et al.*, 2003a). Although the case-fatality rate of FMD is generally below 5% (Rushton *et al.*, 2012), mortality is often high in young animals and debilitating effects are evident through decreases in milk production, weight loss and loss of draught power (Knight-Jones and Rushton, 2013; Knight-Jones *et al.*, 2016). These reductions in productivity, in combination with the severe economic consequences attained through disease control and trade restrictions, have resulted in FMD being listed by the World Organisation for Animal Health (OIE) as the most important animal disease constraining world trade (OIE/FAO, 2012).

FMD is caused by foot-and-mouth disease virus (FMDV), a positive-sense, single-stranded RNA virus belonging to the family *Picornaviridae*, genus *Aphthovirus*. Seven serotypes of FMDV are recognised, defined as antigenically distinct groups (determined by serological testing), namely: O, A, C, Asia 1 and Southern African Territories (SAT) 1-3, which can be further subdivided phylogenetically and geographically into distinct topotypes. FMDV has numerous characteristics of a successful pathogen including: a wide host range (FMDV affects over 70 species [Fenner *et al.*, 1993]), low infectious dose required for infection, rapid replication rate and a high level of viral excretion (Alexandersen *et al.*, 2003a). In addition, multiple routes of transmission are evident. FMDV is present in all excretions from an infected animal, therefore transmission most commonly occurs though direct contact between infected and susceptible animals (Alexandersen *et al.*, 2003b). However, transmission can also occur though the indirect exposure to these excretions and via untreated meat products from infected animals (Figure 1.1) (OIE, 2012). As such, FMD remains the most contagious disease of mammals (OIE, 2012) and requires continuous efforts for control, monitoring and eradication.

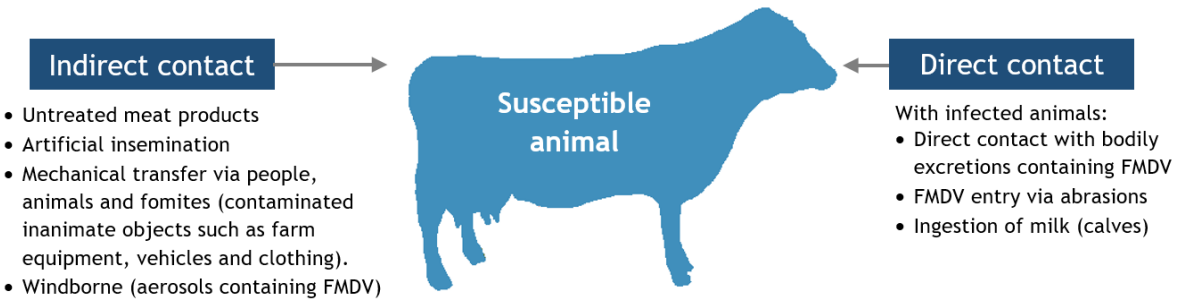

Figure 1.1 Transmission pathways of foot-and-mouth disease virus (FMDV).

1.2.2 Global distribution of FMDV

Historically, FMD has occurred in most livestock-containing areas of the world (Grubman and Baxt, 2004), however current global circulation occurs primarily within three continental epidemiological clusters in Asia, Africa and South America (Figure 1.2). Although Europe, North and Central America, Greenland and Australasia are currently considered FMD-free without vaccination, occasional incursions to such areas have occurred: including outbreaks of FMDV in the UK in 2001 (PanAsia O strain) and 2007 (O_1 British Field Strain [BFS]/1967) (Knowles *et al.*, 2001; Cottam *et al.*, 2008), with the last FMD introduction into Europe occurring in Bulgaria in 2011 (PanAsia-2 O strain) (Valdazo-González *et al.*, 2012; Alexandrov *et al.*, 2013) .

The global distribution of FMDV serotypes is uneven. Serotypes O and A have the widest global distribution and are reported in most endemic regions. Conversely serotype C has not been isolated since 2004 (Kenya and Brazil) (Rweyemamu *et al.*, 2008a). The SAT 1-3 viruses are generally restricted to sub-Saharan Africa and Asia 1 is generally confined to the Asian continent (Paton *et al.*, 2009). Phylogenetic analysis of FMDV circulating worldwide has enabled the definition of seven regional pools (Figure 1.2), each containing regionally specific viral topotypes (Paton *et* al., 2009).

Movement of FMDV strains is observed between the pools (Figure 1.2), and is viewed with concern due to the potential for disease emergence in areas previously naïve to the specific strains. For example, recent long-distance "trans-pool" movements have included the extensive spread of O/ME-SA/Ind-2001d from the Indian sub-continent (pool 2), first west into Gulf States of the Middle East (2013), followed by separate introductions into countries within North Africa (2014), including Tunisia, Algeria and Morocco which had last reported FMD cases in 1999 (Bouguedour and Ripani, 2016). Additional multiple independent movements (2015-6) have occurred within mainland South East Asia and the

Indian Ocean, with the most recent reports including Eastern parts of Russia and South Korea (Subramaniam *et al.*, 2013; Yuvaraj *et al.*, 2013; Knowles *et al.*, 2016). In addition to O/ME-SA/Ind-2001d, outbreaks of the A/ASIA/G-VII lineage (also originally found in pool 2) have occurred in northern regions of pool 3 (Saudi Arabia, Turkey, Iran, Armenia), with initial reports in September 2015 (Das *et al.*, 2016).

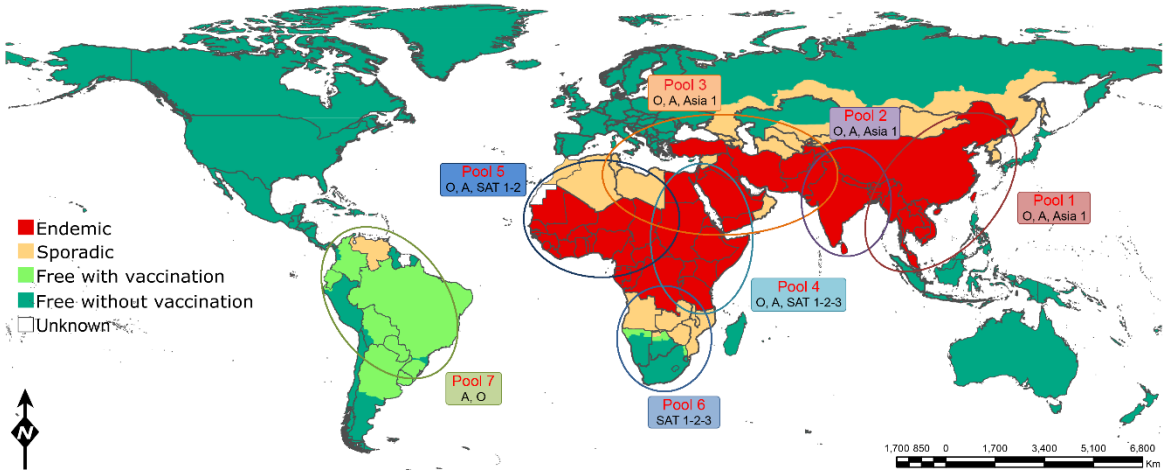

Figure 1.2 Conjectured status of foot-and-mouth disease worldwide in 2017. Foot-and-mouth disease is maintained within three continental clusters: Asia, Africa and South America, which are further subdivided into seven regional pools (annotated on the figure). Courtesy of Dr Antonello Di Nardo (The Pirbright Institute, UK). (SAT) Southern African Territories.

1.2.3 Epidemiological patterns of FMD

FMD is estimated to circulate in 77% of the global livestock population (Rushton *et al.*, 2012), with disease distribution roughly reflecting economic development (Jamal and Belsham, 2013). Epidemiologically, FMD patterns vary enormously between different countries, however the OIE has classified areas into one of the following (OIE, 2016a):

1. FMD-free country where vaccination is not practised
2. FMD-free country where vaccination is practised
3. FMD-free compartment or zone where vaccination is not practised
4. FMD-free compartment or zone where vaccination is practised
5. FMD-infected country or zone

According to the provisions of the OIE (OIE, 2016a), 68 member countries are now recognised as FMD free (66 without vaccination and two with vaccination), with FMD-free zones recognised in a further 22 member countries (13 without vaccination and nine with

vaccination) (OIE, 2017). For instance, zonal FMD-freedom has been achieved within South Africa (Brückner *et al.*, 2002), where FMD infection is restricted to Kruger National Park. Here, African buffalo (*Syncerus caffer*) provide a principal reservoir for FMD for the SAT serotypes (Esterhuysen *et al.*, 1985; Thomson *et al.*, 1992): defined as an ecological system in which the pathogen can be permanently maintained, and from which infection can be transmitted (Haydon *et al.*, 2002).

Within FMD-endemic countries, the epidemiological situation is often incredibly complex. For instance, within East African countries, four FMD serotypes and multiple topotypes are known to be currently circulating (Figure 1.3) (Vosloo *et al.*, 2002; Vosloo *et al.*, 2004; Ayelet *et al.*, 2009; Kasanga *et al.*, 2012; Namatovu *et al.*, 2015), with the occurrence of SAT 3 in wildlife yet to be defined (Dhikusooka *et al.*, 2015).

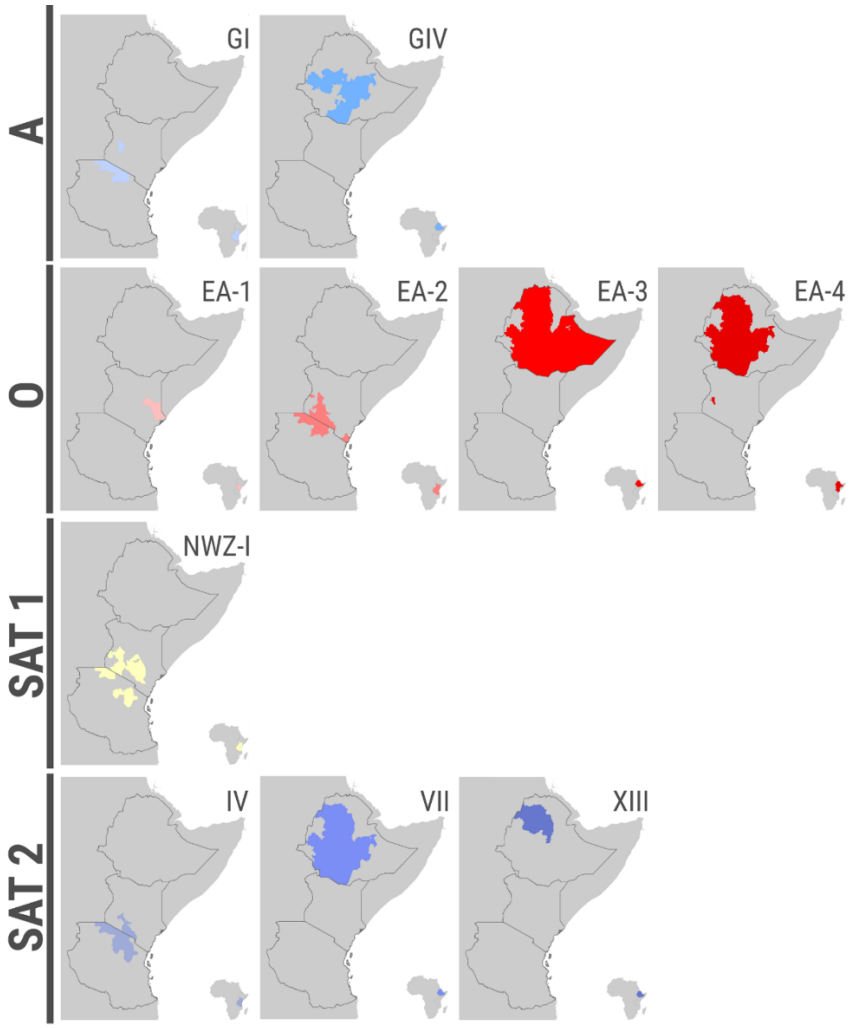

Figure 1.3 The reported occurrences of foot-and-mouth disease viral serotypes and lineages within Kenya, Tanzania and Ethiopia (2010-2017). Based on molecular epidemiology reports produced by the Food and Agriculture Organization World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD). Courtesy of Dr Antonello Di Nardo (The Pirbright Institute, UK).

The epidemiology within FMD-endemic regions is further complicated by agro-pastoral practices resulting in uncontrolled human and animal movements (over 99% of livestock in Tanzania are kept by smallholders [United Republic of Tanzania, 2012], with 73% in communal grazing production systems [Kivaria, 2003]), and the presence of wildlife as a potential FMD reservoir. For instance, a high prevalence of FMDV infection has been shown in African buffalo within East Africa (Anderson *et al.*, 1979; Hamblin *et al.*, 1990; Bronsvoort *et al.*, 2008; Ayebazibwe *et al.*, 2010a; Ayebazibwe *et al.*, 2010b; Mkama *et al.*, 2014), however their role in transmitting FMDV to livestock remains inconclusive (Casey *et al.*, 2013). With the majority of FMDV outbreaks East Africa remaining unrecorded, due to factors including (i) a lack of encouragement to report disease, (ii) poor diagnostic infrastructure and (iii) logistical and cultural barriers associated with transporting samples to centralised facilities, the complete epidemiological situation within these regions remains unknown (Vosloo *et al.*, 2002; Namatovu *et al.*, 2013)

1.3 Impacts and control of FMD

1.3.1 Impacts of FMD

Although a disease of low mortality, FMD is considered one of the most economically devastating diseases of animals worldwide (Sumption *et al.*, 2012). The impacts of FMD can be separated into two types: those that arise from the direct effects of the disease on production, and those that have indirect effects through the costs of disease control, trade restrictions and use of sub-optimal livestock practices (Figure 1.4). However, the costs associated with FMD are not evenly distributed globally. In FMD-free countries, the costs associated with maintaining FMD-free status or regaining FMD-free status following an outbreak (in order to satisfy international trade requirements) can be severe. For example, the UK 2001 FMD outbreak is estimated to have cost the national economy US$ 9.2 billion (FAO, 2002), including US$ 4.2 billion in governmental compensation to the agricultural and food chain industry (Thompson *et al.*, 2002). Furthermore, intensive vaccination in South America, to maintain FMD-free status, is estimated to cost US$ 0.7 billion annually (Knight-Jones and Rushton, 2013).

Production losses are greatest in low to middle income countries (LMIC) with endemic-FMD infection, where communities are often dependent on the health of their livestock (Knight-Jones and Rushton, 2013; Knight-Jones *et al.*, 2016). For instance, at the herd level, FMDV infection has shown to decrease the average daily milk yields by 35% per cow, only recovering after approximately two months (Lyons *et al.*, 2015). Furthermore,

endemically infected countries, with FMD control programs, incur the ongoing costs of disease management (e.g. vaccination, movement restrictions and surveillance) (James and Rushton, 2002). The annual global impact of FMD in terms of production losses and vaccination in endemic regions alone is estimated to amount to between US$6.5 and 21 billion (Knight-Jones and Rushton, 2013).

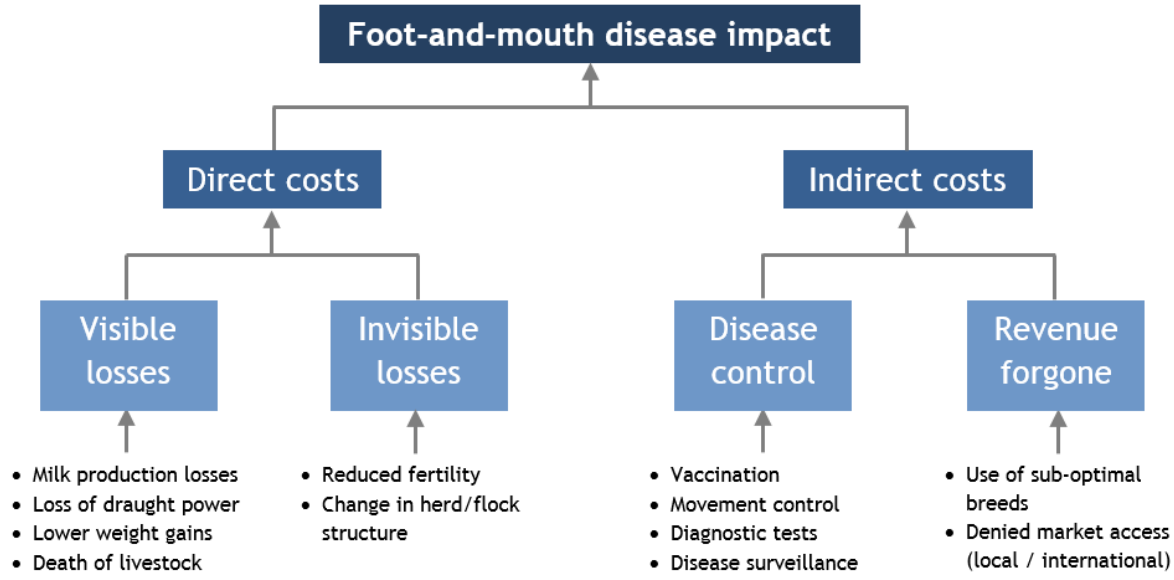

Figure 1.4 The impacts of foot-and-mouth disease. Adapted from Rushton *et al.* (2009).

1.3.2 Control of FMD

The control of FMD has been recognised by the Food and Agricultural Organization of the United Nations (FAO) and OIE as a global priority (Sumption *et al.*, 2012). There are a variety of control measures that can be adopted, which are largely driven by the countries policy, capacity, priorities and FMD status (Paton *et al.*, 2009). For instance, the economic burden associated with FMD, including restricted access to lucrative international markets, results in prosperous FMD-free countries employing rapid measures for disease eradication during outbreaks. For instance, during the UK 2001 FMDV outbreak, control measures included a stamping out policy (culling of suspect and in contact animals: animals were slaughtered on >10,000 farms [Scudamore and Harris, 2002]), combined with strict movement restrictions, zoo-sanitary measures and intensive surveillance (Anderson, 2002).

FMD control in endemic countries however, is often much more complex. Zonal FMD-freedom has been achieved by some endemic countries, such as South Africa and Kazakhstan, through a combination of vaccination, animal movement control and separation of wildlife and livestock (Brückner *et al.*, 2002; OIE, 2016b). However, the level of enforcement of these methods differs between countries. For instance, although vaccination and quarantine are cited as existing FMD control strategies for East Africa (Hunter, 1998; Rweyemamu and Astudillo, 2002), they are only rarely implemented. Movement restrictions are limited by social customs (communal grazing and pastoralism), weak law enforcement on animal movement and the potential role of wildlife as a maintenance host (Vosloo *et al.*, 2002; Ayebazibwe *et al.*, 2010b; Maree *et al.*, 2014). While livestock vaccination campaigns remain a feasible control approach (Rweyemamu *et al*. 2008b), several major challenges remain in the implementing of effective vaccination strategies (Parida, 2009). The immunological diversity present within African serotypes necessitates continuous FMDV characterisation to match field strains with available vaccines, something that is often challenging for low-resource laboratories (Vosloo *et al.*, 2002; Mumford, 2007). Furthermore, access to good quality and affordable FMD vaccines can prove problematic. FMD control therefore still presents multiple challenges throughout endemic countries.

With total FMD control unlikely for East Africa in the near future, due to factors including (i) poor diagnostic infrastructure, (ii) social customs/attitudes and (iii) highly complex epidemiology (Vosloo *et al.*, 2002; Maree *et al.*, 2014), a progressive approach for FMD control is required. In order to address such challenges, the FAO and OIE launched a global strategy in 2012 to assist countries where FMD is endemic to progressively reduce the impact of the disease: The Progressive Control Pathway for FMD (PCP-FMD) (Sumption *et al.*, 2012). This framework is built on the principle that countries are at different stages in the pathway towards FMDV control and eradication, and has been adopted as a working tool by the FAO to help tailor national and regional control programs to specific contexts and needs (Figure 1.5). However, with all stages of the PCP-FMD requiring active monitoring of FMDV circulation and understanding of FMDV epidemiology, progress along the pathway is dependent on countries establishing robust diagnostic testing capacity for the detection and characterisation of FMDV.

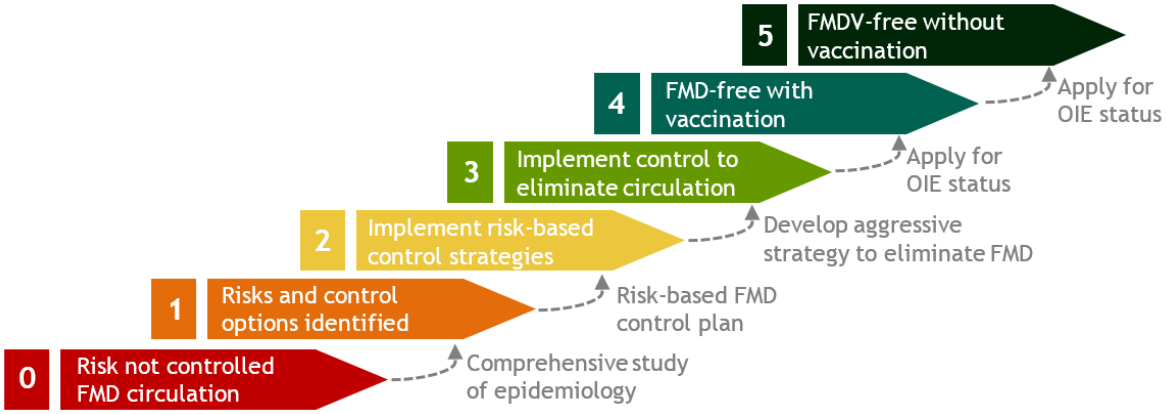

Figure 1.5 Progressive Control Pathway for Foot-and-Mouth Disease (PCP-FMD). Adapted from Sumption *et al.* (2012).

1.4 Current diagnostic strategies

1.4.1 Clinical diagnosis

Initial diagnosis of FMD is based on the observation of clinical signs in susceptible livestock by their owners and/or veterinarians. Initial FMDV replication occurs close to the site of entry (mucosae and lymphoid tissues of the pharyngeal or tonsillar region) with the incubation period (time from infection to clinical signs of disease) ranging from 1-14 days (most commonly between 2-5 days), depending upon factors such as pathogen dose, virus strain, host species, pre-existing immunity and the route of transmission (Alexandersen *et al.*, 2003b). Initial clinical signs include fever, dullness and a drop in milk yield for dairy animals. Viral dissemination into tissues and organs, results in the development of secondary characteristic lesions on the epithelial surfaces of the feet, mouth, nasal region and mammary glands/teats. These vesicular lesions can be aged, in order to estimate the time of initial infection (OIE, 2012) (Figure 1.6).

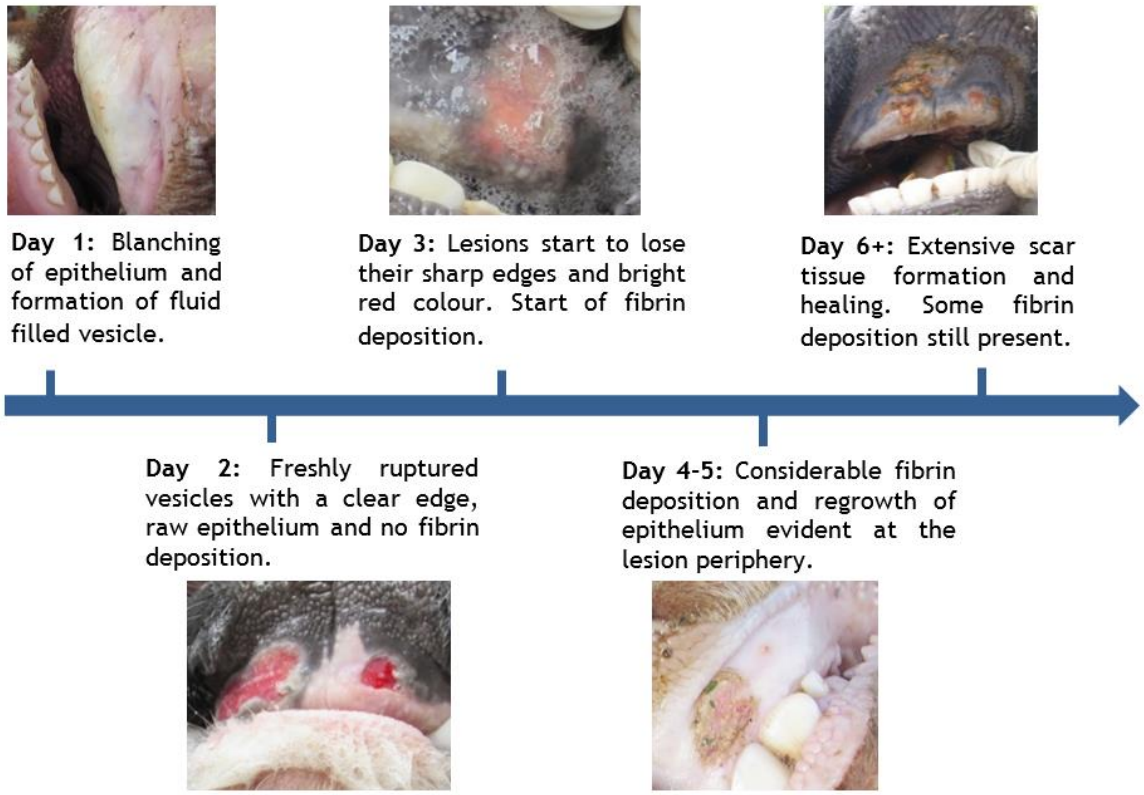

Figure 1.6 Timeline for estimating the age of foot-and-mouth disease lesions. Descriptions are based on those of Kitching and MacKay (1995). Photographs of days 2-6 were taken during field work in East Africa; day 1 photograph courtesy of Dr Veronica Fowler (The Pirbright Institute, UK).

Clinical observations alone however remain insufficient for confirmation of FMD, as clinical signs are indistinguishable from other vesicular diseases including swine vesicular disease (SVD), vesicular stomatitis (VS), Seneca Valley virus 1 (SVV) and vesicular exanthema of swine (VES) (OIE, 2012). Furthermore, the severity of clinical signs varies between species: clinical signs are most apparent in cattle followed by pigs, whereas sheep, goats and other small ruminants commonly display only mild signs (Kitching, 2002a; 2002b; Kitching and Hughes, 2002). As such, misdiagnosis or missed detection has been common in the history of FMD outbreaks. For example the FMD outbreak in Tunisia in 1989 was initially misdiagnosed in sheep as bluetongue and only recognised as FMD after it was transmitted to cattle (Kitching, 2004), whilst the outbreak in Greece in 1994 went unnoticed in sheep for a number of months, again only recognised as FMD following transmission to cattle (Kitching, 1998). Therefore, depending on context, following a suspect FMD case and where resources permit, samples require transportation to national / international reference laboratories (NRL/IRL) for diagnostic confirmation (laboratory confirmation of FMD in endemic settings often does not occur).

1.4.2 Samples for diagnostics

FMDV is present in all excretions and secretions of acutely infected animals, starting shortly before the onset of clinical signs and declining after approximately 5 days of clinical presentation (Alexandersen *et al.*, 2003a) (Figure 1.7). Epithelium and vesicular fluid are the preferred tissues for diagnostics since they contain high viral loads during the acute phase of infection. Where collection of these samples is not possible, for example before clinical presentation, FMDV can be detected in the oesophageal-pharyngeal fluid (OP), blood and nasal fluid (Chase-Topping *et al.*, 2013). Animals in which FMDV persists in the oropharynx for more than 28 days after infection are known as carriers, with virus isolated from OP fluid 28 days post challenge (DPC) used to define these (Zhang and Kitching, 2001; Alexandersen *et al.*, 2002). For cattle, approximately 50% of those infected with FMDV become carriers, however the carrier state usually does not persist for more than 9 months (but can last up to 3 years). African buffalo have been shown to harbour the virus for 5 years (Alexandersen *et al.*, 2003b). In fatal cases, myocardial tissue and blood are the preferred sample type. Samples are collected and transported to laboratories following international regulations and guidelines as stated in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012).

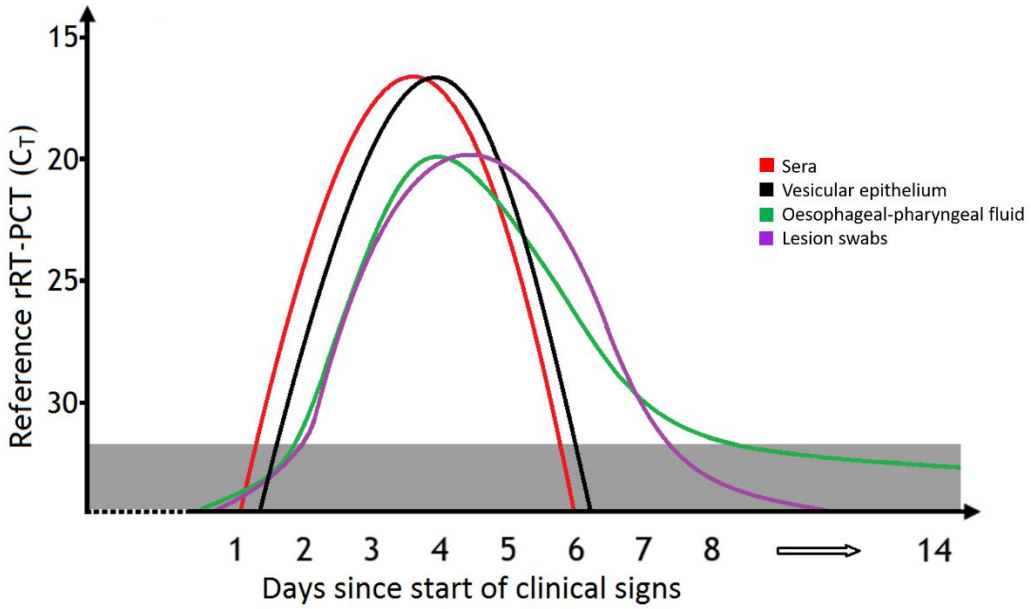

Figure 1.7 Schematic of the foot-and-mouth disease (FMD) diagnostic window. Figure shows the temporal distribution of FMD virus (FMDV) in different biological samples, represented as real-time reverse transcription PCR (rRT-PCR) cycle threshold (C_T) values (grey shaded area represents C_T values over the diagnostic cut-off [Shaw *et al.*, 2007]). (Red) sera; (black) vesicular epithelium; (green) oesophageal-pharyngeal fluid; (purple) lesion swabs; (blue) FMDV-specific antibodies in sera. The dotted x-axis line represents the incubation period, dependent upon: infectious dose, viral strain, animal species and host determinants. Based on data from Alexandersen *et al.* (2003a) and King *et al.* (2012).

1.4.3 Diagnostic targets

The FMDV particle consists of a single copy of an RNA genome, encased within a non-enveloped icosahedral protein capsid, approximately 30 nm in diameter. The *ca.* 8.5 kb RNA genome is composed of a *ca.* 7 kb open reading frame (ORF), flanked by two non-coding regions: the 5' untranslated region (UTR), which contains a number of highly structured regulatory regions, including the internal ribosome entry site (IRES) and long (70-200 nt) poly(C) tract (starting approximately 360 nt from the 5'-end), and the 3' UTR containing a heteropolymetric segment and poly(A) tail (Figure 1.8) (Forss *et al.*, 1984).

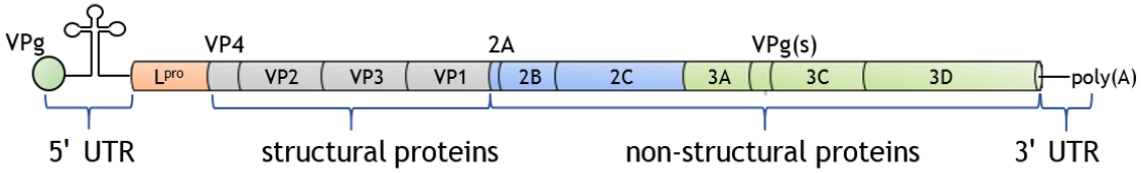

Figure 1.8 Simplified schematic of the foot-and-mouth disease viral genome. Nomenclature and structure based on Rueckert and Wimmer (1984). The FMDV open reading frame can be divided into four regions based on initial cleavage products: (orange) the 5' L region which encodes the N-terminal polyprotein component; (grey) structural protein encoding P1; (blue) non-structural protein encoding region P2; (green) and non-structural protein encoding region P3 (Grubman and Baxt, 2004).

The FMDV RNA genome is translated as a single ORF, which following cleavage by viral encoded proteases, comprises four structural proteins (VP1, VP2, VP3 and VP4) and at least eight non-structural proteins (L^{pro}, 2A, 2B, 2C, 3A, 3B, $3C^{pro}$, $3D^{pol}$). Sixty copies of each structural protein assemble to form the capsid (VP1-3 and internally located VP4) (Acharya *et al.*, 1989), to which immunological diagnostic assays can be targeted (either directly via antigen capture enzyme-linked immunosorbent assays [Ag-ELISA], or indirectly via virus neutralisation tests [VNT] which detect antibodies to these regions). Immunological assays can also be used to identify antibodies generated to non-structural proteins (Mackay *et al.*, 1998), the results of which can be used to determine infection/subclinical infection in non-vaccinated and vaccinated animals. In addition, the replication cycle of FMDV is cytolytic, therefore virus isolation (VI) can be used to indicate the presence of live virus in specialised cell lines based on the appearance of cytopathic effect (CPE).

Similar to other picornaviruses, the FMDV genome has high sequence variability and can evolve rapidly as a consequence of errors introduced during replication (Domingo *et al.*, 1985). However, differential selection pressures have resulted in both highly conserved

(critical for aspects of viral biology) and highly varied (critical for antigenic diversity and immune evasion) regions, which can be targeted by sequencing and/or molecular-based methods. For example, highly conserved regions including the RNA polymerase ($3D^{pol}$-coding region) (Meyer *et al.*, 1991; Callahan *et al.*, 2002) and regulatory 5'-UTR (Reid *et al.*, 2000) enable pan-specific detection of FMDV. Alternatively, highly varied regions of the FMDV genome, including the capsid-coding region, allow for FMDV characterisation via detection and/or sequencing of viral nucleic acid (Knowles and Samuel, 1988; Giridharan *et al.*, 2005; Ahmed *et al.*, 2012; OIE, 2012; Reid *et al.*, 2014; Jamal and Belsham, 2015; Bachanek-Bankowska *et al.*, 2016; Knowles *et al.*, 2016).

1.4.4 Laboratory diagnosis

Laboratory diagnosis is currently performed almost exclusively in dedicated laboratories which meet appropriate requirements for handling highly infectious agents. Diagnostic assays are performed and interpreted by trained personnel, following the recommendations as summarised in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012). Although the diagnostic process varies according to factors such as sample priority and sample type received, a typical diagnostic workflow, as used by the World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) (The Pirbright Institute [TPI]), is depicted below (Figure 1.9; Table 1.1).

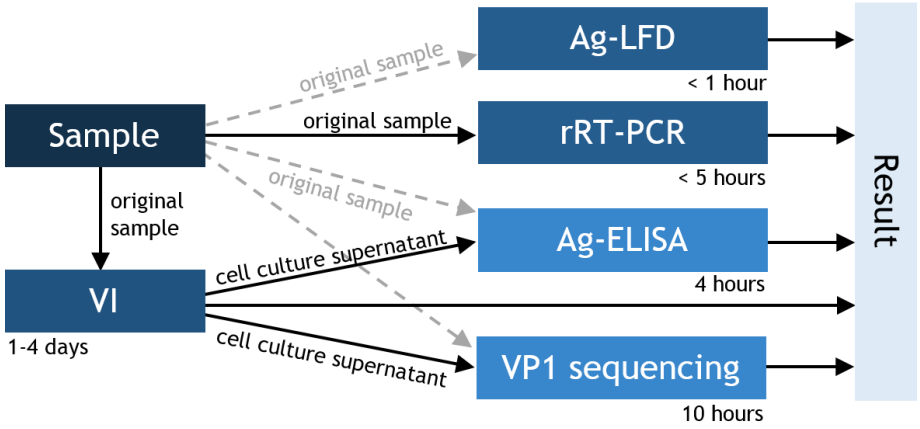

Figure 1.9 Typical laboratory work flow for diagnosis of foot-and-mouth disease carried out at the World Reference Laboratory for Foot-and-Mouth Disease. Black arrows symbolise standard diagnostic procedures, grey arrows symbolise actions for high priority samples (e.g. suspect outbreaks requiring rapid diagnosis and result reporting). Original sample can be either a tissue homogenate/suspension or original fluid (e.g. oesophageal-pharyngeal fluid/cell culture supernatant). (VI) virus isolation; (Ag-LFD) antigen-detection lateral-flow device; (rRT-PCR) real-time reverse transcription PCR; (Ag-ELISA) antigen capture enzyme-linked immunosorbent assay.

Table 1.1 Current diagnostic assays for foot-and-mouth disease

Diagnostic test	Detects	Performance time	Serotype specific	Advantages	Disadvantages
Clinical observations	Clinical signs		N	• No equipment necessary	• Misdiagnosis possible • Low specificity • Experienced staff required • Requires good biosafety practices
Virus isolation	Live virus	1-4 days	N	• OIE gold-standard • Confirms live virus	• Time consuming • Lacks specificity • Need for naïve animal tissue (difficult in endemic countries) • Requires high containment • Cell line variability • Experienced staff required
Ag-ELISA	FMDV antigen	4 hours	Y	• Rapid typing of FMDV	• Lower analytical sensitivity • Only suitable for certain sample types • Use of animals to generate polyclonal antisera • Requires high containment • Experienced staff required
Ag-LFD	FMDV antigen	< 1 hour	N	• Extremely rapid • Simple to use • Portable • No cold storage required	• Lower analytical sensitivity • Only suitable for certain sample types
RT-PCR	FMDV RNA	< 5 hours	N	• High analytical sensitivity • Intact virus not required	• Does not distinguish viable/non-viable virus • Experienced staff and equipment required
Typing RT-PCR	FMDV RNA	< 5 hours	Y	• High throughput • Uses many sample types	
VP1 sequencing	FMDV RNA	1-2 days	Y	• High level of detail	• Costly equipment necessary
Whole genome	FMDV RNA	1-3 days	Y	• Maximum level of detail	• Experienced staff required
Virus neutralisation	FMDV Ab (to SP)	2-3 days	Y	• OIE gold-standard for Ab • Limited cross-reactivity • Strain specific reagents	• Time consuming • Experienced staff required • Cell line variability • Requires high containment
SPC-ELISA LPB-ELISA	FMDV Ab (to SP)	18 hours	Y	• Low containment needed • Simple to perform	• Possible cross-reactivity • Use of animals to generate polyclonal antisera • Experienced staff required
NSP-ELISA	FMDV Ab (to NSP)	20 hours	N	• Simple to perform • Differentiate infection from vaccination	• Possible cross-reactivity • Experienced staff required

(Ag-LFD) antigen-detection lateral-flow devices; (RT-PCR) reverse transcription PCR; (Ag-ELISA) antigen capture enzyme-linked immunosorbent assay; (Ab) antibody; (SP) structural protein; (NSP) non-structural protein; (SPC) solid phase competition; (LPB) liquid phase blocking; (OIE) World Organisation for Animal Health.

1.4.5 Virus isolation

The majority of samples received by FMD reference laboratories will undergo VI, with the principle that when propagated in susceptible cell lines, clinical samples containing viable virus will result in cytopathic effect (CPE) (Figure 1.10). The development of highly sensitive cell culture systems, such as primary bovine thyroid (BTY) cells (Snowdon, 1966), has resulted in the technique being considered gold-standard (OIE, 2012). However, a number of drawbacks are associated with the use of primary cultures (Ferris *et al.*, 2002), including the requirement to regularly source fresh tissue to prepare the cell suspensions. Established cell lines, including BHK-21 (baby hamster kidney) and IB-RS-2 cells (renal swine), are also available (De Castro, 1964; Stoker and Macpherson, 1964), offering simplified culturing requirements. However these have reduced analytical sensitivity to infection (Clarke and Spier, 1980). Although the only way of confirming live virus, VI is time consuming, taking up to four days to generate a result, and variability exists in the sensitivity of cell lines to FMDV. For instance, field isolates from the O/CATHAY topotype grow poorly on BTY cells (Samuel and Knowles, 2001a). Consequently, samples typically require isolation in multiple cell lines to provide confidence in negative results. Furthermore, the observation of CPE in susceptible cell lines alone is not sufficient for a definitive diagnosis of FMD, as CPE may be caused by alternative factors such as contamination with other pathogens. Therefore, confirmation of positives through other diagnostic methods is necessary.

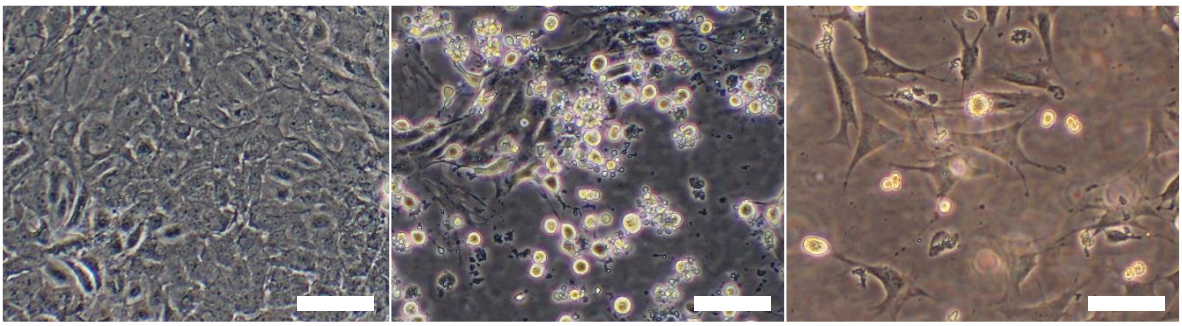

Figure 1.10 Foot-and-mouth disease virus growth in bovine thyroid cells. Photographs (courtesy of the World Reference Laboratory for Foot-and-Mouth Disease) depict virus isolation results: (left) negative; (middle) early positive, showing signs of cytopathic effect; (right) positive, showing clear cytopathic effect. Photographs taken at 10 x magnification, scale bar represents 1 mm.

1.4.6 Detection and characterisation of FMDV antigen

For detection and characterisation of FMDV antigen, immunological methods such as the antigen capture enzyme-linked immunosorbent assay (Ag-ELISA) are typically employed. The indirect-sandwich Ag-ELISA, recommended within the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012), utilises polyclonal antisera raised in rabbits (capture) and guinea pigs (detection) against FMDV antigen from all seven serotypes, enabling identification of serotype in less than four hours (Roeder and Le Blanc Smith, 1987; Ferris and Dawson, 1988). Although this ELISA largely replaced the complement fixation (CF) test, due to improved sensitivity and interpretation (ELISAs are not affected by pro- or anti-complementary factors) (Ferris and Dawson, 1988), limited analytical sensitivity restricts usefulness to samples with high viral concentrations, with positive results obtained for approximately 70-80% of epithelial tissue suspensions that contain virus (Reid *et al.*, 1998; Mohapatra *et al.*, 2007; Jamal and Belsham, 2013). As such, within advanced laboratories, virus within clinical samples is routinely propagated in cell culture prior to Ag-ELISA (with the exception of high priority samples). Furthermore, the use of polyclonal antisera as capture and detector ligands leads to disadvantages including: a finite supply of stocks (replacement stocks often display different reaction characteristics), regular requirement for animals for antibody production and cross-reactivity between serotypes (Ferris *et al.*, 2005). Consequently, monoclonal antibody (MAb)-based Ag-ELISAs have been developed (Morioka *et al.*, 2009; Ferris *et al.*, 2011), with MAbs produced from the immortalised ß-cells of immunised mice. Recently, a sandwich Ag-ELISA based on recombinant αvß6 integrin for use as the FMDV capture ligand has been developed, using serotype-specific MAbs as detectors. When compared with the conventional polyclonal indirect sandwich Ag-ELISA, this integrin-based assay displays increased specificity (less cross-reactivity between serotypes) while retaining test sensitivity (Ferris *et al.*, 2005; 2011), however it is less robust to changes in the FMDV capsid proteins.

Antigen detection has also been incorporated onto immuno-chromatographic strip tests, known as antigen-detection lateral-flow devices (Ag-LFDs) (Ferris *et al.*, 2009). Functioning through the binding of both viral antigen and antibody-coated detector particles to bands of capturing monoclonal antibody on a membrane (Figure 1.11), Ag-LFDs can provide rapid results (*ca.* 10 minutes), and as such are useful for high priority samples. There are now eight publications detailing the development and use of Ag-LFDs for detection of FMDV antigen (Ferris *et al.*, 2009; 2010; Oem *et al.*, 2009; Jiang *et al.*, 2011a; 2011b; Yang *et al.*, 2013; 2015; Morioka *et al.*, 2015). In addition, commercially

available Ag-LFDs are now obtainable for pan-FMDV detection (SVANODIP® FMDV-Ag) through Boehringer Ingelheim (Bracknell, UK) and are moving towards routine use, for instance by the WRLFMD for triaging high priority samples for further characterisation (Figure 1.11). Ag-LFDs are now recognised in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012), however, validation studies show that they only offer equivalent diagnostic sensitivity to laboratory-based Ag-ELISAs (Ferris *et al.*, 2009), which limits their application to the acute clinical phase of disease and samples which contain high amounts of intact virus particles.

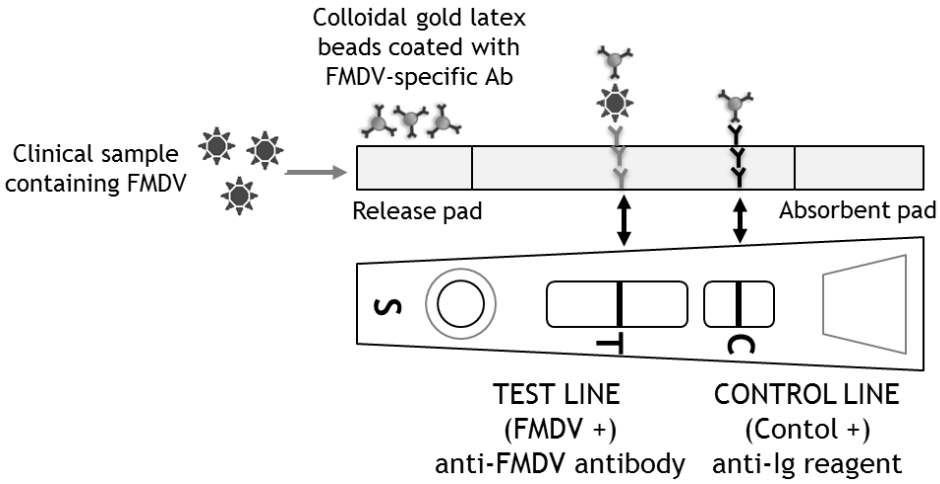

Figure 1.11 The mechanism of antigen-detection lateral-flow devices. A positive result is signified by the presence of two red bands (test and lateral-flow device control line); negative results are indicated by a single band (the lateral-flow device control line). (Ab) antibody; (FMDV) foot-and-mouth disease virus; (Ig) immunoglobulin. *In* Biological threat reduction (T. Beckham, ed.; paper by Howson *et al.*, 2017a).

However, as with all antigen-based detection, there is a continuous requirement to ensure reagents have affinity for new emerging viral strains. Furthermore, testing for intact viral antigen and/or live virus can be problematic when considering differing sample quality and partial degradation that can be caused by poor sample transportation. In addition, all the above tests require the use of animals or animal tissue to produce assay components. With organisations such as the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) dedicated to the promotion of methods that either avoid/replace the use of living animals, reduce the number of animals used, or refine the ways in which the animals are produced (minimise suffering/increase welfare), it is important to also consider alternative diagnostic procedures (Richmond, 2002).

1.4.7 Detection and characterisation of FMDV nucleic acid

The limitations associated with VI and antigen-based detection has led to the development and utilisation of molecular assays in order to complement immunological diagnostic methods. Reverse transcription PCR (RT-PCR) has become an established laboratory-based tool for the highly sensitive detection and characterisation of FMDV RNA (Knowles and Samuel, 1988; Callahan *et al.*, 2002; Reid *et al.*, 2002; Shaw *et al.*, 2007; OIE, 2012), relying upon thermocycling, a pair of oligonucleotide primers, the activities of two enzymes (reverse transcriptase and DNA polymerase) and amplicon detection. Fluorescence-based detection (real-time RT-PCR [rRT-PCR]) has superseded analysis by gel-electrophoresis due to decreasing the risk of cross-contamination between assays and enabling real-time quantification. Importantly, rRT-PCR can detect low amount of FMDV RNA extracted from numerous sample types, including tissues, OP fluid, blood, swabs, faeces, aerosols and milk, and is therefore operational across a large clinical window. Furthermore, rRT-PCR does not require intact virus particles, therefore can still be used for poorly stored samples that exhibit partial degradation. However, this ability results in rRT-PCR not being able to distinguish between viable and non-viable virus, which can be problematic in situations such as distinguishing between carrier animals and delayed viral clearance. When coupled to automatic nucleic extraction, rRT-PCR exhibits increased assay throughput and at least equal diagnostic sensitivity to that of VI (Reid *et al.*, 2003). For instance, rRT-PCR was employed during the UK 2007 FMDV outbreak to test 99.1% of 3246 diagnostic samples submitted to the UK National Reference Laboratory for FMD (TPI), and is able to detect FMDV in pre-clinical cattle (Reid *et al.*, 2009).

In order to study the molecular (nucleotide) epidemiology of FMD, nucleotide sequence analysis is used to compare the genetic differences between viruses (Knowles and Samuel, 1988; OIE, 2012). For instance, sequencing of the VP1-coding region, which displays *ca.* 30-50% nucleotide sequence difference between the seven serotypes, enables phylogenetic analysis to be performed for reasons such as tracing the origin of outbreaks and spread of FMD viruses (Beck and Strohmaier, 1987; Dopazo *et al.*, 1988; Samuel and Knowles, 2001b; Knowles and Samuel, 2003). Comparison of the whole FMDV genome can provide further discrimination between closely related viruses, and although not used routinely for sample analysis, has enabled retrospective tracing of transmission pathways for outbreak investigations (Cottam *et al.*, 2008; Logan *et al.*, 2014). There have also been efforts to design serotype-specific assays (Giridharan *et al.*, 2005; Ahmed *et al.*, 2012; Reid *et al.*, 2014; Jamal and Belsham, 2015; Bachanek-Bankowska *et al.*, 2016; Knowles *et al.*, 2016). Although these do not provide the detail gained through sequencing

techniques, these have the potential to help rapidly identify circulating FMDV strains (useful for basic epidemiology and informing vaccine matching studies), thus improving upon current lengthy laboratory-based typing techniques such as Ag-ELISA (Bachanek-Bankowska *et al.*, 2016).

1.4.8 Serological tests

FMD diagnostic laboratories also employ serological assays in order to: (i) certify animals for import or export, (ii) demonstrate previous FMDV infection or vaccination through the presence / absence of anti-FMDV antibodies, (iii) perform antigenic matching between vaccine and field strains (OIE, 2012) and (iv) for routine serosurveillance. Serological assays are of two types: those which detect antibodies to FMDV structural proteins (SPs) and those which detect antibodies to non-structural proteins (NSPs). Assays that detect antibodies to SPs are serotype-specific and signify either FMDV infection or vaccination. The current gold-standard assay for detection of antibodies to SPs is the virus neutralisation test (VNT), which detects anti-FMDV antibodies through the inhibition of viral infectivity in cell culture (Golding *et al.*, 1976). However, VNT assays pose several challenges. Different cell lines result in variable degrees of sensitivity and, in contrast to other serological tests that use inactivated virus (e.g. purified antigen), the requirement for large amounts of live virus means that the test needs to be performed in restrictive biocontainment facilities. Alternative assays such as the solid phase competition ELISA (SPCE) (Brocchi *et al.*, 1990; Mackay *et al.*, 1998; Chénard *et al.*, 2003; Paiba *et al.*, 2004) and liquid phase blocking ELISA (LPBE), (Hamblin *et al.*, 1986; 1987) are therefore commonly used to support testing (Table 1.1).

Assays which detect antibodies to NSPs are pan-specific and signify previous FMDV infection, regardless of vaccination status (assuming purified vaccines are used containing only SPs). Several assays, including ELISAs, have been established for detection of antibodies to FMDV NSPs such as 2C, 3AB and 3ABC involved in viral replication (Shen *et al.*, 1999; Bergmann *et al.*, 2000). Although initial NSP-ELISAs required species-specific conjugates, these have been updated through the years to be host species-independent and utilise monoclonal antibodies (Sørensen *et al.*, 1998; 2005). An LFD for detection of antibodies to 3ABC (SVANOVIR® FMDV 3ABC-Ab) has since been developed and is commercially available through Boehringer Ingelheim, although further validation is required for inclusion in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012).

1.4.9 Disadvantages of current diagnostic strategies

Although laboratory-based tests can provide rapid and accurate results, several factors affect the utility and practicality of these tests for guiding critical decision-making, particularly in low-resource settings. First, transportation of samples to laboratories can negatively affect the quality of the specimens and delay or hinder the processes of immediate critical decision-making and disease control. This is particularly problematic in countries with poor transport infrastructure, but even in the UK alternative control methods have been used to gain rapid control of outbreaks by bypassing laboratory confirmation, such as the UK 2001 "slaughter on suspicion" policy (a subjective method that relied entirely upon clinical observation with retrospective laboratory analysis showing no evidence of FMDV on 23% of suspected premises [Ferris *et al.*, 2006]). Secondly, many diagnostic procedures necessitate well-equipped laboratories that meet restrictive biocontainment requirements. This can be difficult for many countries with endemic FMD infection, which often lack infrastructure and financial/human resources for sample collection, sample submission, veterinary diagnostics and disease surveillance (Vosloo *et al.*, 2002; Paton *et al.*, 2009; Namatovu *et al.*, 2013). As such, laboratories within these areas often lack the ability to diagnose FMD adequately (Vosloo *et al.*, 2002; Paton *et al.*, 2009).

The development of simple, portable, diagnostic devices is now considered a priority for FMD to (i) help reduce the response time during outbreaks in FMD-free countries (recommended within independent reports that have followed outbreaks [Anderson, 2002; Anon, 2002a; 2002b]), and (ii) improve the diagnostic capacity of endemic laboratories (Namatovu *et al.*, 2013).

1.5 Future diagnostic strategies

Within the last decade, many technological advances have been made in the development of improved diagnostics for infectious diseases of public health importance. This has included the development of point-of-care tests (POCT) for rapid disease diagnosis in decentralised settings, such as public health centres and local laboratories. For instance, diagnostic assays based on the detection of antigens, antibodies or nucleic acid molecules have been developed and successfully introduced for routine surveillance and decentralised testing of human diseases such as human immunodeficiency virus infection / acquired immune deficiency syndrome (HIV/AIDS), tuberculosis and malaria (Maltha *et al.*, 2013; Reid *et al.*, 2013; Weyer *et al.*, 2013; Morris *et al.*, 2015). In addition to providing a rapid diagnosis to expedite medical decision-making, POCTs offer advantages for public health including expanded testing capabilities (e.g. access to hard to reach communities), reduced potential for sample deterioration and improved patient outcomes through reduced patient handling times (Makusha *et al.*, 2015).

The advantages associated with POCTs have resulted in calls for rapid and simple diagnostic tests in response to emergency situations by The World Health Organisation (WHO), such as the most recent outbreak of Ebola virus disease (EVD) in West Africa (WHO, 2014; Broadhurst *et al.*, 2015; Kurosaki *et al.*, 2016; Semper *et al.*, 2016). It has been estimated that if such tests had been available throughout the EVD outbreak, despite published assays displaying reduced test accuracy comparatively to laboratory equivalents, their use in combination with confirmatory PCR testing could have reduced the epidemic in Sierra Leone by over a third (Nouvellet *et al.*, 2015)

Capitalising on these advances, the veterinary industry is now exploiting the use of similar technologies for the detection of important livestock pathogens. The global veterinary diagnostics market is subsequently estimated to expand at a compound annual growth rate of 8.6% from 2016, to reach US$ 6.71 billion by 2021 (marketsandmarkets.com, 2016). A powerful illustration of the effectiveness of rapid POCT diagnostics for veterinary diseases was during the latter stages of the rinderpest eradication programme. The use of a simple, disposable, antigen-detection test (Ag-LFD) proved invaluable in countries such as Pakistan and Somalia, empowering field veterinarians to stamp out the last remaining pockets of infection (Brüning *et al.*, 1999).

The use of a similar strategy for FMD could aid in both FMD-free countries for rapid implementation of control measures and prevention of unnecessary culling (Anderson, 2002), and in endemic countries for establishing robust laboratory testing capacity (Namatovu *et al.*, 2015). As such, there have been numerous advances in decentralised diagnostics for FMD in the veterinary field, mainly focusing on antigen/antibody and nucleic acid detection technologies. This section describes some of these approaches.

1.5.1 Antigen/antibody detection systems

Ag-LFDs display many of the characteristics required for POCT use, including being portable, disposable, simple to use and relatively inexpensive. Consequently, Ag-LFDs have been successfully trialled for decentralised testing during the 2007 UK FMD outbreak on an infected premises to confirm FMD-positive cases (Ryan *et al.*, 2008). However, Ag-LFDs display reduced analytical sensitivity in comparison to molecular diagnostic techniques, making confirmation of FMD-negative cases difficult (a combined approach with laboratory-based methods would be required). Furthermore, Ag-LFDs present a simple alternative method for transport of samples to reference laboratories for further testing. For instance, recent studies have shown that nucleic acid can be recovered from LFDs following long-term storage at room temperature. This has permitted further downstream characterisation, including rRT-PCR, Sanger sequencing and recovery of live virus following electroporation (Fowler *et al.*, 2014; Romey *et al.*, 2017).

1.5.2 Real-time reverse transcription PCR

Due to its routine laboratory use and high sensitivity, much recent progress has been made in transitioning rRT-PCR onto POCT platforms (Belák *et al.*, 2010), with a number of technologies now commercially available (Table 1.2). For instance, there are now 11 publications detailing portable rRT-PCR assays for detection of FMDV, with potential for use in decentralised laboratories and field settings (Donaldson *et al.*, 2001; Callahan *et al.*, 2002; Hearps *et al.*, 2002; Moniwa *et al.*, 2007; King *et al.*, 2008; Paixão *et al.*, 2008; Reid *et al.*, 2010; Pierce *et al.*, 2010; Madi *et al.*, 2012; Ambagala *et al.*, 2016; Goller *et al.*, 2017).

Perhaps some of the most promising technologies, although costly, are those that are fully automated, integrating nucleic acid extraction, thermal cycling and result reporting onto a single instrument. By streamlining assay setup, advantages of such platforms

include reduced opportunities for cross-contamination and the requirement for minimal onsite expertise. For instance, the prototype Enigma® Field Laboratory (FL) (Enigma® Diagnostics, Porton Down, UK), a robust 19 kg platform which can be powered using a vehicle auxiliary, combines silica paramagnetic-bead-based nucleic acid extraction with rRT-PCR reagents in a single-cartridge (Madi *et al.*, 2012). Its commercially available successor, the MiniLab™ (ML) (Enigma® Diagnostics), is aimed at decentralised locations (e.g. hospitals /veterinary clinics). Using similar technology to the FL, the ML has increased throughput, with the option to add up to six processing modules (Goldenberg and Edgeworth, 2015; Goller *et al.*, 2017). Lyophilised assays have been developed for FMDV using this platform, in addition to a number of other veterinary diseases of importance, including African swine fever virus and classical swine fever virus (Enigma® Diagnostics). The use of lyophilised reagents increases compatibility for decentralised testing by improving stability, storage and transportability as reagents can remain at ambient temperatures. However, at present such reagents remain commercially unavailable.

Not all mobile PCR platforms incorporate nucleic acid extraction. For instance, commercially available platforms, including the Cepheid SmartCycler® (Cepheid), T-COR™ 8 (Tetracore), genesig® (Primerdesign Ltd.), R.A.P.I.D® (Idaho technologies) and Genedrive® (Epistem Limited, Manchester, UK) combine amplification and fluorescence-based detection in a single tube, with sample preparation required separately (Table 1.2). Some of these platforms have developed their own simple sample preparation methods, for example the Genedrive® uses a paper-based extraction approach (Niemz and Boyle, 2012). However, the majority of validation exercises on these platforms have been undertaken using nucleic acid extracted using either manual kits or robotic platforms, which are often unsuitable for decentralised testing in the field or low-resource laboratories. For example, the SmartCycler has been successfully trialled for the detection of FMDV, in combination with manual nucleic acid extraction kits, in laboratory settings (Hearps *et al.*, 2002; Moniwa *et al.*, 2007; Paixão *et al.*, 2008). The adoption of PCR assays that are less prone to inhibition (Kermekchiev *et al.*, 2009; Zhang *et al.*, 2010) could further increase demand by negating the requirement for complex sample preparation, offering an advantage over laboratory-based assays and opening up the real possibility for rRT-PCR use in decentralised settings.

Lyophilised FMDV-specific real-time PCR assay kits are now commercially available for a number of these POCT platforms, with both Tetracore and genesig® (Primerdesign Ltd., Southampton, UK) offering assays for FMDV (Boyle *et al.*, 2004). However, these kits are

only licensed for research purposes at present. Further development and evaluation is required for these to enter the diagnostic market.

Table 1.2 Examples of molecular point-of-care testing platforms

Platform	Manufacturer	Method	Well capacity	Integrated sample preparation	Detection channels	Assay time	Power
*Enigma® FL	Enigma Diagnostics, UK	PCR	1	✓	6	1 hour	Car auxiliary
*Enigma® ML	Enigma Diagnostics, UK	PCR	1 - 6	✓	6	1 hour	Mains
SmartCycler®	Cepheid, USA	PCR	16-96	X	4	< 1 hour	Mains
Genedrive®	Epistem Ltd., UK	PCR	3	X	1	< 1 hour	Battery
R.A.P.I.D®	Idaho Tech., USA	PCR	32	X	3	30 min	Mains
POCKIT™	GeneReach, USA	iiPCR	8	X	2	< 1 hour	Mains
*BioSeeq™	Smiths Detection, UK	LATE-PCR	5	✓	4	2 hours	Battery
T-COR™ 8	Tetracore, USA	PCR	8	X	6	1 – 2 hours	Battery
genesig q16	genesig, UK	PCR	16	X	2	1 – 2 hours	Mains
Genie® II Genie® III	OptiGene Ltd., UK	LAMP	16 8	X	1 2	< 30 min	Battery
T-8	TwistDx, UK	RPA	8	X	8	< 30 min	Battery

*Platform no longer available. (iiPCR) insulated isothermal PCR; (LAMP) loop-mediated isothermal amplification; (RPA) recombinase polymerase amplification; (LATE) linear-after-the-exponential; (FL) field laboratory; (ML) mini laboratory. Adapted from previous publications (Holland and Kiechle, 2005; Dineva *et al.*, 2007).

1.5.3 Alternative PCR chemistries

Whilst the transferral of routine rRT-PCR assays onto POCT platforms maintains some level of consistency and test confidence between laboratory and POCT assays, numerous drawbacks are still evident. For instance, in order to maintain the precise thermal regulation required for PCR, sophisticated instrumentation is still necessary, which can be expensive and difficult to decontaminate between premises. This requirement has resulted in an inevitable trade-off between portability and sample-throughput capabilities (Table 1.2). As such, alternative PCR chemistries such as insulated isothermal PCR (iiPCR) and Linear-After-The-Exponential PCR (LATE-PCR) have been explored as potential veterinary diagnostic solutions.

In iiPCR, a horizontal temperature gradient is created across a PCR vessel by heating it from the bottom at a fixed temperature, with the top cooled by surrounding air. By negating the requirement for conventional thermal cycling, iiPCR reactions can be performed using relatively simple, portable platforms such as the commercially available POCKIT™ nucleic acid analyser (Table 1.2) (GeneReach, MA, USA) (Tsai *et al.*, 2012). Recently, POCKIT™-compatible lyophilised real-time RT-iiPCR assays have been developed and evaluated for pan-specific detection of FMDV in laboratory settings (Ambagala *et al.*, 2016), with comparable sensitivity to laboratory-based rRT-PCR. Furthermore, direct detection of FMDV from vesicular fluid samples has been shown to yield positive results, somewhat negating the requirement for sample preparation and increasing compatibility with field use (Ambagala *et al.*, 2016). The use of other clinical materials is yet to be determined. Although rapid, with detectable levels of amplicon generated within 30 minutes (Chang *et al.*, 2012), platforms such as the POCKIT™ are binary end-point systems (report samples only as positive or negative), which, although beneficial if used by less-skilled operators (results require no interpretation), reduces assay flexibility (quantitative interpretation is not possible).

LATE-PCR is an advanced form of asymmetric PCR, in which primers are designed for use at unequal concentrations to preferentially amplify one strand of the target DNA. Therefore following exponential amplification, reactions switch to linear amplification, during which single-stranded amplicons are generated for additional thermal cycles, thereby increasing the detection signal comparatively to symmetric PCR (Pierce *et al.*, 2005; Sanchez *et al.*, 2009). Beneficially, LATE-PCR probes can be designed to be mismatch-tolerant (beneficial when considering the high mutation rate of RNA viruses [Domingo *et al.*, 1985; Drake and Holland, 1999]), aiding the development of reverse transcription LATE-PCR (RT-LATE-PCR) assays for pan-FMDV detection (Pierce *et al.*, 2010; Reid *et al.*, 2010). In addition to enabling end-point quantification, and sharing equivalent analytical sensitivity to rRT-PCR, these assays are compatible with the BioSeeq™-Vet (Smiths Detection, London, UK), a portable diagnostic platform which incorporates sample preparation. However, this platform has never been made commercially available (Pierce *et al.*, 2010).

1.5.4 Isothermal chemistries

An alternative approach to reducing the size, cost and complexity of molecular POCT platforms is to eliminate the requirement for precise thermal regulation and perform reactions at a single fixed temperature using isothermal chemistries. Numerous isothermal methodologies have been developed (Craw and Balachandran, 2012), with four to date applied for the detection of FMDV, namely: loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), nucleic acid sequence based amplification (NASBA) and helicase dependent amplification (HDA).

LAMP is a rapid nucleic acid amplification technique that utilises a strand-displacing polymerase, multiple primers and auto-cycling under isothermal conditions (Notomi *et al.*, 2000; Nagamine *et al.*, 2002). Due to high sensitivity and rapid detection, a number of reverse transcription (RT-LAMP) assays have been designed for the detection of FMDV (Dukes *et al.*, 2006; Li *et al.*, 2009; Shao *et al.*, 2010; Chen *et al.*, 2011a; 2011b; Yamazaki *et al.*, 2013; Guan *et al.*, 2013; Madhanmohan *et al.*, 2013; Ding *et al.*, 2014; Ranjan *et al.*, 2014; Farooq *et al.*, 2015). However, none are yet commercially available in a kit format. Much progress has been made in transitioning LAMP onto portable platforms. This is partly due to LAMP chemistry having increased tolerance of inhibitors compared to PCR. For example, LAMP has shown higher sensitivity than rRT-PCR when detecting SVD virus from porcine faecal RNA extractions (Blomström *et al.*, 2008) and has been used to detect FMDV RNA directly from epithelial dilutions and aerosol samples (Waters *et al.*, 2014). Furthermore, numerous detection methods are possible. Low-cost, objective, end-point detection can be performed using molecular lateral-flow devices (LFDs) (Figure 1.12) (Waters *et al.*, 2014) or commercially available portable fluorimeters can be used for real-time detection (Genie® II / III: OptiGene Ltd.) (Table 1.2; Figure 1.13).

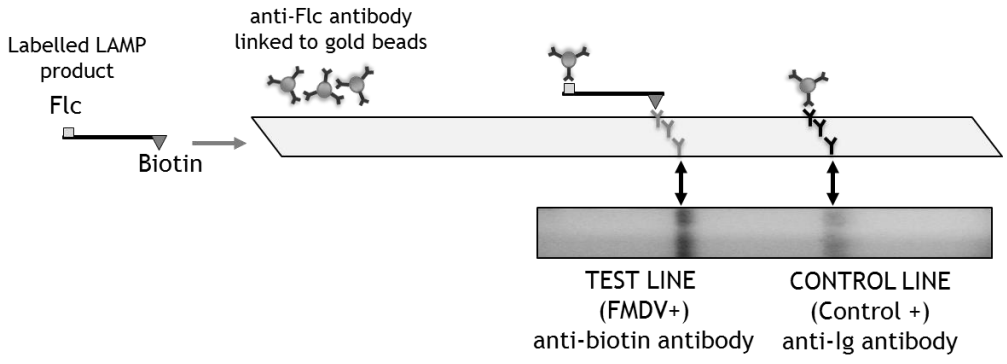

Figure 1.12 Illustration of reverse transcription loop-mediated isothermal amplification using end-point lateral-flow detection. A positive result is signified by the presence of two blue bands (test and lateral-flow device [LFD] control); negative results are indicated by a single band (LFD control). (Flc) fluorescein; (Ab) antibody; (Ig) immunoglobulin. *In* Biological threat reduction (T. Beckham, ed.; paper by Howson *et al.*, 2017a).

Although assays for characterisation of FMDV by RT-LAMP have been published (Chen *et al.*, 2011b; Madhanmohan *et al.*, 2013; Ding *et al.*, 2014; Farooq *et al.*, 2015), their design is complicated through the requirement for six to eight target regions in highly varied regions of the FMDV genome, including the capsid-coding region.

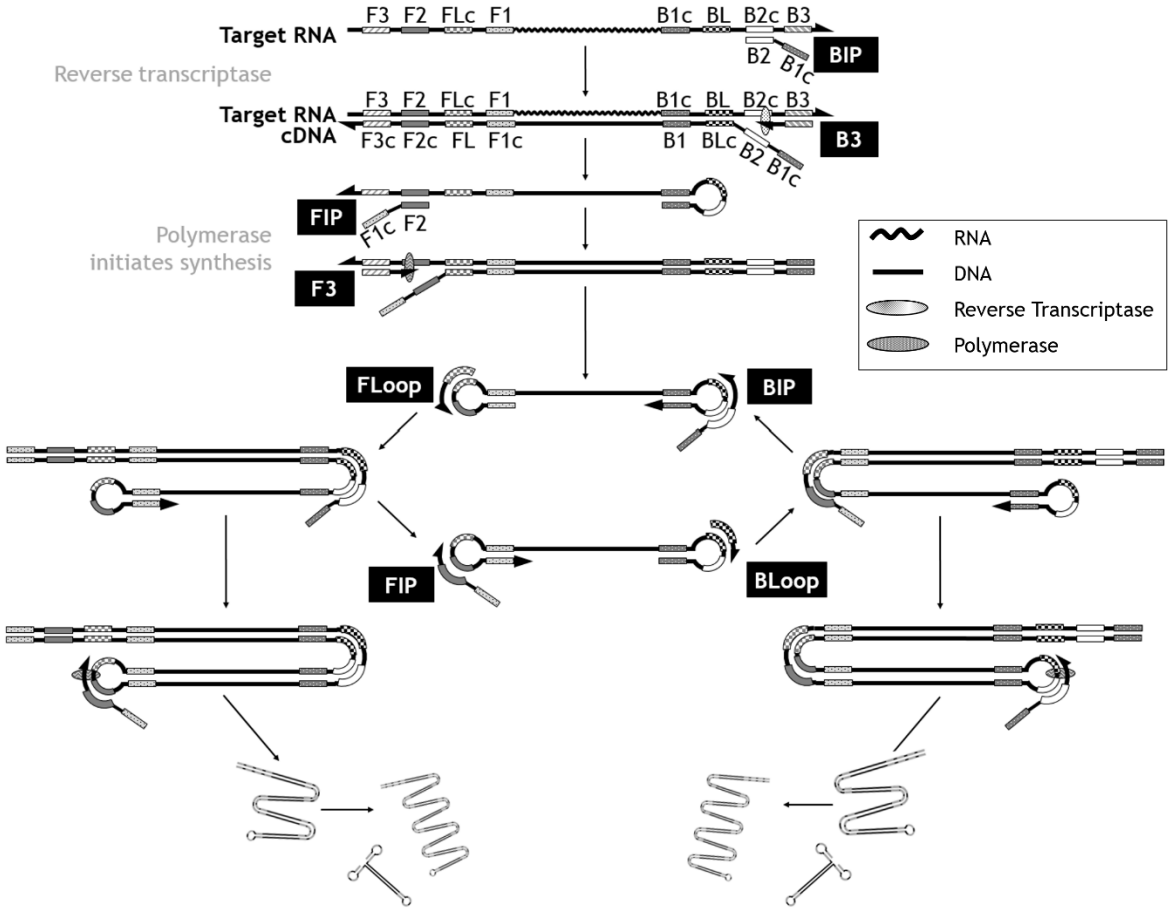

Figure 1.13 Schematic of loop-mediated isothermal amplification. F1(c)-F3(c) and B1(c)-B3(c) represent primer binding regions on the target sequence. (c) complementary; (FIP) forward internal primer; (BIP) backwards internal primer; (F3) forward external primer; (B3) backward external primer; (FLoop) forward loop primer; (BLoop) backward loop primer. Arrows represent the 5'-3' nucleotide sequence direction. *In* Biological threat reduction (T. Beckham, ed.; paper by Howson *et al.*, 2017a).

In RPA, isothermal amplification of template is achieved through the coupling of recombinase-driven primer targeting with strand-displacement synthesis (Figure 1.14) (Piepenburg *et al.*, 2006). Tube scanners from TwistDx™ (Cambridge, UK) and Qiagen (ESEQuant TS2), in combination with lyophilised regents (TwistDx™), have enabled RPA to enter the POCT market. For instance, during the 2012 FMDV Egypt outbreak, a pan-FMDV assay and portable reverse transcription RPA (RT-RPA) laboratory was trialled with manual RNA extraction (Dynabeads® Silane viral nucleic acid kit, Invitrogen™), achieving 98% sensitivity over 45 samples, with an analytical sensitivity of 1436 FMDV RNA molecules (Abd El Wahed *et al.*, 2013). However, many available nucleic acid extraction procedures are complex and time consuming (Dineva *et al.*, 2007), consequently, validation of RT-RPA on simple sample preparations is important for transitioning the chemistry into a realistic field-ready format.

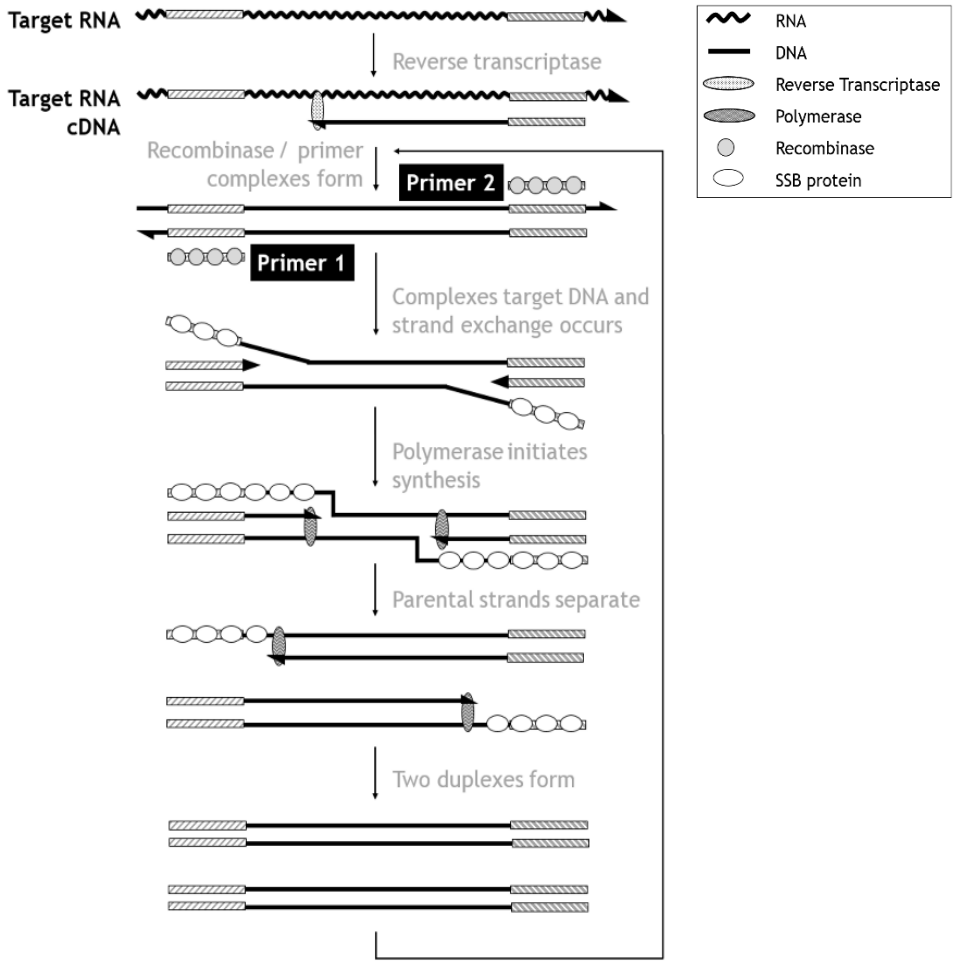

Figure 1.14 Schematic of recombinase polymerase amplification. Arrows represent the 5'-3' nucleotide sequence direction. *In* Biological threat reduction (T. Beckham, ed.; paper by Howson *et al.*, 2017a).

Nucleic Acid Sequence Based Amplification (NASBA) is a continuous isothermal technique used primarily to detect single-stranded RNA. The reaction depends on the activities of three enzymes: a reverse transcriptase, T7 RNA polymerase and ribonuclease-H, in addition to a single pair of specially-designed oligonucleotide primers (Figure 1.15), with optimal conditions allowing for 10^9-10^{12} fold amplification after two hours (Compton, 1991; Lau *et al.*, 2006). Using real-time detection methods, assays have been developed for numerous animal viruses, including FMDV (Lau *et al.*, 2008), with sensitivity reported to be equivalent to that of rRT-PCR. However, to date, validation of these tests remain in the research and development phase, using extracted nucleic acid, therefore further work is required to transition this chemistry into a POCT format.

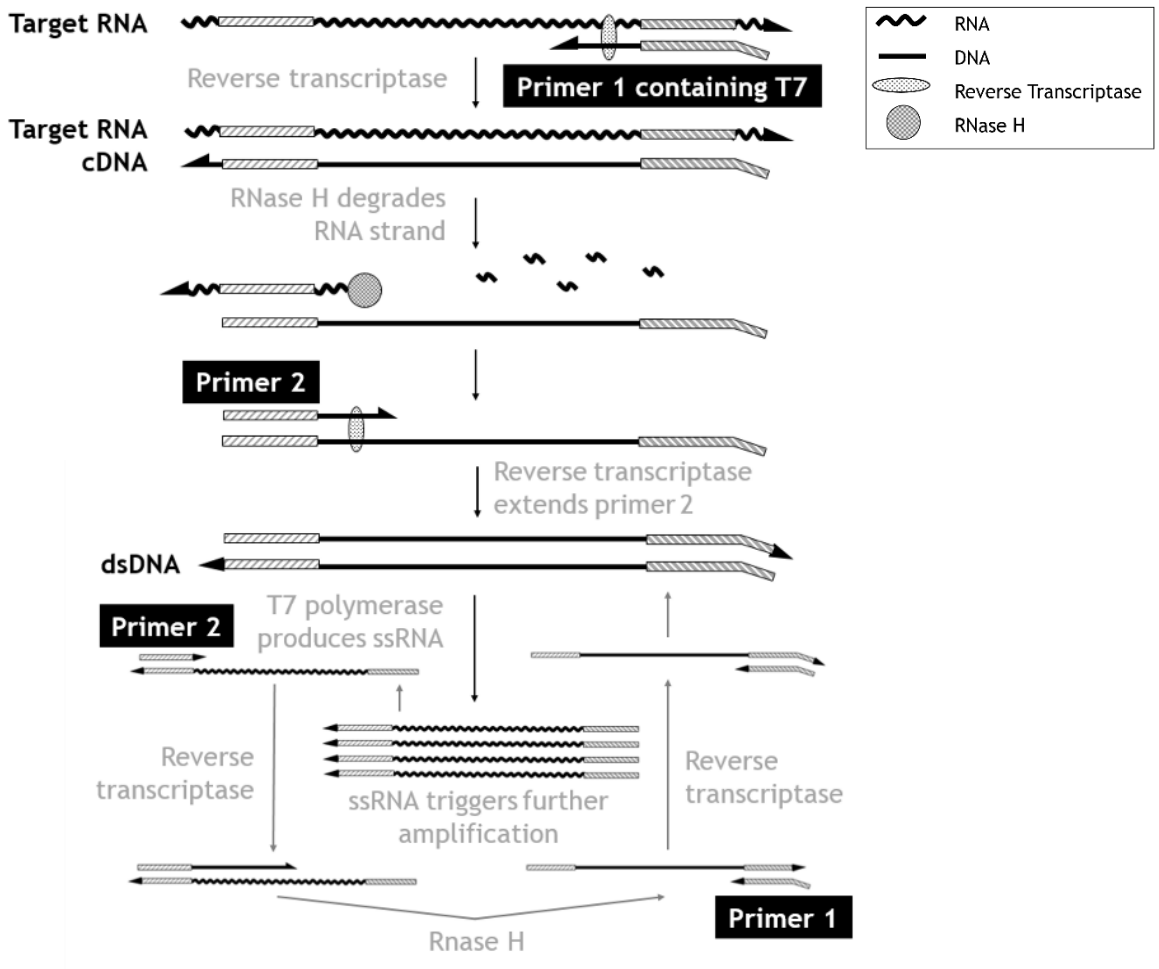

Figure 1.15 Schematic of nucleic acid sequence based amplification. Arrows represent the 5'-3' nucleotide sequence direction. *In* Biological threat reduction (T. Beckham, ed.; paper by Howson *et al.*, 2017a).

Helicase dependent amplification (HDA) is an isothermal amplification method that is similar to conventional PCR, but utilises the action of a helicase enzyme, rather than heat, to denature nucleic acids (Figure 1.16) (Vincent *et al.*, 2004). The addition of intercalating dyes has enabled HDA to be performed in real-time (Goldmeyer *et al.*, 2007), generally taking between 30–90 minutes for relatively short targets (70–120 bp) (Boonham *et al.*, 2014). Reverse transcription HDA (RT-HDA) assays for the detection of FMDV have been developed and tested in laboratory settings (Jingwei *et al.*, 2014), with detection limits reported as 10-fold higher than conventional agarose-gel based RT-PCR. However, as yet these assays have not been transferred onto portable platforms, therefore more work is required in transitioning and validating this chemistry as a POCT.

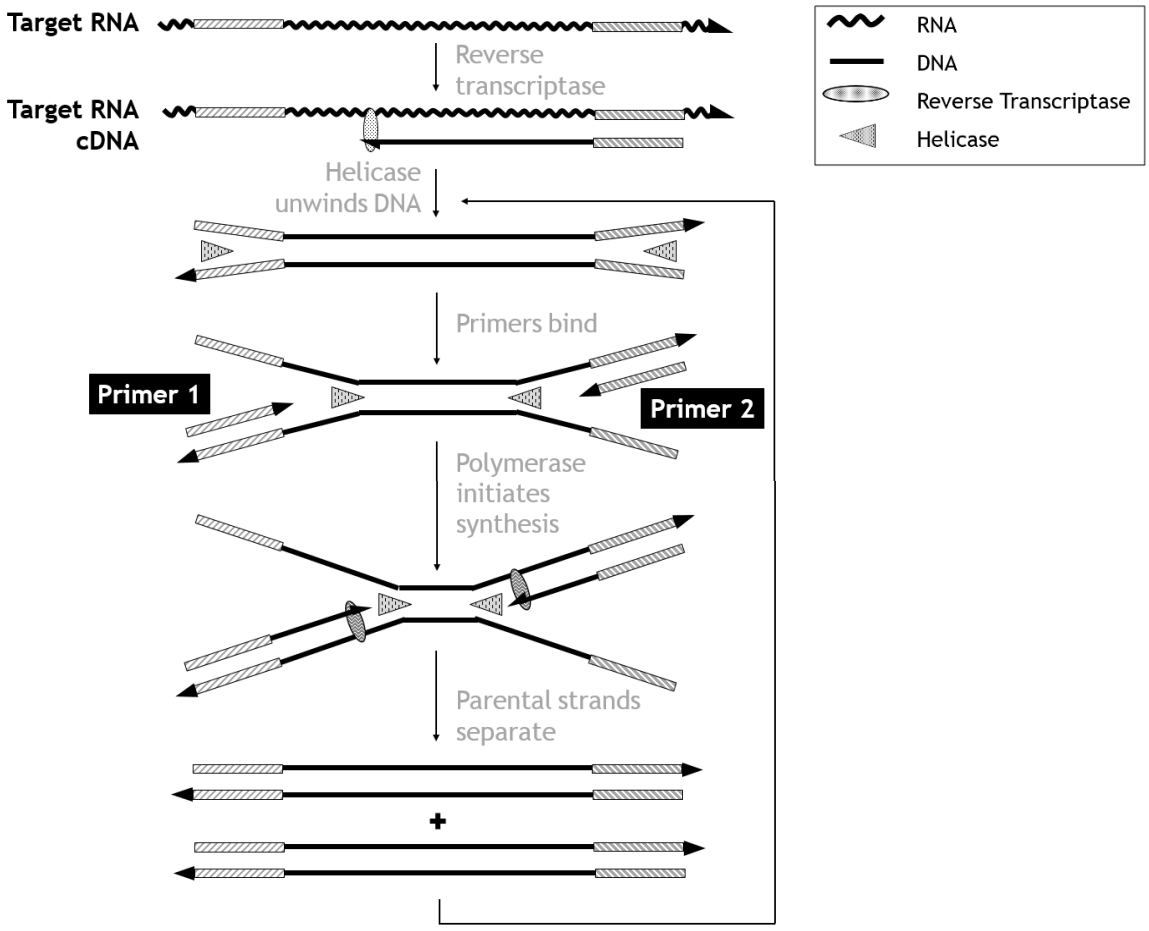

Figure 1.16 Schematic of helicase dependent amplification. Arrows represent the 5'-3' nucleotide sequence direction. *In* Biological threat reduction (T. Beckham, ed.; paper by Howson *et al.*, 2017a).

1.5.5 Mobile sequencing

The availability of novel sequencing methods may change the way diagnosis is performed in the future, offering a potential way to perform epidemiological tracing in low-resource settings. For instance, the MinION (Oxford Nanopore, Oxford, UK) is a commercially available, portable sequencer (requires connection to a laptop) that can be used for real-time biological analysis. This platform was recently applied in the field of human diagnostics for real-time genomic surveillance of Ebola virus in local laboratory settings, with sequencing results generated in less than 24 hours after receiving Ebola-positive samples (Quick *et al.*, 2016). Although not used for initial confirmation of disease presence, sequencing was performed on a total of 142 clinical samples, providing a new tool for informing epidemiological investigations.

Sequencing technologies currently still in the development stage (e.g. developments by Genapsys, CA, USA and QuantuMDX, Newcastle upon Tyne, UK) may in the future enable decentralised sequencing of targeted pathogen genome regions, complete genomes or metagenomes, although it is presently unclear if and when they will become commercially available. For instance, Oxford Nanopore are currently developing the SmidgION, with expected release in late 2017 (Lu *et al.*, 2016), which uses the same nanopore sensing technology as the MinION but has been designed for use with smartphones for mobile field-based sequence analyses. The future availability of field-based sequencing technologies, combined with current knowledge of simple sample preparation strategies, such as elution of nucleic acid from LFDs (Fowler *et al.*, 2014), could make *in situ* sequencing a real possibility in the next decade. For FMD, the rapid generation of sequencing data could be valuable in epidemiological outbreak tracing and informing potential vaccination responses.

1.6 Scientific aims

The benefits of transitioning diagnostic assays into POCT-formats is clear in terms of accelerating diagnostic confirmation, strengthening veterinary surveillance systems and providing increased flexibility in disease control. However, despite these benefits and numerous technological advances, the uptake of molecular POCTs remains low for the detection of FMDV, with promising new assays remaining in the research and development phase.

Although assays are regularly reported as suitable for use in low-resource environments, there is limited validation of FMDV-specific assays within these settings, with the majority validated in laboratories using formats unsuitable for decentralisation. For instance, assays typically utilise 'wet' reagents, which contain temperature-sensitive enzymes, and utilise nucleic acid extracted using either robots or manual extraction kits, both incompatible with decentralised use. The first aim of this thesis was therefore to compare multiple FMDV-specific assays and protocols, in order to define their current capabilities, and secondly transition the most promising of these into formats suitable for POCT. These assays were subsequently deployed into endemic settings (field and low-resource laboratories) to assess their potential use as real-time surveillance tools (Figure 1.17).

Most evaluation of POCT platforms has been performed using pan-specific assays, which enable confirmation of the presence or absence of FMDV, but lack the ability to distinguish between FMDV serotypes. Although critical for managing outbreak scenarios, where rapid pan-FMDV confirmation of clinical signs is necessary, such assays have reduced applicability for settings where FMD is endemic. In these situations, the disease can usually be detected quite accurately on the basis of clinical signs, which are well recognised by livestock-keepers and veterinary officers. However, rapid identification of serotypes could be very useful in informing specific responses to control, such as reactive and targeted vaccination in response to serotype-specific outbreaks. The transfer of typing assays (Bachanek-Bankowska *et al.*, 2016) onto portable platforms could provide necessary tools for FMDV characterisation in decentralised settings, subsequently forming the third aim of the project.

By targeting the more varied regions of the FMDV genome, there is a continuous need to re-evaluate molecular assays to ensure that primers designed sufficiently match emerging strains. Due to the logistics associated with doing this via laboratory-evaluation (in terms of cost, time and acquisition of suitable samples), the final aim of this thesis was to

develop a novel bioinformatics tools to rapidly predict the performance of molecular assays *in silico*.

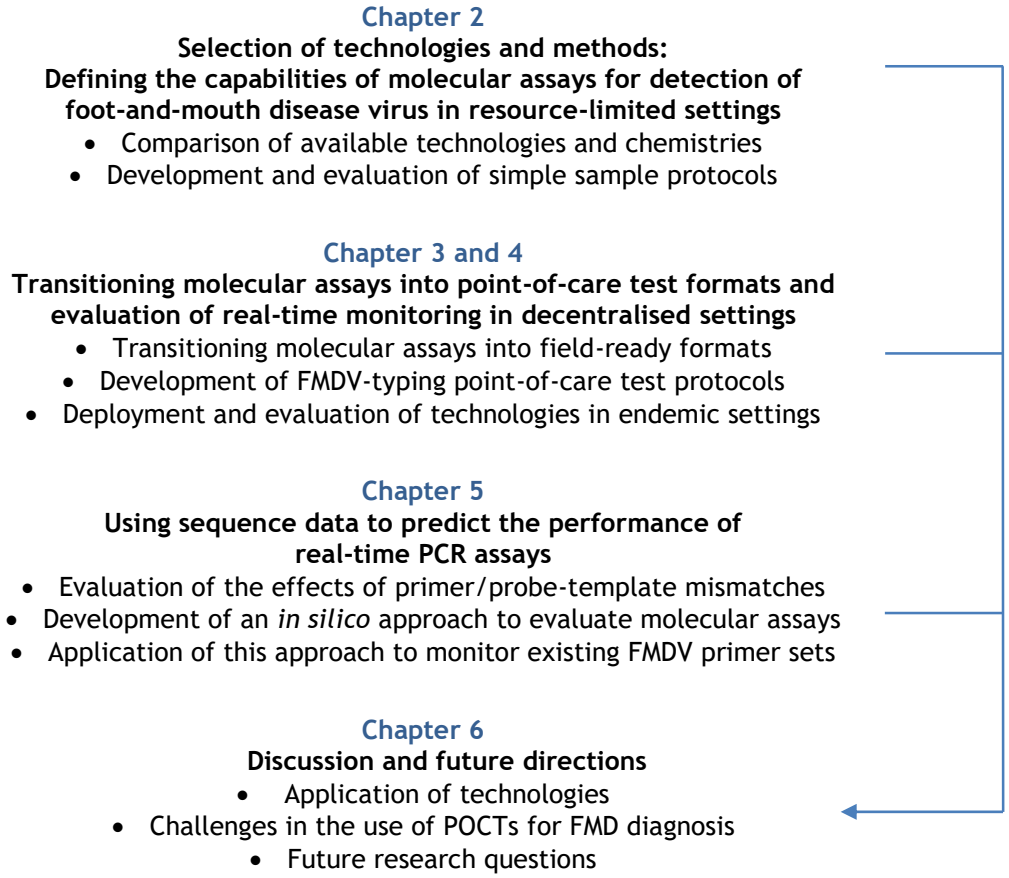

Figure 1.17 Thesis overview.

CHAPTER 2

Selection of technologies and methods: Defining the capabilities of molecular assays for detection of foot-and-mouth disease virus in resource-limited settings

Elements of this chapter have been published in the following peer-reviewed article:

Howson, E.L.A., Kurosaki, Y., Yasuda, J., Takahashi, M., Goto, H., Gray, A.R., Mioulet, V., King, D.P. and Fowler, V.L. (2017b). Defining the relative performance of isothermal assays that can be used for rapid and sensitive detection of foot-and-mouth disease virus. *J Virol Methods*. doi: 10.1016/j.jviromet.2017.08.013 [Epub ahead of print].

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Defining the relative performance of isothermal assays that can be used for rapid and sensitive detection of foot-and-mouth disease virus

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ABSTRACT

This study describes the first multiway comparison of portable isothermal assays for the detection of foot-and-mouth disease virus (FMDV), benchmarked against real-time reverse transcription RT-PCR (rRT-PCR). The selected isothermal chemistries included reverse transcription loop-mediated isothermal amplification (RT-LAMP) and reverse transcription recombinase polymerase amplification (RT-RPA). The analytical sensitivity of RT-LAMP was comparable to rRT-PCR (10^1 RNA copies), while RT-RPA was one $\log_{10}$ less sensitive (10^2 RNA copies). Diagnostic performance was evaluated using a panel of 35 samples from FMDV-positive cattle and eight samples from cattle infected with other vesicular viruses. Assay concordance for RT-LAMP and RT-RPA was 86–98% and 67–77%, respectively, when compared to rRT-PCR, with discordant samples consistently having high rRT-PCR cycle threshold values (no false-positives were detected for any assay). In addition, a hierarchy of sample preparation methods, from robotic extraction to simple dilution of samples, for epithelial suspensions, serum and oesophageal-pharyngeal (OP) fluid were evaluated. Results obtained for RT-LAMP confirmed that FMDV RNA can be detected in the absence of RNA extraction. However, simple sample preparation methods were less encouraging for RT-RPA, with accurate results only obtained when using RNA extraction. Although the evaluation of assay performance is specific to the conditions tested in this study, the compatibility of RT-LAMP chemistry with multiple sample types, both in the presence and absence of nucleic acid extraction, provides advantages over alternative isothermal chemistries and alternative pen-side diagnostics such as antigen-detection lateral-flow devices. These characteristics of RT-LAMP enable the assay to be performed over a large diagnostic detection window, providing a realistic means to rapidly confirm positive FMD cases close to the point of sampling.

1. Introduction

Molecular diagnostic assays such as PCR are now recognised as reliable detection methods for many viral pathogens, including foot-and-mouth disease (FMD) virus (FMDV: family *Picornaviridae*, genus *Aphthovirus*) (The World Organisation for Animal Health [OIE, (2012)], 2012). Endemic across many countries in Asia and Africa and parts of South America, FMD is highly contagious, with outbreaks in previously disease-free areas incurring severe economic damage (Knight-Jones and Rushton, 2013). Accurate and early diagnosis is therefore critical for the rapid enforcement of monitoring, control and eradication strategies. Laboratory-based real-time reverse transcription PCR (rRT-PCR) has become a widely accepted molecular tool for the detection and quantification of FMDV RNA, with diagnostic rRT-PCR assays developed for detection of all seven FMDV serotypes (A, O, C, Asia 1 and Southern African Territories [SAT] types 1–3) across a wide range of sample types (Callahan et al., 2002; Reid et al., 2002). Although relatively quick to perform, only taking a few hours to produce results (Shaw et al., 2007), sample transportation to laboratories is required which can be lengthy and consequently delay critical decision making.

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2.1 Summary

Despite technological advances in the development of point-of-care tests (POCTs) for diagnosis of foot-and-mouth disease (FMD), uncertainties still remain about test performance and further validation is required to increase confidence in their use. This chapter describes the first multiway comparison of assays for the detection of FMD virus (FMDV), to define their capabilities for use in resource-limited settings, benchmarked against a reference real-time reverse transcription PCR (rRT-PCR). The chemistries evaluated included POCT-adapted protocols for rRT-PCR, reverse transcription loop-mediated isothermal amplification (RT-LAMP) and reverse transcription recombinase polymerase amplification (RT-RPA). In this study, the analytical sensitivities of rRT-PCR and RT-LAMP were comparable to the reference rRT-PCR (10^1 RNA copies), while RT-RPA was one $\log_{10}$ less sensitive (10^2 RNA copies). Diagnostic performance was evaluated using a panel of 35 clinical samples from FMDV-positive animals and eight samples from animals infected with other vesicular viruses. Assay concordance for POCT-ready rRT-PCR, RT-LAMP and RT-RPA was 100%, 86-88% and 67-77%, respectively, when compared to the reference rRT-PCR. Discordant samples (false-negatives) consistently had high rRT-PCR cycle threshold values (no false-positives were detected for any assay). In order to assess the suitability of these methods for field diagnosis, a hierarchy of sample preparation methods, from robotic extraction to simple dilution of samples, for epithelial tissue suspensions, serum and oesophageal-pharyngeal (OP) fluid were evaluated. Results obtained for POCT-ready rRT-PCR and RT-LAMP confirmed that FMDV RNA can be detected in the absence of RNA extraction. However, simple sample preparation methods (such as the dilution of samples in nuclease-free water), were less encouraging for RT-RPA and the reference rRT-PCR, with accurate results only obtained when using RNA extraction. Although this evaluation is specific to the assays tested in this study, the performance of POCT-ready rRT-PCR and RT-LAMP, in terms of sensitivity and compatibility with multiple sample types, provides advantages over alternative pen-side diagnostics such as antigen-detection lateral-flow devices (Ag-LFDs). As such, POCT-ready RT-LAMP and rRT-PCR were selected as the most promising assays for diagnosis of FMD in the field and low-resource laboratory settings, and were taken forward for subsequent development, evaluation and field testing.

2.2 Introduction

The recent drive to develop decentralised diagnostics for foot-and-mouth disease (FMD) has led to a number of research initiatives prioritising the transition of laboratory-based assays into formats suitable for use in resource-limited settings. As such, there have been numerous publications, in peer-reviewed journals, outlining the development and deployment of point-of-care tests (POCTs) for the detection of FMD virus (FMDV) (Belák *et al.*, 2010; Sammin *et al.*, 2010; King *et al.*, 2012). Although the advantages of these assays are clear in terms of accelerating diagnostic confirmation during FMDV outbreaks (Anderson, 2002) and establishing robust laboratory testing capacity in endemic countries (Namatovu *et al.*, 2013), uptake of such assays remains low, remaining almost exclusively in the research and development phase.

One of the main barriers preventing the routine use of decentralised diagnostic assays for FMD is the lack of validation. Assays are typically evaluated through pairwise concordance testing against an established reference test, with molecular assays regularly compared against real-time reverse transcription PCR (rRT-PCR). Whilst this approach is useful for understanding the diagnostic performance of an assay in isolation, it does not capture the variability in test performance that may arise as a result of the use of different reference rRT-PCR reagents, extraction protocols, testing conditions and test cut-off values. Selection of the most appropriate chemistry / assay for decentralised diagnostics is therefore difficult and guidelines for the use of such assays are not yet included within the manual of recommended tests of The World Organisation for Animal Health (OIE) (OIE, 2012), with the exception of antigen-detection lateral-flow devices (Ag-LFDs).

Furthermore, although assays are reported as suitable for use in decentralised settings (in the field or low-resource laboratories) (Callahan *et al.*, 2002; Hearps *et al.*, 2002; Moniwa *et al.*, 2007; King *et al.*, 2008; Abd El Wahed *et al.*, 2013; Ambagala *et al.*, 2016), the majority of FMDV-specific assays have been validated using protocols only suitable for laboratory-based testing. For instance, molecular assays are typically evaluated using nucleic acid extracted by either robots or manual extraction kits, unsuitable for use in resource-limited settings. A number of alternative simple sample preparation methods have been developed: the preparation of epithelial tissue suspensions by dilution in nuclease-free water (NFW) has shown to produce accurate reverse transcription loop-mediated isothermal amplification (RT-LAMP) results (Waters *et al.*, 2014), simple elution from disposable immuno-chromatographic strip tests has been successful for preparation of epithelial tissue suspensions prior to rRT-PCR (Fowler *et al.*, 2014) and Chelex® 100 has

been reported to remove inhibitors of rRT-PCR from serum (Ochert *et al.*, 1994; Walsh *et al.*, 2013). Although these methods provide potential solutions for resource-limited settings, further evaluation and comparison of these methods for this purpose is required.

The transition of decentralised diagnostic assays into current diagnostic pipelines (see Chapter 1.4.4, Figure 1.9) also needs to be considered to evaluate how these assays could work in parallel with (or replace) existing methodologies. For instance, in order to fully understand the epidemiology of circulating FMDV strains, even in the presence of POCTs for rapid virus detection, FMD samples would still require shipment to suitable laboratories for characterisation purposes (e.g. whole genome sequencing and vaccine matching). Therefore, POCTs do not completely eliminate the requirement for sample submission to laboratories, which is hindered by shipment costs, cold chain maintenance, potential sample degradation and packaging requirements for diagnostic material. As such, recent publications have proposed Ag-LFDs as a potential non-hazardous transport solution as they preserve nucleic acid for downstream characterisation, with preliminary data suggesting that, after periods of storage, it is not possible to recover live virus from the devices (Fowler *et al.*, 2014; Romey *et al.*, 2017). Similarly, FTA® cards have been implemented as a sampling device for the collection, shipment, storage and detection of FMDV, as again preliminary data suggests that while live virus is not recoverable, characterisation of FMDV RNA is possible (Muthukrishnan *et al.*, 2008; Madhanmohan *et al.*, 2015). Consequently, such methods could be used to support decentralised diagnostics, with further evaluation of their potential required.

This chapter presents the first multiway comparison of different molecular assay formats for the detection of FMDV, benchmarked against a reference rRT-PCR. Assays included POCT-adapted representatives of rRT-PCR, RT-LAMP and reverse transcription recombinase polymerase amplification (RT-RPA). In addition, a selection of sample preparation methods, including those which could support simplified sample transportation to laboratories, were evaluated for use with each of these chemistries in order to define their capabilities. These chemistries, assay formats and sample preparation methods were selected based on existing literature (Boyle *et al.*, 2004; Polichronova *et al.*, 2010; Abd El Wahed *et al.*, 2013; Waters *et al.*, 2014) and because they represent realistic options for FMDV-specific decentralised diagnostics.

2.3 Materials and Methods

2.3.1 Ethics statement

Clinical samples utilised in this chapter (including negative oesophageal-pharyngeal [OP] fluid) were either archival samples from previous experimental studies approved by The Pirbright Institute (TPI) Ethical Review Committee under the Animal Scientific Procedures Act (ASPA) 1986 (as amended), or comprised samples submitted by endemic countries to the Food and Agricultural Organization (FAO) World Reference Laboratory for FMD (WRLFMD) at TPI. Negative bovine epithelium and blood (serum) were obtained from a UK abattoir under the requirements of Article 23 of the Animal By-products Regulation (EC) No. 1069/2009 as approved by the Animal and Plant Health Agency (APHA) (reference U1230581/ABP).

2.3.2 Reference real-time reverse transcription PCR

The one-step rRT-PCR, used as the reference test, employed primers and probes as previously described to target the highly conserved $3D^{pol}$-coding region of the FMDV genome (Callahan *et al.*, 2002), with reagents, parameters and thermal cycling as previously reported (Shaw *et al.*, 2007). rRT-PCR reactions were performed in duplicate on a bench-top real-time PCR machine (Stratagene Mx3005P™: Agilent Technologies, CA, USA) (Table 2.1). A positive rRT-PCR reaction was indicated by an exponential increase in fluorescence (δR) and the cycle threshold (C_T) was called when reactions reached δR 1000. This rRT-PCR method is recommended within The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012).

Table 2.1 Molecular assay formats compared

Assay	Reagents	Reagent form	Primers and Probes	Platform
Reference rRT-PCR	Shaw *et al.*, 2007	Wet	Callahan *et al.*, 2002	Mx3005P™
T-COR™ 8 (#1) T-COR™ 8 (#2)	SSIII-based TC-9092-064	Lyophilised	Proprietary primer/probe mix targeting $3D^{pol}$-coding region	T-COR™ 8
rRT-LAMP	ISO-001 + AMV	Wet*	Dukes *et al.*, 2006	Genie® II
RT-LAMP-LFD	ISO-001 + AMV	Wet*	Dukes *et al.*, 2006	Genie® II + LFD
rRT-RPA-exoRT	TwistAmp® exo RT	Lyophilised	Abd El Wahed *et al.*, 2013	Genie® II
rRT-RPA-nfo	TwistAmp® nfo + RT	Lyophilised	Abd El Wahed *et al.*, 2013	Genie® II

(rRT-PCR) real-time reverse transcription PCR; (rRT-LAMP) real-time reverse transcription loop-mediated isothermal amplification; (RT-LAMP-LFD) RT-LAMP combined with lateral-flow detection; (rRT-RPA) real-time reverse transcription recombinase polymerase amplification; (AMV) Avian Myeloblastosis Virus; (RT) reverse transcriptase. *Only available in a wet format.

2.3.3 Portable real-time reverse transcription PCR

Two field-ready rRT-PCR assays from Tetracore Inc. (MD, USA) were evaluated: T-COR™ 8 (#1) and T-COR™ 8 (#2). T-COR™ 8 (#2) reagents are commercially known as "FMDV 2.0 reagents with inhibition control" (catalogue number TC-9092-064). These lyophilised assays differed in chemistry (Table 2.1), however they contain the same proprietary primers and probes, which target two areas within the FMDV $3D^{pol}$-coding region (probes labelled with 6-fluorescein amidite [FAM]). In addition, these assays both contain an exogenous internal control with corresponding primers and probes (probe labelled with Cy®5).

To each reaction, 20 µl of re-suspension buffer and 5 µl sample were added to give 25 µl total volume. Reactions were performed in duplicate on a T-COR™ 8 (Tetracore Inc.) and cycling conditions for both assays were as follows: 48°C for 15 minutes, 95°C for 2 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 40 seconds. C_T was defined automatically by the T-COR™ 8 software. Positive reactions were defined as those which gave a detectable C_T in duplicate assays.

2.3.4 Reverse transcription loop-mediated isothermal amplification

Previously published FMDV-specific real-time RT-LAMP (rRT-LAMP) and RT-LAMP combined with lateral-flow detection (RT-LAMP-LFD) assays require reagents from multiple suppliers and numerous pipetting stages for assay set-up (Dukes *et al.*, 2006; Waters *et al.*, 2014). In order to simplify reactions, and thus increase compatibility with decentralised settings, rRT-LAMP and RT-LAMP-LFD in this chapter were performed using a commercial master mix from OptiGene Ltd. (Horsham, UK). Due to the change in reagents, conditions were initially optimised with regards to the following: master mix selection, final primer concentrations, reaction time and the addition of reverse transcriptase (Table 2.2) (Appendix 1a). The final protocols used are stated below.

Table 2.2 Optimisation of RT-LAMP conditions

Condition	Rationale
Master mix selection	Two commercially available LAMP master mixes (ISO-001 and ISO-004) from OptiGene Ltd. (Horsham, UK) were tested. These were selected for their compatibility with decentralised settings: reactions require minimal pipetting stages and there was potential for reagent lyophilisation at OptiGene Ltd.
Primer concentrations	Four primer concentration ratios were tested (IPs [µM]: Loops [µM]: EPs [µM]): 1) 2.0 : 1.0 : 0.2 (Dukes *et al.*, 2006; Ding *et al.*, 2014) 2) 1.6 : 0.8 : 0.2 (Shao *et al.*, 2010; Guan *et al.*, 2013; Yamazaki *et al.*, 2013) 3) 1.2 : 0.6 : 0.2 4) 0.8 : 0.4 : 0.2 (Nagamine *et al.*, 2002).
Reaction time	The recommended reaction time for ISO-001 and ISO-004 is 30 minutes. RT-LAMP was also evaluated with an extended incubation due to previously published RT-LAMP assays (using alternative reagents) reporting 60 minute incubations (Dukes *et al.*, 2006; Shao *et al.*, 2010; Waters *et al.*, 2014).
Reverse transcriptase	Master mixes ISO-001 and ISO-004 contain a proprietary DNA polymerase (isolated from a *Geobacillus* species) known to contain residual reverse transcriptase activity. RT-LAMP reactions were evaluated with and without additional AMV.

(RT-LAMP) reverse transcription loop-mediated isothermal amplification; (IPs) internal primers; (Loops) loop primers; (EPs) external primers; (AMV) Avian Myeloblastosis Virus.

Real-time RT-LAMP (rRT-LAMP): rRT-LAMP (wet reagents) was performed in a total reaction mixture of 25 µl, containing: 15 µl isothermal master mix ISO-001 (OptiGene Ltd.), primers and concentrations as per Dukes *et al.* (2006), 2 U Avian Myeloblastosis Virus (AMV) reverse transcriptase (New England Biolabs, MA, USA), 5 µl template and made up to total volume with NFW (Table 2.1). Reactions were performed in duplicate and incubated at 65˚C for the manufacturer's recommended 30 minutes using a Genie® II (OptiGene Ltd.). ISO-001 contains an intercalating dye, enabling results to be visualised using fluorescence collected at one minute intervals: time to positivity (T_P) was automated using Genie® Explorer v0.2.1.1 software (OptiGene Ltd.).

To confirm that amplicons were FMDV specific, annealing analysis was performed. RT-LAMP products were heated to 98˚C for one minute, then cooled to 80˚C ramping at 0.05˚C per second. Anneal temperature (T_a) calculations were automated using Genie® Explorer v0.2.1.1 software. The primer-specific T_a was initially identified through analysis of 210 FMDV-positive rRT-LAMP reactions (88.5˚C was the average T_a over 210 FMDV-positive RT-LAMP reactions, with 98.55% of reactions within +/- 1˚C). From these results, rRT-LAMP reactions were called positive if amplification had occurred and the LAMP product annealed in the temperature range of 87.5-89.5˚C.

RT-LAMP combined with lateral-flow detection (RT-LAMP-LFD): rRT-LAMP-LFD was performed by modifying Dukes *et al.* (2006) RT-LAMP primers as previously described (Waters *et al.*, 2014): the 5' termini of the inner LAMP primers (forward internal primer [FIP] and backward internal primer [BIP]) were labelled with fluorescein (Flc) and biotin (Btn), respectively. Reactions were incubated at 65˚C for 30 minutes using a Genie® II

(Table 2.1), followed by assay inactivation at 85°C for 5 minutes. All samples were tested in duplicate and results were visualised using PCRD-2™ lateral-flow devices (Abingdon Health, York, UK) as per manufacturer's instructions. A positive result was signified by the presence of two blue bands (test [T-Line] and LFD control line [C-line]); negative results were indicated by the presence of a single band at the C-line (Figure 2.1). If no bands were visible, the test was invalid and thus repeated. A T_P value is not given for RT-LAMP-LFD due to the interference of Flc with amplicon detection.

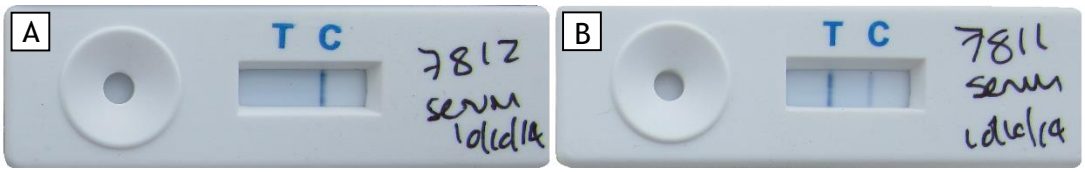

Figure 2.1 Interpreting the results of molecular lateral-flow devices (LFD). (A) Negative results are indicated by the presence of an LFD control line only (C-line); (B) positive results are indicated by the presence of both a test line (T-line) and C-line.

2.3.5 Real-time reverse transcription recombinase polymerase amplification

TwistAmp® exo RT kit (rRT-RPA-exoRT): rRT-RPA was performed in duplicate using the TwistAmp® exo RT kit as manufacturer's instructions (TwistDx Ltd., Cambridge, UK), with primers and probes for FMDV as previously published (Abd El Wahed *et al.*, 2013). Reactions were performed in a total reaction volume of 50 µl (containing 5 µl sample) and incubated at 42°C for 20 minutes using a Genie® II, with inversion at five minutes to mix. T_P was defined when reactions reached a threshold increase of δR 1500 (Table 2.1).

TwistAmp® nfo kit (rRT-RPA-nfo): the TwistAmp® nfo kit (TwistDx Ltd.) was used as manufacturer's instructions, including the addition of 10 U RT Transcriptor (Roche, Mannheim, Germany). Reactions were performed in a total reaction volume of 50 µl (containing 5 µl sample) with primers and probes as previously published (Abd El Wahed *et al.*, 2013). rRT-RPA-nfo was performed in duplicate, incubating each reaction at 39°C for 40 minutes using a Genie® II, with inversion at four minutes to mix. T_P was calculated as above (Table 2.1).

2.3.6 Comparison in the performance of assays

Two different approaches were used to determine analytical sensitivity: an artificial RNA standard and RNA extracted from a FMDV cell culture isolate.

For the artificial RNA standard, the FMDV $3D^{pol}$-coding region was amplified using previously described primers 5'-GGA CAG GAC ATG CTC TCA G-3' and 5'-CAG GAA ACA GCT ATG ACT TTT TTT TTT TTT TTT TTT TTG-3' (Valdazo-González *et al.*, 2012) from cell culture isolate O/UKG/35/2001 (topotype ME-SA, lineage PanAsia). The subsequent PCR product was purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, IL, USA) and inserted into a pGEM®-T vector (Promega, WI, USA). Synthetic viral RNA transcripts were produced by *in vitro* transcription (MEGAscript® T7 Transcription Kit: Invitrogen™, Thermo Fisher Scientific, MA, USA) followed by DNase digestion using TURBO™ DNase (Ambion®, Thermo Fisher Scientific). Transcripts were purified using MEGAclear™ Transcription Clean-Up Kit (Ambion®) prior to quantification at A_{260} using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). A dilution series of the RNA standard (10^6 to 10^0 copies) was prepared in 0.1 µg/ml carrier RNA (Ambion®).

For the FMDV RNA, RNA was extracted from a FMDV cell culture isolate, consisting of clarified bovine thyroid cell lysate infected with an epithelial tissue suspension of FMDV O/UAE/2/2003 (topotype ME-SA, lineage Iran-2001). A decimal dilution series (10^{-1} to 10^{-8}) of this isolate was performed in bovine epithelial tissue suspensions, prepared from cattle tongues (obtained from an abattoir) (10% [w/v] in M25 phosphate buffer: 35 mM Na_2HPO_4, 5.7 mM KH_2PO_4, pH 7.6). RNA was extracted from each dilution using a MagMAX™-96 Viral RNA Isolation Kit (Thermo Fisher Scientific), following an automated procedure on a KingFisher™ Flex (Thermo Fisher Scientific). RNA was extracted from 50 µl sample and eluted in a final volume of 90 µl MagMAX™ elution buffer (Thermo Fisher Scientific).

To determine the relative diagnostic performance of the different assays, RNA was extracted (MagMAX™ / KingFisher™ Flex system as described above) from a panel of clinical FMDV samples that had previously been submitted to WRLFMD (Table 2.3). These samples comprised 35 FMDV-positive samples, representing five serotypes (O, A, Southern African Territories [SAT] 1, SAT 2 and Asia 1) from ten countries, and eight epithelial tissue suspension samples representing viruses that cause similar characteristic lesions to FMDV: swine vesicular disease virus (SVD: UKG/24/1972; UKG/50/1972; UKG/51/1972;

UKG/68/1972) and vesicular stomatitis virus (vesicular stomatitis Indiana virus [VSIV] n = 2; vesicular stomatitis New Jersey virus [VSNJV] n = 2).

Table 2.3 FMDV clinical samples used for comparison of assays

Virus	Serotype	Sample	Topotype	Lineage	Location	Year	Sample Type
FMDV	O	HKN/12/2010	SEA	Mya-98	Hong Kong	2015	ES
		IRN/26/2015	ME-SA	PanAsia-2^{BAL-09}	Iran	2015	ES
		KUW/1/2016	ME-SA	PanAsia-2^{BAL-09}	Kuwait	2016	ES
		KUW/4/2016	ME-SA	PanAsia-2^{BAL-09}	Kuwait	2016	ES
		PAK/30/2015	ME-SA	PanAsia-2^{BAL-09}	Pakistan	2015	ES
		PAK/32/2015	ME-SA	PanAsia-2^{BAL-09}	Pakistan	2015	ES
		PAK/34/2015	ME-SA	PanAsia-2^{BAL-09}	Pakistan	2015	ES
		PAT/4/2015	ME-SA	PanAsia	Palestine	2015	ES
	A	IRN/21/2015	ASIA	G-VII	Iran	2015	ES
		IRN/24/2015	ASIA	Iran-05^{SIS-10}	Iran	2015	ES
		SAU/8/2015	ASIA	G-VII	Saudi Arabia	2015	ES
		PAK/31/2015	ASIA	Iran-05^{FAR-11}	Pakistan	2015	ES
		PAK/56/2015	ASIA	Iran-05^{FAR-11}	Pakistan	2015	ES
		TAN/15/2013	AFRICA	G-I	Tanzania	2013	ES
		TAN/71/2012	AFRICA	G-I	Tanzania	2012	ES
	SAT 1	TAN/22/2014	I (NWZ)	unnamed	Tanzania	2014	ES
		TAN/29/2013	I (NWZ)	unnamed	Tanzania	2013	ES
		TAN/23/2013	I (NWZ)	unnamed	Tanzania	2013	ES
		TAN/50/2012	I (NWZ)	unnamed	Tanzania	2012	ES
		KEN/26/2008	I (NWZ)	unnamed	Kenya	2008	ES
		KEN/9/2009	I (NWZ)	unnamed	Kenya	2009	ES
		KEN/12/2009	I (NWZ)	unnamed	Kenya	2009	ES
	SAT 2	ZIM/9/2015	II	unnamed	Zimbabwe	2015	ES
		ZIM/21/2015	II	unnamed	Zimbabwe	2015	ES
		TAN/3/2011	IV	IV	Tanzania	2011	ES
		TAN/64/2012	IV	unnamed	Tanzania	2012	ES
		TAN/14/2012	IV	unnamed	Tanzania	2012	ES
		TAN/19/2012	IV	unnamed	Tanzania	2012	ES
		KEN/2/2007	IV	unnamed	Kenya	2007	ES
		SUD/7/2014	VII	Alx-12	Sudan	2015	ES
	Asia 1	IRN/20/2015	ASIA	Sindh-08	Iran	2015	ES
		PAK/33/2015	ASIA	Sindh-08	Pakistan	2015	ES
		PAK/28/2015	ASIA	Sindh-08	Pakistan	2015	CC
		PAK/29/2015	ASIA	Sindh-08	Pakistan	2015	OF
		PAK/43/2015	ASIA	Sindh-08	Pakistan	2015	ES

*(ES) epithelial tissue suspension; (CC) cell culture isolate; (OF) original fluid (unknown type); (SAT) Southern African Territories.

2.3.7 Comparison of sample preparation methodologies

To compare sample preparation methods, three decimal dilution series (10^{-1} to 10^{-9}) of FMDV (cell culture isolate O/UAE/2/2003) were prepared as described above in 10% (w/v) bovine epithelial tissue suspension, bovine serum (obtained from a UK abattoir) and bovine OP fluid (archival FMDV-negative experimental samples from TPI). Aliquots were stored at -80°C until use. All sample preparation methods were performed on the reference rRT-PCR, T-COR™ 8 (#1) and (#2) reagents, rRT-LAMP and rRT-RPA-exoRT. Of each preparation method, 5 µl of sample was added to each assay.

A hierarchy of sample preparation methods were selected for comparison, ranging from sophisticated laboratory nucleic extraction methods (MagMax™, MagMax™ [manual] and QIAamp®) to simpler methods such as dilution of samples in NFW. Alternate methods, such as the use of a syringe filter, Chelex® 100 and elution from Ag-LFDs were selected as they

had been previously described as simple techniques for PCR inhibitor removal (Ochert *et al.*, 1994; Wu and Kado, 2004; Walsh *et al.*, 2013; Fowler *et al.*, 2014).

MagMax™: This protocol was used as the reference sample preparation procedure. RNA extraction was performed as described previously (Chapter 2.3.6). RNA was extracted from 50 µl sample and eluted in 90 µl of MagMAX™ elution buffer.

MagMax™ (manual): RNA was extracted following manufacturer's manual guidelines with a DynaMag™-Spin magnet (Thermo Fisher Scientific). RNA was extracted from 50 µl sample and eluted in 90 µl of MagMAX™ elution buffer.

QIAamp®: The QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used according to manufacturer's guidelines. RNA was extracted from 140 µl of sample (using the spin column protocol) and eluted in a final volume of 60 µl QIAamp® buffer AVE (Qiagen).

Neat: Sample was added directly to reactions.

Dilutions: Samples were processed by diluting either 1 in 5, 1 in 10 or 1 in 20 in NFW (Waters *et al.*, 2014).

Filter: Samples were diluted 1 in 5 and 1 ml of sample passed through a Acrodisc® 25 mm syringe filter (w/ 0.1 µm Supor® Membrane) (Pall Life Sciences, MI, USA).

Chelex® 100: 50 µl of 50% (w/v) Chelex® 100 was added to 500 µl of diluted sample (1 in 5). Samples were vortexed, allowed to settle for five minutes and the supernatant used in assays. For Chelex® 100 (heat) samples were heated at 56°C for 10 minutes prior to processing as above, in order to denature the FMDV capsid and release RNA prior to inhibitor removal.

LFD: (epithelium only) 200 µl of each sample was added to SVANODIP® FMDV-Ag LFDs (Boehringer Ingelheim, Bracknell, UK), which were incubated at room temperature (25°C) for 72 hours to mimic extreme field conditions. Nucleic acid was extracted from the loading pad and wicking strip of the Ag-LFDs as previously described (Fowler *et al.*, 2014).

2.3.8 Alternatives for sample collection and transportation

Use of Ag-LFDs for molecular detection of FMDV:

To ascertain whether Ag-LFDs can be used for long-term transport and storage of FMDV, rRT-PCR (reference and T-COR™ 8 [#1] and [#2]), rRT-LAMP and rRT-RPA-exoRT assays were performed on nucleic acid template extracted from 20 archival Ag-LFDs. These Ag-LFDs had been collected within the Serengeti District, Tanzania in August to September 2013 (kindly provided by Paulo F. Raphael [Sokoine University of Agriculture, Tanzania] and Tiziana Lembo [The University of Glasgow, UK]). Epithelial samples, from cows displaying acute signs of FMD, had been processed in the field using the SVANODIP® FMDV-Ag Extraction kit (Boehringer Ingelheim) and applied to the SVANODIP® FMDV-Ag LFDs according to manufacturer's guidelines. These Ag-LFDs were stored at The Nelson Mandela African Institution of Science and Technology (Arusha, Tanzania), in individual fabric pouches at room temperature for two years (dry conditions), prior to shipment to WRLFMD. Nucleic acid was recovered as previously described from the loading pad and wicking strip of the Ag-LFDs as previously described (Fowler *et al.*, 2014), by suspending the sections in 100 µl of RNase inhibitor (20 U/µl, Applied Biosystems™, Thermo Fisher) at 1:50 in NFW for five minutes. Tubes were spun at 3489 x g for two minutes to obtain the eluate, which quality checked using a NanoDrop ND-1000 spectrophotometer (at A_{260}) and Agilent Bioanalyzer 2100 RNA nanochip (Agilent Technologies) and used as template in reactions.

FTA® cards for non-invasive saliva sampling:

To assess the potential of FTA® cards for saliva sampling, FTA® DMPK-B cards were evaluated (catalogue number WB129242: Whatman™, GE Healthcare). FTA® DMPK-B cards were selected as they contain chemicals to lyse cells and denature proteins on contact, and have a larger sample collection area than alternative DMPK cards. For initial analysis of extraction methods, a decimal dilution series (10^{-1} to 10^{-8}) of FMDV (cell culture isolate O/UAE/2/2003) was prepared in bovine epithelial tissue suspensions as above (Chapter 2.3.6). Of each dilution, 200 µl was loaded onto individual FTA® cards and dried at room temperature for three hours in dark settings. Three extraction methods were compared (Table 2.4) for use in the reference rRT-PCR.

To determine the ability of FTA® DMPK-B cards for non-invasive saliva swabbing, samples were collected from two unvaccinated intradermolingual needle challenged cattle (FMDV

challenge virus: A/IRAN/22/2015 [topotype ASIA, lineage G-VII]), by swabbing the cards around the external oral regions of cattle (lips and chin). Samples were collected daily from the day of challenge to five days post challenge. FTA® DMPK-B cards were dried and stored for approximately one week at room temperature. RNA was extracted from cards following the QIAamp® extraction protocol (Table 2.4), with the eluate used as template in the reference rRT-PCR.

Table 2.4 FTA® DMPK-B card extraction methods compared

Name	Protocol	Reference
Extraction	RNA was extracted from the FTA® cards using a QIAamp® Viral RNA Mini Kit. In brief, eight punches of the FTA® card (each with a 2 mm diameter) were incubated overnight (4˚C) in 140 µl NFW plus 560 µl of QIAamp® lysis buffer. Extraction was performed as manufacturer's instructions (using the spin column protocol), eluting in a final volume of 60 µl QIAamp® buffer AVE.	QIAamp® Viral RNA Mini Handbook (Fourth edition, December 2014)
Elution	A small section of the FTA® card (1 cm^2) was cut and suspended in 100 µl of RNase inhibitor (20 U/µl, Applied Biosystems™, Thermo Fisher) at 1:50 in NFW. After five minutes of soaking the tubes were spun at 3489 x g for two minutes. The elution wash was used as template in reactions.	Adapted from Fowler *et al*. (2014)
Punch	A small punch of the FTA® card (diameter of 2 mm) was washed three times with 200 µl Whatman™ FTA® purification Reagent (GE Healthcare) and then further three times with 200 µl TE buffer (Invitrogen™). Each wash consisted of a five minute incubation. Punches were dried for one hour at room temperature, before being used directly as the template in reactions.	Muthukrishnan *et al*. (2008)

Initial extraction methods trialled for use of FTA® DMPK-B cards

2.4 Results

2.4.1 Optimisation of rRT-LAMP and RT-LAMP-LFD

Both master mixes (ISO-001 and ISO-004) displayed comparable analytical sensitivity in rRT-LAMP and RT-LAMP-LFD when used in a wet format, using the recommended incubation time of 30 minutes. Both consistently detected down to 10^{-5} of a dilution series of FMDV RNA, with ISO-001 detecting down to 10^{-6} in one of the two replicates (Appendix 1a). Increasing the incubation time to 60 minutes led to a one $\log_{10}$ improvement in analytical sensitivity for ISO-001 reagents in one of the two replicates (to match that of ISO-004), however non-specific amplification (shown by T_a analysis) was evident from 50 minutes in 1/4 rRT-LAMP FMDV-negative assays for ISO-001 and 2/4 rRT-LAMP FMDV-negative assays for ISO-004 (Appendix 1a). Further optimisation for ISO-001 rRT-LAMP reagents revealed that reactions with a primer ratio of 2 µM internal primers: 1 µM loop primers: 0.2 µM external primers exhibited a two $\log_{10}$ increase in analytical sensitivity when compared to other ratios tested (Appendix 1a). Furthermore, by omitting AMV RT for ISO-001 rRT-LAMP reagents, the detection limit was reduced by two $\log_{10}$ (Appendix 1a).

In light of these results, all further analysis of rRT-LAMP and RT-LAMP-LFD was performed using the conditions as stated in Table 2.5.

Table 2.5 Optimised rRT-LAMP and RT-LAMP-LFD conditions

Component	Working Stock	µl per reaction	Final concentration
ISO-001 master mix		15	
Forward external primer (F3)	5 pmol/µl	1	0.2 µM
Backward external primer (B3)	5 pmol/µl	1	0.2 µM
Forward loop primer (FLoop)	25 pmol/µl	1	1.0 µM
Backward loop primer (BLoop)	25 pmol/µl	1	1.0 µM
Forward internal primer[1] (FIP)	50 pmol/µl	1	2.0 µM
Backward internal primer[2] (BIP)	50 pmol/µl	1	2.0 µM
*AMV reverse transcriptase	10,000 U/ml	0.2	2 U
Incubation time			30 minutes

All primer sequences were as published in Dukes *et al*. (2002). (rRT-LAMP) real-time reverse transcription loop-mediated isothermal amplification; (RT-LAMP-LFD) RT-LAMP combined with lateral-flow detection; (AMV) Avian Myeloblastosis Virus. Internal primers for the RT-LAMP-LFD assays were modified according to Waters *et al*. (2014) by labelling the 5' termini with either fluorescein[1] or biotin[2].

2.4.2 Comparison in the performance of assays

Using an artificial RNA standard, the analytical sensitivities of rRT-PCR (all formats), RT-LAMP (all formats) and rRT-RPA-exoRT were 10^1, 10^1, and 10^2 copies, respectively (Figure 2.2A). A $\log_{10}$ reduction in analytical sensitivity was evident for rRT-RPA-nfo compared to rRT-RPA-exoRT, detecting down to 10^3 copies (Figure 2.2A). A T_P value was not defined for RT-LAMP-LFD due to the interference of the fluorescein-labelled inner primer (required for molecular LFD detection) with the intercalating dye used for real-time amplicon detection on the Genie® II.

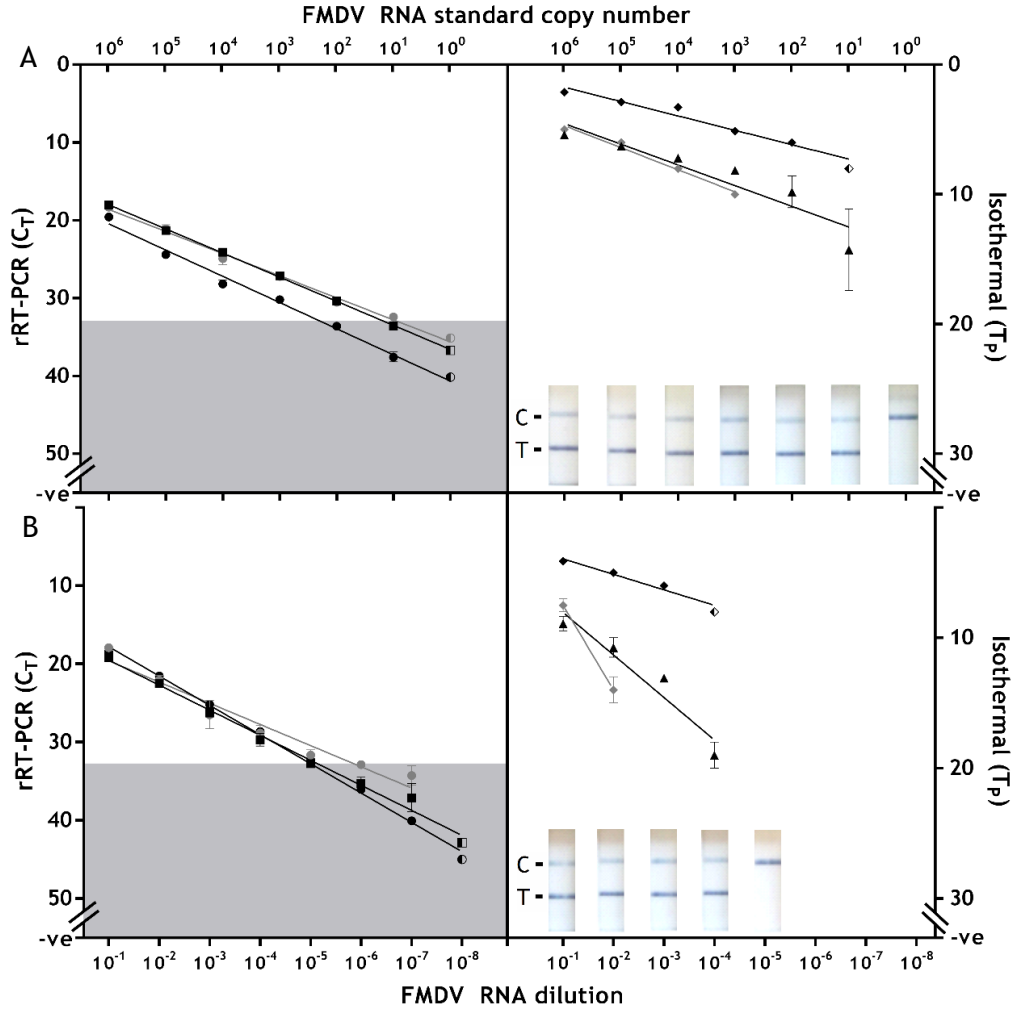

Figure 2.2 Analytical sensitivity of molecular assay formats. (A) RNA standard; (B) FMDV RNA (extracted) diluted in bovine epithelium. (●) Reference real-time reverse transcription PCR (rRT-PCR); (●) T-COR™ 8 (#1); (■) T-COR™ 8 (#2); (▲) real-time reverse transcription loop-mediated isothermal amplification (rRT-LAMP); (◆) real-time reverse transcription recombinase polymerase amplification (rRT-RPA)-exoRT; ◆ rRT-RPA-nfo. Grey shaded area (reference rRT-PCR only) represents cycle thresholds values over the diagnostic cut-off of C_T < 32 (Shaw *et al.*, 2007). Each point represents the mean of two duplicates; half shaded points represent duplicates where one was positive and the other negative; error bars display the range. For RT-LAMP combined with lateral-flow detection (RT-LAMP-LFD): (C)-line and (T)-line = positive result; (C)-line only = negative result.

Both sets of T-COR™ 8 reagents (#1 and #2) gave equivalent analytical sensitivity with the artificial RNA standard, however the change in fluorescence detected in the FAM channel (FMDV-detection) was consistently higher for T-COR™ 8 (#2) reagents (Figure 2.3). Furthermore, the internal control was consistently amplified in all T-COR™ 8 (#2) reactions, whereas internal control amplification was inhibited in T-COR™ 8 (#1) reactions with a high copy number of FMDV RNA standard (Figure 2.3).

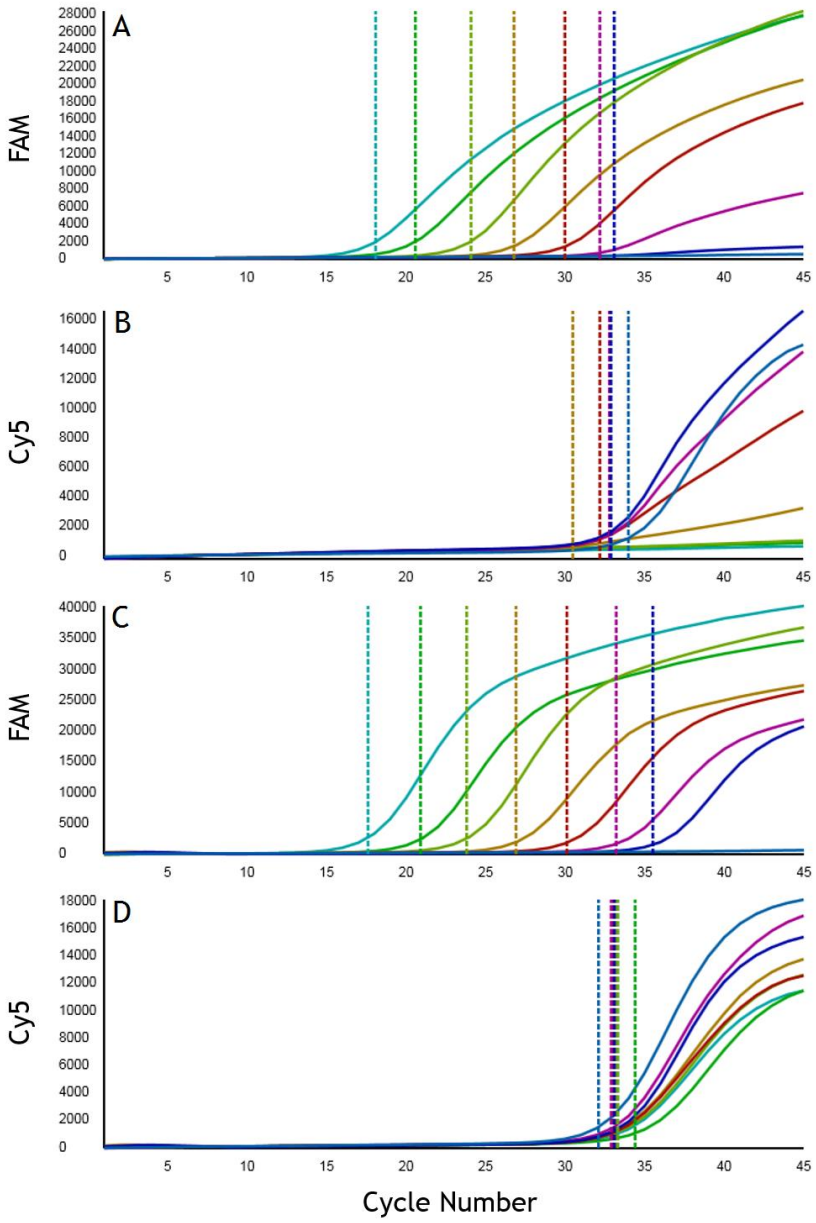

Figure 2.3 Amplification plots for the T-COR™ 8 (#1 and #2) assays formats. Analytical sensitivity was determined using an artificial foot-and-mouth disease virus (FMDV) RNA standard (one duplicate shown). (A) T-COR™ 8 (#1) FMDV-detection plot; (B) T-COR™ 8 (#1) internal control detection plot; (C) T-COR™ 8 (#2) FMDV-detection plot; (D) T-COR™ 8 (#2) internal control detection plot. Assays were performed on an RNA standard (copies/µl), 10^6 (light blue); 10^5 (green); 10^4 (yellow); 10^3 (orange); 10^2 (red); 10^1 (pink); 10^0 (purple); negative (dark blue). (FAM) with 6-fluorescein amidite; (Cy5) Cy®5.

When using RNA extracted from a dilution series of a cell culture of FMDV isolate O/UAE/2/2003 spiked into epithelial tissue suspensions to determine assay performance, the analytical sensitivities of isothermal assays were reduced in comparison to rRT-PCR (total detection limit of 10^{-7} of the FMDV dilution series): rRT-LAMP (all formats): 10^{-4}; rRT-RPA-exoRT: 10^{-3}; rRT-RPA-nfo: 10^{-2} (Figure 2.2B). Both T-COR™ 8 assays (#1 and #2) displayed equivalent analytical sensitivity to the reference rRT-PCR (10^{-7}), however the internal control was inhibited in the T-COR™ 8 (#1) reactions in 10^{-1} and 10^{-2} dilutions (data not shown).

To determine if the reduction in analytical sensitivity was a consequence of mismatches in primer/probe binding regions, a sequence comparison of $3D^{pol}$-coding regions between O/UKG/35/2001 (artificial RNA standard [GenBank accession number AJ539141.1]) and O/UAE/2/2003 (FMDV RNA dilution [unpublished data from The Pirbright Institute: TPI]) was performed. For rRT-PCR (Callahan *et al.*, 2002), no mismatches were present in the primer/probe binding regions for either sequence (Table 2.6). For rRT-LAMP, eight nucleotide differences were present between O/UKG/35/2001 and O/UAE/2/2003 within the primer target regions (Table 2.7), one of which affected two rRT-LAMP primers (total of nine primer-template mismatches for O/UAE/2/2003). Five mismatches were present within the rRT-RPA target regions (Abd El Wahed *et al.*, 2013), however these mismatches were consistent between O/UKG/35/2001 and O/UAE/2/2003 (table 2.8).

Table 2.6 rRT-PCR target sequence comparison

Primer	Genome position*	Sequence (Primers in the 5'-3')
Forward primer	7863-7884	ACTGGGTTTTACAAACCTGTGA
O/UKG/35/2001		ACTGGGTTTTACAAACCTGTGA
O/UAE/2/2003		ACTGGGTTTTACAAACCTGTGA
Probe	7914-7933	TCCTTTGCACGCCGTGGGAC
O/UKG/35/2001		TCCTTTGCACGCCGTGGGAC
O/UAE/2/2003		TCCTTTGCACGCCGTGGGAC
Reverse primer	7953-7969	GCGAGTCCTGCCACGGA
O/UKG/35/2001(rc)		GCGAGTCCTGCCACGGA
O/UAE/2/2003(rc)		GCGAGTCCTGCCACGGA

(rRT-PCR) real-time reverse transcription PCR. Nucleotide sequences in black indicate the Callahan *et al.* (2002) primers/probe in the 5'-3' direction. Nucleotide sequences in grey indicate the target regions within O/UKG/35/2001 (GenBank accession number AJ539141.1 [nucleotides 7847-8026]) and O/UAE/2/2003 (unpublished sequence, personal communication from The Pirbright Institute). (rc) sequence shown in the reverse complement; *genome position according to the FMD virus strain O/UKG/35/2001 (Genbank Accession number AJ539141.1).

Table 2.7 rRT-LAMP target sequence comparison

Primer	Genome position*	Sequence (Primers in the 5'-3')
Forward external primer	7850-7869	CATGGACTATGGAACTGGGT
O/UKG/35/2001		CATGGACTATGGAACTGGGT
O/UAE/2/2003		CATGGACTATGGAACTGGGT
Backward external primer	8006-8022	GGCCCTGGAAAGGCTCA
O/UKG/35/2001 (rc)		GGCCCTGGAAAGGCTCA
O/UAE/2/2003 (rc)		GCCCCTGGAAGGGTTCA
Forward internal primer (F1c)	7909-7929	CACGGCGTGCAAAGGAGAGGA
O/UKG/35/2001 (rc)		CACGGCGTGCAAAGGAGAGGA
O/UAE/2/2003 (rc)		CACGGCGTGCAAAGGAGAGGA
Forward internal primer (F2)	7873-7892	ACAAACCTGTGATGGCTTCG
O/UKG/35/2001		ACAAACCTGTGATGGCTTCG
O/UAE/2/2003		ACAAACCTGTGATGGCCTCA
Backward internal primer (B1c)	7940-7961	GGAGAAGTTGATCTCCGTGGCA
O/UKG/35/2001		GGAGAAGTTGATCTCCGTGGCA
O/UAE/2/2003		GGAGAAGTTGATCTCCGTGGCA
Backward internal primer (B2)	7988-8005	AAGAGACGCCGGTACTCG
O/UKG/35/2001 (rc)		AAGAGACGCCGGTACTCG
O/UAE/2/2003 (rc)		AAGAGACGCCGGTACTCG
Forward loop primer	7891-7908	TAGCCTCGAGGGTCTTCG
O/UKG/35/2001 (rc)		TAGCCTCGAGGGTCTTCG
O/UAE/2/2003 (rc)		TAGCCTCAAGAGTCTTTG
Backward loop primer	7962-7979	GGACTCGCCGTCCACTCT
O/UKG/35/2001		GGACTCGCCGTCCACTCT
O/UAE/2/2003		GGACTCGCTGTCCACTCT

(rRT-LAMP) real-time reverse transcription loop-mediated isothermal amplification. Nucleotide sequences in black indicate the Dukes *et al.* (2006) primers in the 5'-3' direction. Nucleotide sequences in grey indicate the target regions within O/UKG/35/2001 (GenBank accession number AJ539141.1 [nucleotides 7847-8026]) and O/UAE/2/2003 (unpublished sequence, personal communication from The Pirbright Institute). (rc) sequence shown in the reverse complement; *genome position according to the FMD virus strain O/UKG/35/2001 (Genbank Accession number AJ539141.1); red underlined bases indicate primer-template mismatches.

Table 2.8 rRT-RPA target sequence comparison

Primer	Genome position*	Sequence (Primers in the 5'-3')
Forward primer	7850-7883	CATGGATTATGGAACTGGGTTTTACAAACCTGTG
O/UKG/35/2001		CATGGACTATGGAACTGGGTTTTACAAACCTGTG
O/UAE/2/2003		CATGGACTATGGAACTGGGTTTTACAAACCTGTG
Probe (part 1)	7903-7934	GGTCCCACGGCGTGCGAAGGAGAGGATGGCTT
O/UKG/35/2001 (rc)		GGTCCCACGGCGTGCAAAGGAGAGGATAGCCT
O/UAE/2/2003 (rc)		GGTCCCACGGCGTGCAAAGGAGAGGATAGCCT
Probe (part 2)	7938-7953	AGATCAACTTCTCCTG
O/UKG/35/2001 (rc)		AGATCAACTTCTCCTG
O/UAE/2/2003 (rc)		AGATCAACTTCTCCTG
Reverse primer	7942-7964	TCCTGCCACAGAGATCAACTTCT
O/UKG/35/2001(rc)		TCCTGCCACGGAGATCAACTTCT
O/UAE/2/2003(rc)		TCCTGCCACGGAGATCAACTTCT

(rRT-RPA) real-time reverse transcription recombinase polymerase amplification. Nucleotide sequences in black indicate the Abd El Wahed *et al.* (2013) primers/probe in the 5'-3' direction. Nucleotide sequences in grey indicate the target regions within O/UKG/35/2001 (GenBank accession number AJ539141.1 [nucleotides 7847-8026]) and O/UAE/2/2003 (unpublished sequence, personal communication from The Pirbright Institute). (rc) sequence shown in the reverse complement; *genome position according to the FMD virus strain O/UKG/35/2001 (Genbank Accession number AJ539141.1); red underlined bases indicate primer/probe-template mismatches.

Initial assessment of relative diagnostic performance showed the agreement between each assay and the reference rRT-PCR for RNA extracted from clinical samples were as follows (n = 43): T-COR™ 8 (#1): 43/43 (100%); T-COR™ 8 (#2): 43/43 (100%); rRT-LAMP: 38/43 (88%); RT-LAMP-LFD: 37/43 (86%); rRT-RPA-exoRT: 33/43 (77%); rRT-RPA-nfo: 29/43 (67%). False-negative samples consistently displayed high rRT-PCR C_T values (Figure 2.4). All assays yielded negative results against SVDV, VSNJV and VSIV isolates.

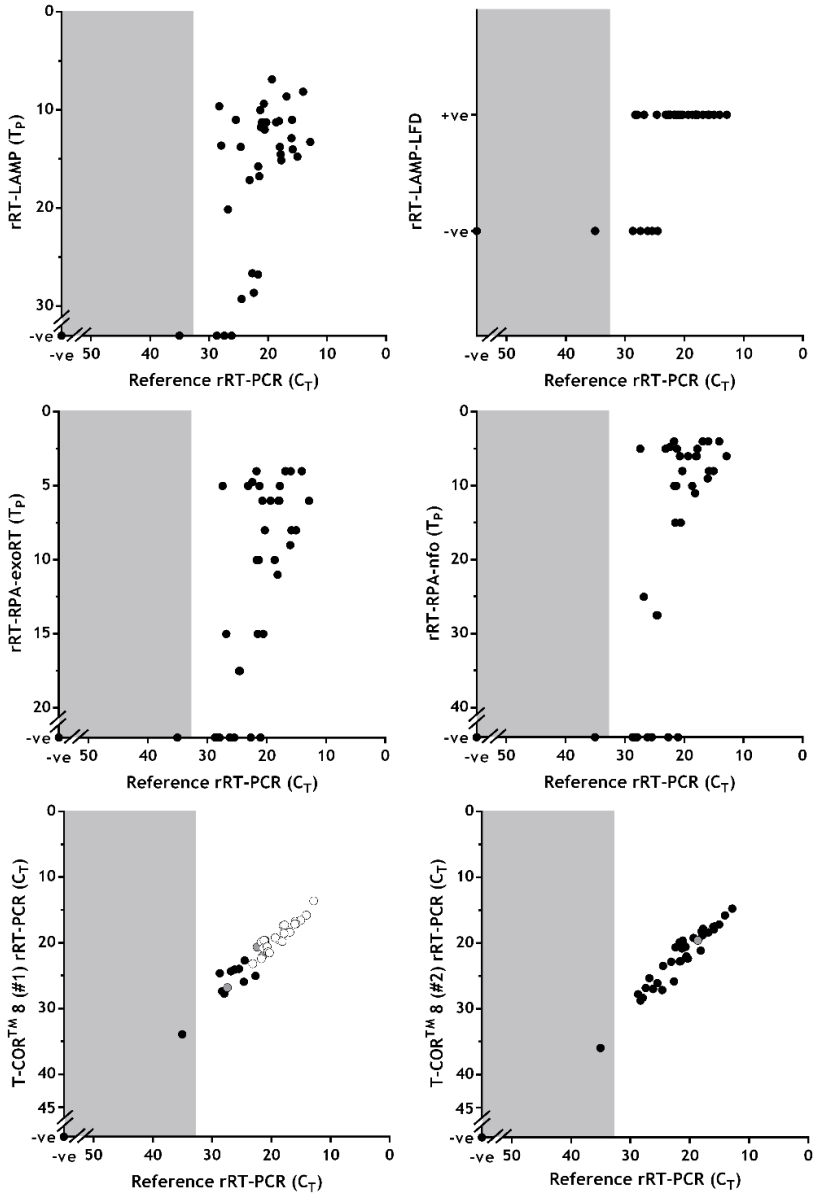

Figure 2.4 Initial assessment of relative diagnostic performance (n = 43). The grey shaded area highlights reference real-time reverse transcription PCR (rRT-PCR) cycle threshold (C_T) values over the FMDV diagnostic threshold of C_T < 32 (Shaw *et al.*, 2007). For T-COR™ 8: (black) internal controls positive; (grey) internal control positive in one duplicate; (white) internal controls negative. Each point represents the mean of two duplicates, however samples that share the same C_T or time to positivity (T_p) appear as a single point. (rRT-LAMP) real-time reverse transcription loop-mediated isothermal amplification; (RT-LAMP-LFD) RT-LAMP combined with lateral-flow detection; (rRT-RPA) real-time reverse transcription recombinase polymerase amplification.

The exogenous internal control (detected in the Cy®5 channel) was positive in both duplicates for 19/43 samples tested using the T-COR™ 8 (#1) assay. Assays where the internal control failed to be amplified all displayed low C_T values for FMDV detection (C_T values between 13.2 and 23.2) (Figure 2.4), with failure likely due to amplification of the FMDV target outcompeting that of the internal control. When using the T-COR™ 8 (#2) assay, only one sample displayed a negative internal control result for one duplicate (FMDV detected at C_T 19.5), with all other samples displaying positive exogenous internal control results in both duplicates (Figure 2.4).

2.4.3 Comparison of sample preparation methodologies

Eleven simple methods were compared for preparation of samples prior to molecular analysis, using the MagMax™ / KingFisher™ system as the reference sample preparation procedure. Sample preparation methods were performed on the reference rRT-PCR, T-COR™ 8 (#1) and (#2) reagents, rRT-LAMP and rRT-RPA-exoRT (rRT-RPA-nfo was not tested due to poor analytical sensitivity). The use of simple extraction kits for all assays (QIAamp® and MagMax™ [manual]) achieved comparable analytical sensitivity across all sample types to automated nucleic acid extraction (Figure 2.5, Appendix 2).

Using epithelial tissue suspensions, all simple sample preparation methods resulted in complete inhibition of the reference rRT-PCR. For the T-COR™ 8 reagents (#1 and #2) and rRT-LAMP, dilution of epithelial tissue suspensions in NFW resulted a one $\log_{10}$ reduction in analytical sensitivity comparatively to the use of extracted RNA, with the use of both Chelex® 100 and syringe filters further decreasing the limit of detection (LOD). A one $\log_{10}$ reduction was also evident for rRT-RPA-exoRT when using diluted epithelial tissue suspensions, however non-specific amplification was evident. This was removed by pre-processing of samples through a syringe filter. Ag-LFDs displayed a reduced LOD comparatively to molecular methods, however elution from Ag-LFDs produced positive results in all molecular assays (Figure 2.5, Appendix 2).

Serum could be added neat to the reference rRT-PCR, T-COR™ 8 reagents and rRT-LAMP, however dilution in NFW and/or the use of Chelex® 100 or a syringe filter was required to minimise the reduction in LOD (either a one or two $\log_{10}$ reduction in LOD was evident). For rRT-RPA-exoRT, all simple serum preparation methods resulted in complete assay inhibition with nucleic acid extraction required, using any of the three RNA extraction methods tested, in order for amplification to be observed (Figure 2.5, Appendix 2).

When using OP fluid, a one to two log_{10} reduction in analytical sensitivity was evident for all rRT-PCR assays and rRT-LAMP when OP fluid was diluted in NFW prior to analysis: 1 in 10 dilutions resulted in the smallest decrease in LOD. The additional use of syringe filters or Chelex® 100 resulted in a further decrease. For rRT-RPA-exoRT, extraction of RNA from OP fluid was required, with the use of simple OP fluid preparations resulting in complete assay inhibition (Figure 2.5, Appendix 2).

The exogenous internal control was positive in T-COR™ 8 (#2) assays for all sample types, across all sample preparation methods, with the exception of when samples were added neat to reactions. Detection of the internal positive control was inhibited in the T-COR™ 8 (#1) assays both when samples were added to reactions neat, or when reactions were strongly positive for FMDV (low C_T values for FMDV detection) (Figure 2.5).

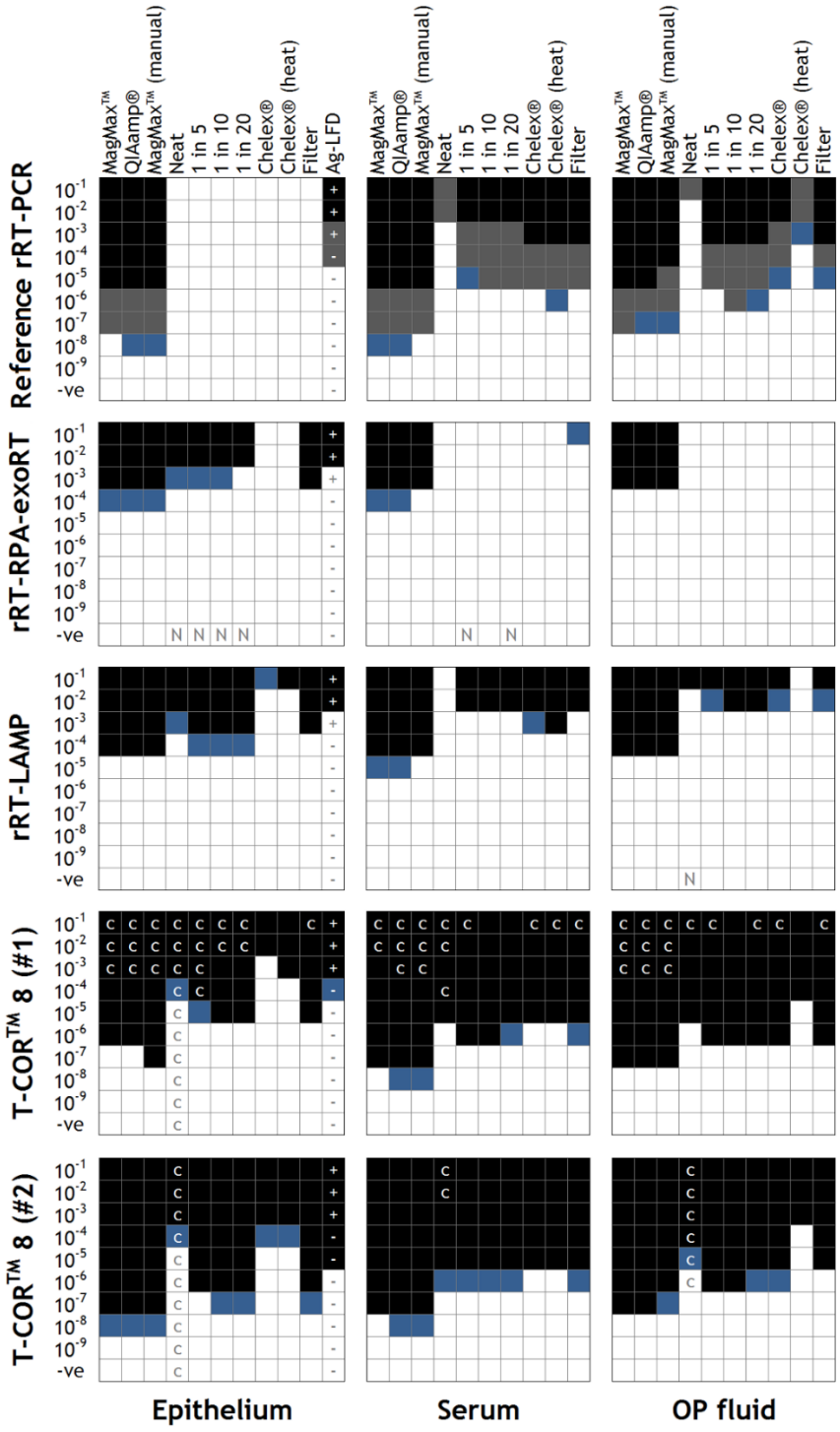

Figure 2.5 Comparison of sample preparation methods. The Y-axis represents the decimal dilution series (10^{-1} to 10^{-9}) of foot-and-mouth disease virus (FMDV) in either epithelial tissue suspensions, sera or oesophageal-pharyngeal fluid. Black squares represent FMDV-positive reactions; blue squares represent dilutions where one duplicate was FMDV-positive; white squares represent FMDV-negative reactions. For the reference real-time reverse transcription PCR (rRT-PCR) only, grey boxes highlight cycle threshold values over the FMDV diagnostic threshold of $C_T < 32$ (Shaw *et al.*, 2007). (N) non-specific application; (c) for T-COR™ 8 assays the exogenous internal control was negative in at least one duplicate; +/- represent antigen-detection lateral-flow device (Ag-LFD) results. (rRT-LAMP) real-time reverse transcription loop-mediated isothermal amplification; (rRT-RPA) real-time reverse transcription recombinase polymerase amplification.

2.4.4 Alternatives for sample collection and transportation

Use of Ag-LFDs for molecular detection of FMDV:

From the 20 archival Ag-LFDs collected within the Serengeti District, Tanzania (August to September 2013), FMDV RNA was detected by rRT-PCR (reference, T-COR™ 8 [#1] and [#2]) in the elution wash from all Ag-LFDs (Figure 2.6C; Figure 2.6D). For rRT-LAMP, FMDV RNA was detected in the elution wash from 19/20 Ag-LFDs (false-negative had a reference rRT-PCR C_T value of 27.12) (Figure 2.6A). For rRT-RPA-exoRT, 18/20 Ag-LFDs gave FMDV-positive results (two false-negatives had reference rRT-PCR C_T values of 27.12 and 25.79) (Figure 2.6B).

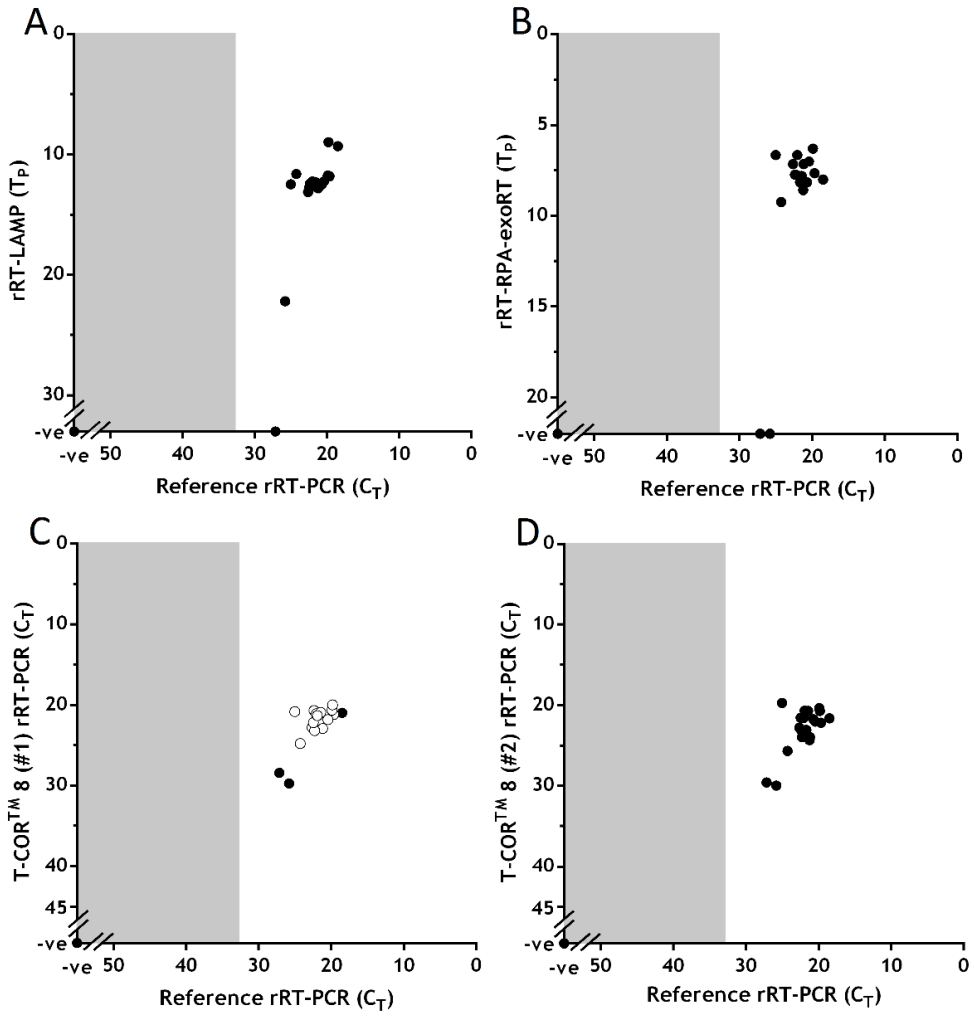

Figure 2.6 Use of antigen-detection lateral-flow devices for storage of foot-and-mouth disease virus. Comparison between the reference real-time reverse transcription PCR (rRT-PCR) and point-of-care test (POCT)-ready assays across 20 Ag-LFDs. The grey shaded area highlights reference rRT-PCR cycle threshold values over the diagnostic threshold of C_T < 32 (Shaw *et al.*, 2007). For T-COR™ 8 assays: (black) internal controls positive; (white) internal controls negative. Each point represents the mean of two duplicates. (rRT-LAMP) real-time reverse transcription loop-mediated isothermal amplification; (rRT-RPA) real-time reverse transcription recombinase polymerase amplification.

FTA® cards for non-invasive saliva sampling:

When evaluating FTA® DMPK-B cards, QIAamp® nucleic acid extraction enabled the highest analytical sensitivity to be achieved out of the three extraction methods trialled, detecting down to 10^{-7} of a FMDV RNA dilution series (reactions performed on the reference rRT-PCR). A two log_{10} reduction was evident for elution from FTA® DMPK-B cards in 1:50 RNase inhibitor to NFW comparatively to QIAamp® extraction (detecting down to 10^{-5}), with the use of punched disks in assays leading to a three log_{10} reduction in analytical sensitivity (reliably detecting down to 10^{-4}, with one duplicate detected at 10^{-5}) (data not shown).

When using FTA® DMPK-B cards used for collection of clinical saliva samples (external oral swabs from unvaccinated intradermolingual needle challenged cattle), the reference rRT-PCR (in combination with QIAamp® extraction) resulted in FMDV being detected in samples from both cattle from one day post challenge (DPC) until sampling was concluded at five DPC (Figure 2.7).

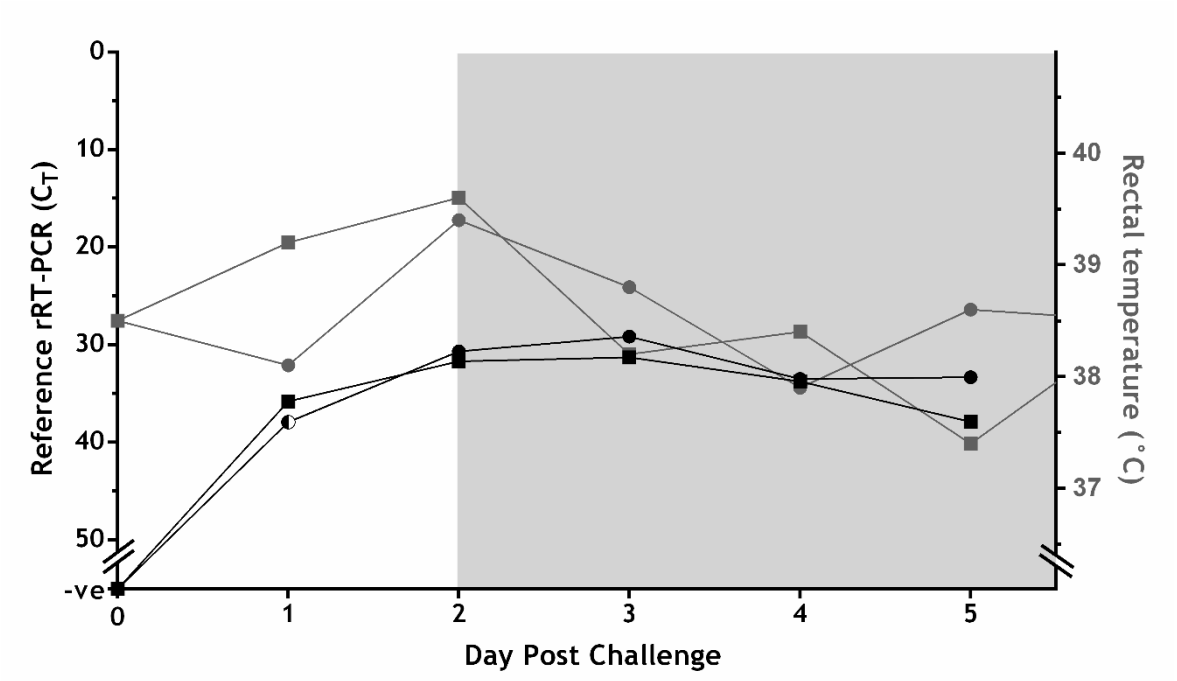

Figure 2.7 Determination of the clinical detection window for FTA® DMPK-B cards used as oral swabs. Samples originated from two unvaccinated cattle needle-challenged cattle with FMDV A/IRAN/22/2015: (circle) animal number one; (square) animal number two. (black) real-time reverse transcription PCR results, each point represents the mean of two replicates (range bars were not visible below the points); (grey) rectal temperature of each animal in °C; (grey box) onset of clinical signs. Half shaded points represent duplicates where one was positive and the other negative

2.5 Discussion

The requirement for decentralised detection of FMDV in field and low-resource settings has led to an increased interest in evaluating simple-to-use diagnostic platforms that can be deployed closer to farms that are experiencing clinical suspicion of FMD. However, the lack of publications comparing multiple FMDV-specific assays, in addition to a lack of standardisation between laboratory evaluations, has led to difficulties in defining the relative performance of new assays and technologies. Furthermore, published POCTs often use nucleic acid extraction protocols incompatible with low-resource settings. This chapter details a comparison of multiple POCT-ready assays (including representatives of rRT-PCR, RT-LAMP and rRT-RPA) and POCT-compatible sample preparation procedures, in order to define their current capabilities and select the most appropriate technologies and protocols for subsequent evaluation and field testing.

The analytical sensitivities of POCT-ready assays were equivalent to previously published results when using an artificial FMDV RNA standard, with rRT-PCR and RT-LAMP assay formats displaying comparable analytical sensitivity to the reference rRT-PCR (Callahan *et al.*, 2002; Shaw *et al.*, 2007; Waters *et al.*, 2014) and rRT-RPA-exoRT one $\log_{10}$ less sensitive (Abd El Wahed *et al.*, 2013). Although other studies have employed longer RT-LAMP incubation periods of 60 minutes to enable assays to run to completion (Dukes *et* al., 2006; Waters *et al.*, 2014), results in this chapter showed that the extension of RT-LAMP from 30 to 60 minutes led to a decrease in assay specificity (as determined by incorrect T_a). Due to the costs associated with responding to a false-positive FMD diagnosis, in addition to the eventual aim of deploying RT-LAMP in field and low-resource settings (require compatibility with crude sample preparation methods and minimal expertise), a time threshold of 30 minutes was used to reduce the risk of non-specific amplification. The poor performance of the TwistAmp® nfo kit is likely due to the absence of Exonuclease III (present within the TwistAmp® exo RT kit), which contains 3'-5' exonuclease activity to enhance fluorescent probe degradation during amplification.

During evaluation of analytical sensitivity, it was apparent that the template of choice impacted upon apparent assay performance, with more variation evident between assays when using RNA extracted from cell culture isolate O/UAE/2/2003 spiked into epithelial tissue suspensions comparatively to artificial RNA standards. The reduction in analytical sensitivity for RT-LAMP could be due to an increased number of primer/template mismatches evident for O/UAE/2/2003 (FMDV RNA dilution) compared to O/UKG/35/2001 (artificial RNA standard). However for rRT-RPA, the sequence differences between

O/UAE/2/2003 and O/UKG/35/2001 did not affect primer/probe target regions. Furthermore, when evaluating relative diagnostic sensitivity (using FMDV RNA isolated from clinical samples), similar variation between assays was evident. This variation between assays therefore could be related to the presence of host-derived nucleic acid and/or nucleic acid structures present in RNA extracted from clinical samples (absent from RNA standards), however further work is required to confirm this. These results should be considered when evaluating new assays, to prevent the false overestimation of assay performance if determined using an RNA standard only.

Sample preparation remains the bottleneck of POCT nucleic acid detection, with current procedures being complex, time-consuming and requiring both dedicated laboratory spaces and specialised equipment (Dineva *et al.*, 2007). Both manual nucleic extraction kits gave comparable analytical sensitivity to automated procedures for each of the POCT-ready assays, however the requirement for multiple stages and basic equipment limits their use to laboratory settings. Therefore, although suitable for low-resource laboratories, more simplistic sample preparation methods are still required for field-based detection of FMDV.

Previous publications have shown that RT-LAMP is able to detect FMDV from air sampler fluid and epithelial tissue suspensions following dilution in NFW (Waters *et al.*, 2014), and PCR assays are able to amplify DNA from crude clinical samples using mutants of *Taq* polymerase (Kermekchiev *et al.*, 2009; Zhang *et al*. 2010). Results obtained in this study confirmed this for rRT-LAMP and rRT-PCR (T-COR^{TM} 8 assays) using OP fluid, serum and epithelial tissue suspensions. This offers advantages over current POCTs such as Ag-LFDs (only evaluated for use with epithelium and vesicular fluid) by being applicable across a larger diagnostic window of detection. However, the use of simple sample preparation procedures resulted in slight reductions in analytical sensitivity, with dilution in NFW resulting in a one to two $\log_{10}$ decrease in LOD. Although a one $\log_{10}$ decrease in LOD is consistent with the dilution factor, a two $\log_{10}$ decrease in LOD suggests partial assay inhibition, in addition to the effects of dilution factor. However, even in the absence of RNA extraction, T-COR^{TM} 8 assays consistently displayed analytical sensitivity comparable to that of the reference rRT-PCR when considering a diagnostic threshold (Shaw *et al.*, 2007).

Simple sample preparation methods were less encouraging for RT-RPA-exoRT, with both sera and OP fluid inhibiting assays when RNA extraction was omitted. When using epithelial tissue suspensions, amplification in the rRT-RPA-exoRT assay was evident following dilution in NFW, however syringe filters were required to prevent non-specific

amplification. Similarly, the use of simple epithelial tissue suspension preparations resulted in complete inhibition of the reference rRT-PCR.

An important point of consideration for POCTs, especially with the use of crude sample preparations, is to ensure that assays are not affected by potential inhibitors present within the sample. As such, the T-COR™ 8 assays contain an exogenous internal control. Throughout evaluation, the T-COR™ 8 (#2) assay displayed superior performance to the T-COR™ 8 (#1) assay when considering amplification of the internal control, which was repeatedly inhibited in the T-COR™ 8 (#1) assay when samples were strongly positive for FMDV (likely due to competitive inhibition differentially affecting the two chemistries). Furthermore, amplification efficiency was consistently higher for T-COR™ 8 (#2) reagents, with an increased detected change in fluorescence in the FAM channel (FMDV-detection) compared to T-COR™ 8 (#1) reagents.

Although Ag-LFDs displayed reduced analytical sensitivity compared to molecular detection methods for FMDV (consistent with Ferris *et al*. [2009]), data presented in this chapter supports the use of Ag-LFDs beyond that of a POCT tool. The ability to detect FMDV from these devices following lengthy storage, in addition to Ag-LFDs having previously been implicated as a possible transport solution (no requirement for special temperature storage requirements) (Fowler *et al.*, 2014), suggests that they may have potential use as a simple way to archive samples for later FMDV detection and characterisation. For example, they could be useful for the dry, non-hazardous transportation of samples from field-settings and FMD-endemic countries to international reference laboratories. Similarly, data presented supports the use of FTA® cards for non-invasive saliva collection, increasing the number of sample types they have displayed compatibility with (previous FMDV-specific studies have implicated their use with cell culture supernatant and impression smears from epithelium) (Muthukrishnan *et al.*, 2008; Madhanmohan *et al.*, 2015). However, samples were only collected up to 5 DPC (for animal welfare reasons). In order to ascertain whether FTA® cards are still applicable for later stage infection, further evaluation is required. Nonetheless, such methods could help to integrate POCTs into current FMD diagnostic strategies, as they provide a means to retrospectively confirm the diagnosis made in decentralised settings, by overcoming many of the pitfalls associated with sample transportation to centralised laboratories. However, further validation is required to assure FMDV inactivation for these alternative sample types

In conclusion, this chapter presents the first analysis of multiple POCT-ready assay formats in order to define their current capabilities, benchmarked against the reference

rRT-PCR. Although this evaluation is specific to the conditions/primers tested in this analysis, current POCT-ready rRT-PCR assays display comparable diagnostic performance to the reference rRT-PCR (which is maintained without extraction and assuming a diagnostic cut-off), forming a viable option for diagnosis of FMD in both field settings and low-resource laboratories. RT-LAMP is comparable to the diagnostic rRT-PCR when using nucleic acid extraction methods and considering a diagnostic cut-off value, however if used in the absence of these may be subject to reductions in analytical sensitivity. Consequently, although RT-LAMP is suitable for rapid confirmation of FMD-positives in the field, its application to FMDV surveillance may be better suited to low-resource laboratories where RNA extraction is possible. The inability to perform rRT-RPA in the absence of extraction limits its use to laboratory settings. Based on these analyses, RT-LAMP and rRT-PCR T-COR™ 8 (#2) reagents were selected as the most appropriate assays for POCT, therefore taken forward in this project for subsequent development, evaluation and field testing.

CHAPTER 3

Transitioning isothermal assays into point-of-care test formats and evaluation of these tests in decentralised settings

Elements of this chapter have been published in the following peer-reviewed article:

Howson, E.L.A., Armson, B., Madi, M., Kasanga, C.J., Kandusi, S., Sallu, R., Chepkwony, E., Siddle, A., Martin, P., Wood, J., Mioulet, V., King, D.P., Lembo, T., Cleaveland, S. and Fowler, V.L. (2017c). 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(3): 861-71. doi: 10.1111/tbed.12451.

Results from this chapter have been presented at:

EuFMD Research Group Open Session 2014, Cavtat, Croatia. Oral presentation: Realising the potential of simple isothermal molecular tools for field diagnosis of foot-and-mouth disease.

Transboundary and Emerging Diseases

Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Evaluation of Two Lyophilized Molecular Assays to Rapidly Detect Foot-and-Mouth Disease Virus Directly from Clinical Samples in Field Settings

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Keywords:
foot-and-mouth disease; foot-and-mouth disease virus; diagnostics; rRT-PCR; RT-LAMP; lyophilized

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Summary

Accurate, timely diagnosis is essential for the control, monitoring and eradication of foot-and-mouth disease (FMD). Clinical samples from suspect cases are normally tested at reference laboratories. However, transport of samples to these centralized facilities can be a lengthy process that can impose delays on critical decision making. These concerns have motivated work to evaluate simple-to-use technologies, including molecular-based diagnostic platforms, that can be deployed closer to suspect cases of FMD. In this context, FMD virus (FMDV)-specific reverse transcription loop-mediated isothermal amplification (RT-LAMP) and real-time RT-PCR (rRT-PCR) assays, compatible with simple sample preparation methods and *in situ* visualization, have been developed which share equivalent analytical sensitivity with laboratory-based rRT-PCR. However, the lack of robust 'ready-to-use kits' that utilize stabilized reagents limits the deployment of these tests into field settings. To address this gap, this study describes the performance of lyophilized rRT-PCR and RT-LAMP assays to detect FMDV. Both of these assays are compatible with the use of fluorescence to monitor amplification in real-time, and for the RT-LAMP assays end point detection could also be achieved using molecular lateral flow devices. Lyophilization of reagents did not adversely affect the performance of the assays. Importantly, when these assays were deployed into challenging laboratory and field settings within East Africa they proved to be reliable in their ability to detect FMDV in a range of clinical samples from acutely infected as well as convalescent cattle. These data support the use of highly sensitive molecular assays into field settings for simple and rapid detection of FMDV.

3.1 Summary

In order to detect foot-and-mouth disease virus (FMDV) RNA in decentralised settings, it is anticipated that assays must fulfil the three steps of a molecular test (sample preparation, amplification and detection), be in the format of a 'ready-to-use kit' and utilise reagents compatible with the environmental conditions found in the countries of deployment. In this context, FMDV-specific reverse transcription loop-mediated isothermal amplification (RT-LAMP) assays compatible with simple sample preparation and visualisation methods have been developed, which share equivalent analytical sensitivity with laboratory-based real-time reverse transcription PCR (rRT-PCR). However, these tests have not yet been transitioned into stabilised formats, which limits their deployment into low-resource laboratories or field settings, as maintenance of a cold-chain is required. To address this gap, this chapter describes the transfer of RT-LAMP assays into lyophilised formats, development of robust field-ready protocols and evaluation of these within FMD-endemic settings. Lyophilisation of reagents did not adversely affect the performance of RT-LAMP using either real-time analysis (portable fluorimeter) or end-point molecular lateral-flow detection. Furthermore, robust field-ready protocols enabled RT-LAMP assays to detect FMDV RNA from clinical samples taken from cattle as early as one day post infection, both in the presence and absence of nucleic acid extraction. Importantly, when these RT-LAMP protocols were deployed into challenging laboratory and field settings within East Africa, RT-LAMP results (n = 145) were consistent with clinical observations and a reference rRT-PCR, with FMDV RNA detected in a range of clinical samples (serum, epithelium and oesophageal-pharyngeal fluid) from acutely infected, as well as convalescent cattle. These data support the deployment of RT-LAMP into decentralised settings to improve local diagnostic capacity and provide methods for simple and rapid confirmation of foot-and-mouth disease in the field.

3.2 Introduction

The analytical sensitivity, rapidity and easy operation of loop-mediated isothermal amplification (LAMP) have resulted in numerous publications detailing the design of diagnostic assays for diseases of veterinary importance. For instance, LAMP and reverse transcription-LAMP (RT-LAMP) assays have been developed to detect numerous livestock pathogens, including African swine fever virus (James *et al.*, 2010), swine vesicular disease virus (SVDV) (Blomström *et al.*, 2008), vesicular stomatitis New Jersey virus (VSNJV) (Fowler *et al.*, 2016), bluetongue virus (Mulholland *et al.*, 2014; Mohandas *et al.*, 2015; Maan *et al.*, 2016) and foot-and-mouth disease virus (FMDV) (Dukes *et al.*, 2006; Li *et al.*, 2009; Shao *et al.*, 2010; Chen *et al.*, 2011a; 2011b; Yamazaki *et al.*, 2013; Guan *et al.*, 2013; Madhanmohan *et al.*, 2013; Waters *et al.*, 2014; Ding *et al.*, 2014; Ranjan *et al.*, 2014; Farooq *et al.*, 2015).

As discussed in Chapter 1, efforts have been made in transitioning some of these FMDV-specific RT-LAMP assays into point-of-care test (POCT) formats, suitable for use in decentralised settings (low-resource laboratories or field settings). Previous publications (Dukes *et al.*, 2006; Waters *et al.*, 2014) and results detailed in Chapter 2, demonstrate that current FMDV-specific RT-LAMP assays have the potential for use in challenging environments, as they are compatible with simple sample preparation, amplification and detection protocols. For instance, FMDV-specific RT-LAMP assays can be performed in the presence or absence of RNA extraction, are compatible with multiple sample types and objective analysis of results can be achieved either in real-time, using a portable fluorimeter, or at end-point using molecular lateral-flow devices (LFDs) (Waters *et al.*, 2014).

However, currently published assays, even those proposed for use in field settings, have been validated using 'wet' reagents which contain temperature sensitive enzymes, incompatible for storage in low-resource laboratories or field deployment (Dukes *et al.*, 2006; Shao *et al.*, 2010; Yamazaki *et al.*, 2013; Waters *et al.*, 2014). Furthermore, FMDV-specific RT-LAMP assays are not yet in commercially available kit formats, requiring multiple reagents (often from multiple suppliers) and pipetting stages for reaction preparation. Methods are now available to lyophilise reagents, already tested in a number of LAMP assays (Boehme *et al.*, 2007; Mair *et al.*, 2013), with benefits including improved stability, storage, transportability and ease of use.

To date, evaluations of FMDV-specific RT-LAMP assays have been restricted to laboratory settings, using protocols incompatible with field-use (assays require RNA extraction).

Consequently, the capabilities of current RT-LAMP assays for use in decentralised settings are poorly defined due to a lack of dedicated validation studies. In order to develop robust protocols for successful decentralisation of FMDV-specific assays, evaluation of assays in the intended scenarios of use is required (both FMDV surveillance in low-resource laboratories and rapid confirmation of FMD in the field).

This chapter describes the (i) transfer of RT-LAMP assays (Dukes *et al.*, 2006; Waters *et al.*, 2014) into lyophilised formats, (ii) development of robust field-ready protocols and (iii) evaluation of these in decentralised settings. In order to ascertain the diagnostic clinical window of detection (time points post-infection where FMDV RNA can be detected), the compatibility of RT-LAMP with different sample types was evaluated (epithelial tissue suspensions, serum, oesophageal-pharyngeal [OP] fluid). Assays were performed both in low-resource laboratory and field settings in East Africa (Tanzania and Kenya), and results were compared against existing POCTs.

3.3 Materials and Methods

3.3.1 Ethics statement

Clinical samples utilised in this chapter were either archival samples from previous experimental studies approved by The Pirbright Institute (TPI) Ethical Review Committee under the Animal Scientific Procedures Act (ASPA) 1986 (as amended), or comprised samples submitted by endemic countries to the Food and Agricultural Organization (FAO) World Reference Laboratory for FMD (WRLFMD) at TPI, UK. Negative bovine epithelium was obtained from a UK abattoir as described previously (Chapter 2.3.1).

Field sampling was carried out in accordance with ASPA guidelines and local country rules. For Tanzania, permission was granted from the Tanzania Commission for Science and Technology (permit no. 2014-368-ER-2005-141). For Kenya, sampling was carried out as part of a training programme run by The European Commission for the Control of Foot-and-Mouth Disease (EuFMD), with permission from the Kenyan Director of Veterinary Services.

3.3.2 Reference real-time reverse transcription PCR

Laboratory-based: The one-step rRT-PCR, used as the reference test, was performed as stated previously (Chapter 2.3.2). This rRT-PCR method is recommended within World Organisation for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012).

Decentralised settings: For the reference test in decentralised settings, rRT-PCR was performed as previously described (Madi *et al.*, 2012) using an Enigma® Field Laboratory (FL) instrument (Enigma Diagnostics Ltd., Salisbury, Wiltshire). This platform integrates automated nucleic acid extraction (from 500 µl of sample), thermal cycling and result reporting. rRT-PCR reagents were provided in a lyophilised format by Enigma Diagnostics Ltd. Within field settings, the Enigma® FL was powered via a 15 V connection with a vehicle auxiliary (Figure 3.1), within low-resource laboratory settings, the Enigma® FL was mains powered.

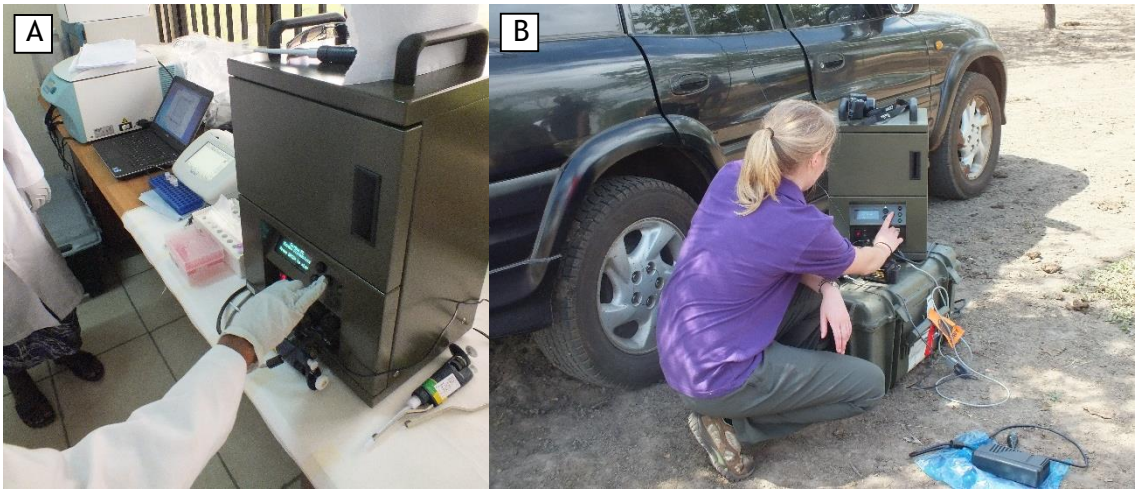

Figure 3.1 Real-time reverse transcription PCR in decentralised settings. (A) low-resource laboratory (Sokoine University of Agriculture, Morogoro, Tanzania) and (B) field-settings in a Maasai herd (Morogoro, Tanzania).

3.3.3 Reverse transcription loop-mediated isotheral amplification

Laboratory-based: Real-time RT-LAMP (rRT-LAMP) and RT-LAMP combined with lateral-flow detection (RT-LAMP-LFD) were performed as stated previously (Chapter 2.3.4). Reactions were incubated using either a Genie® II (OptiGene Ltd., Horsham, UK) (Figure 3.2) or bench-top real-time PCR machine (Stratagene Mx3005P™: Agilent Technologies, CA, USA). Following incubation, all annealing analysis was performed as previously stated using the Genie® II (Chapter 2.3.4) (Figure 3.2).

Lyophilised reagents were developed by OptiGene Ltd. using isothermal master mix ISO-001 with the addition of primers (as stated previously in Chapter 2.3.4), stabilising sugars and Avian Myeloblastosis Virus (AMV) reverse transcriptase. Lyophilised pellets were re-suspended with 15 µl of re-suspension buffer, 5 µl sample and made up to 25 µl total volume with nuclease-free water (NFW). Reactions were performed in duplicate on the Genie® II, using the parameters as described above.

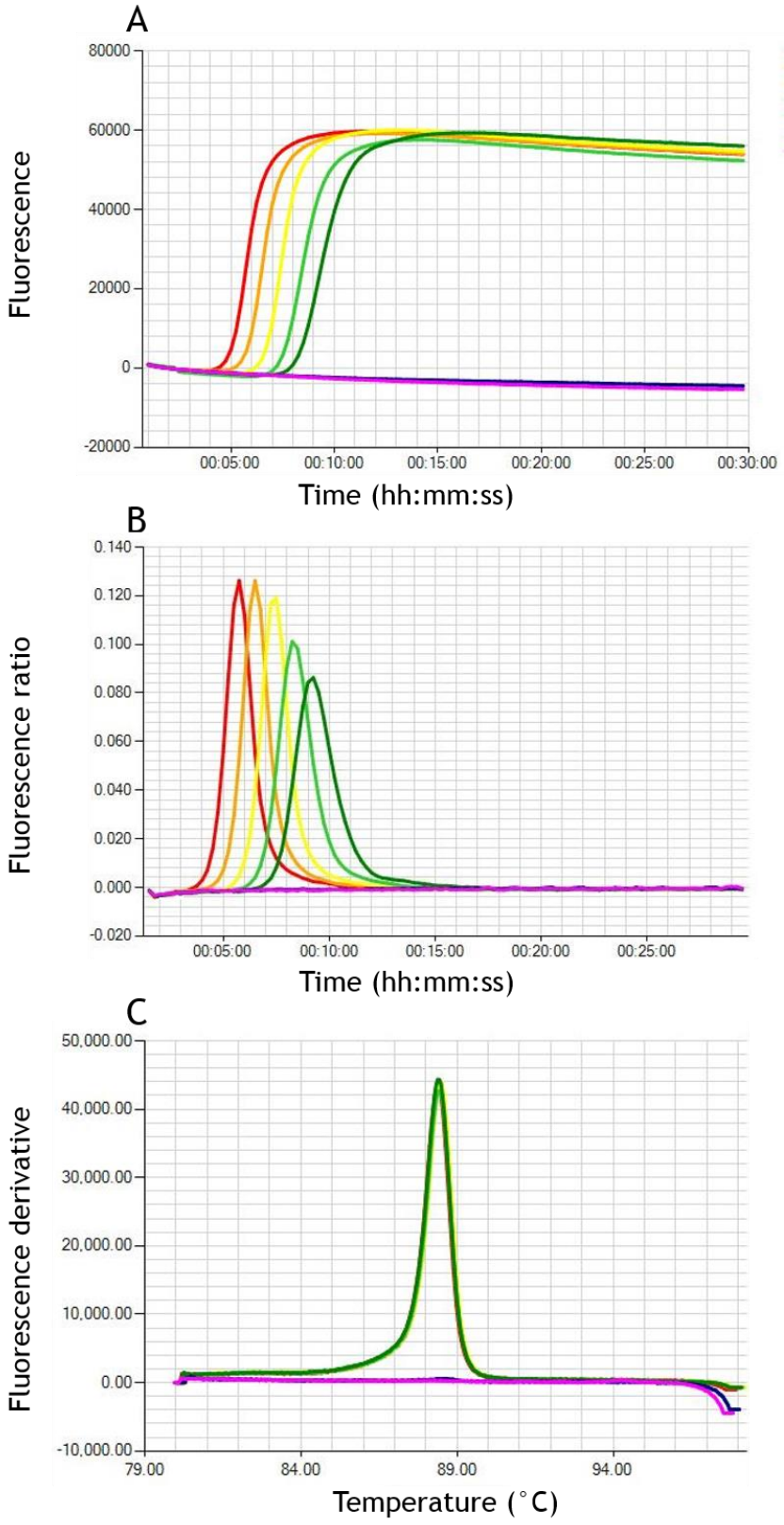

Figure 3.2 Real-time reverse transcription loop-mediated isothermal amplification (rRT-LAMP) reactions viewed using Genie® Explorer v0.2.1.1 software (OptiGene Ltd.). (A) rRT-LAMP amplification curve; (B) rRT-LAMP amplification rate (time to positivity is called at the peak rate of amplification); (C) rRT-LAMP amplicon anneal derivative (anneal temperature is called at the peak of change in fluorescence).

Decentralised settings: In decentralised settings (Figure 3.3), RT-LAMP and RT-LAMP-LFD were performed on the Genie® II using the lyophilised reagents as described above.

Figure 3.3 Real-time reverse transcription loop-mediated isothermal amplification during field evaluation in a Maasai herd (Morogoro, Tanzania).

3.3.4 Antigen-detection lateral-flow devices

In the field, in addition to a reference rRT-PCR (Engima® FL), rRT-LAMP and RT-LAMP-LFD results were compared against an existing, commercially available POCT, the SVANODIP® FMDV-Ag LFD (Boehringer Ingelheim, Bracknell, UK). Six drops of homogenised epithelium from the SVANODIP® FMDV-Ag Extraction Kit (Ag-LFD) (Boehringer Ingelheim) were added to the Ag-LFD as previously published (Ferris *et al.*, 2009) and following manufacturer's guidelines. Ag-LFDs were incubated for 10 minutes at ambient temperature prior to interpretation of results. A positive result was indicated by the presence of two visual bands (FMDV-test line and LFD-control line, Figure 3.4A), a negative result was indicated by the presence of a single band (LFD control line, Figure 3.4B).

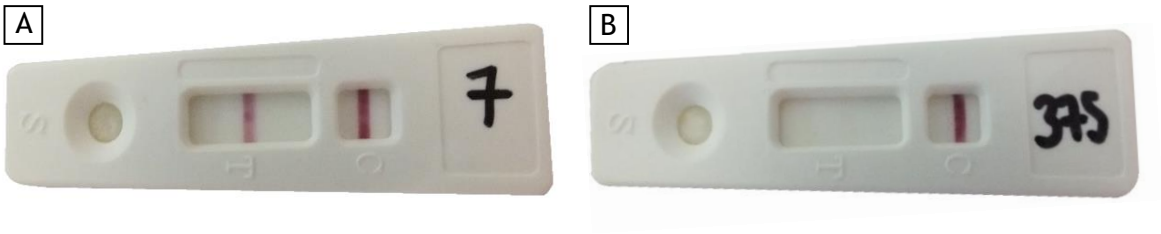

Figure 3.4 Interpreting the results of antigen-detection lateral-flow devices. (A) foot-and-mouth disease virus (FMDV)-positive results were indicated by the presence of both a test line (T-line) and a control line (C-line); (B) FMDV-negative results were indicated by the presence of a control line only.

3.3.5 Laboratory evaluation of rRT-LAMP and RT-LAMP-LFD reagents

All samples for laboratory evaluation of rRT-LAMP and RT-LAMP-LFD are summarised in Appendix 3a.

Initial determination of assay performance:

The analytical and diagnostic sensitivities of the wet rRT-LAMP and RT-LAMP-LFD reagents had been previously evaluated and described in Chapter 2.4.2. To select the best reagents for lyophilisation, an initial comparison of ISO-001 and ISO-004 master mixes (OptiGene Ltd.) were compared in wet and lyophilised formats using a dilution series of RNA extracted from a FMDV cell culture isolate (as described in Chapter 2.3.6) (Appendix 1b). The analytical sensitivities of the selected lyophilised rRT-LAMP and RT-LAMP-LFD reagents were then established using both an artificial RNA standard and RNA extracted from a FMDV cell culture isolate (as described in Chapter 2.3.6).

Determination of the diagnostic window of detection:

The diagnostic window of detection (time points post-infection when FMDV can be detected within clinical samples) was determined using archival experimental bovine sera (n = 19) and OP fluid samples (n = 21). In these studies, calves were either challenged directly (via intradermolingual injection) or indirectly (via housing with an infected donor displaying clinical signs of FMD) with FMDV (isolate O/UKG/34/2001) (DEFRA grant SE2814). Samples from this transmission study were collected daily from initial infection until six days post challenge (DPC), where cattle reached their experimental end-point (lesions present on three feet), and stored at -80°C. Nucleic acid was extracted fresh from 200 µl of each sample using the MagNA Pure LC and Total Nucleic Acid Isolation Kit (Roche, Burgess Hill, UK) as manufacturer's instructions. Nucleic acid was eluted in a final volume of 50 µl MagNA Pure elution buffer (Roche), which was used as template in rRT-LAMP (wet) and the reference rRT-PCR. These samples were also used for further evaluation of the simple sample preparation procedures (dilution of samples in NFW) developed in Chapter 2 on clinical samples (Chapter 2.3.7).

Archival field epithelial tissue suspensions (prepared at 10% [w/v] in M25 phosphate buffer: 35 mM Na_2HPO_4, 5.7 mM KH_2PO_4, pH 7.6) were selected based on previous RT-LAMP validation studies (Waters *et al.*, 2014) and used to evaluate the performance of

rRT-LAMP and RT-LAMP-LFD (lyophilised) on epithelial samples (Table 3.1). Again, rRT-LAMP was evaluated using both extracted RNA (extracted using the MagNA Pure LC / Total Nucleic Acid Isolation Kit as above) and using the simple sample preparation procedures developed in Chapter 2 (Chapter 2.3.7).

Table 3.1 Clinical epithelial samples used for initial validation of lyophilised reagents

Serotype	Sample	Topotype	Lineage	Location	Year
Asia 1	TUR/2/2013	ASIA	Sindh-08	Turkey	2013
A	IRN/24/2012	ASIA	Iran-05^{SIS-10}	Iran	2012
	TUR/7/2013	ASIA	Iran-05^{SIS-10}	Turkey	2013
	TUR/4/2013	ASIA	Iran-05^{SIS-10}	Turkey	2013
SAT 1	TAN/50/2012	I (NWZ)	unnamed	Tanzania	2012
SAT 2	TAN/14/2012	IV	unnamed	Tanzania	2012
	BOT/15/2012	III	unnamed	Botswana	2012

(SAT) Southern African Territories; (NWZ) North West Zimbabwe.

3.3.6 Evaluation of rRT-LAMP on FMDV surveillance samples

Chapter 2 demonstrated that RT-LAMP was suitable for rapid confirmation of FMD-positives in the field (using simple sample preparation methods) and FMDV surveillance in low-resource laboratories (where RNA extraction is possible). Therefore, the performance of rRT-LAMP was evaluated using RNA extracted from FMDV surveillance samples (not all samples would be from animals displaying clinical signs of FMD). Archival cattle OP fluid field samples (n = 158), collected between September 2011 and November 2013 (serotypes O, A, Southern African Territories [SAT] 1 and SAT 2), were received from Dr Tiziana Lembo and Prof. Sarah Cleveland (The University of Glasgow) from BBSRC project BB/H009302/1 (a Combating Infectious Diseases of Livestock for International Development [CIDLID] research initiative assessing new approaches for strategic control of FMD in East Africa). In addition, archival buffalo samples (serum = 38; OP fluid = 50) were received from Dr Nicholas Juleff and Dr Eva Perez (TPI) as part of an African buffalo FMDV persistence study within Kruger National Park (KNP) (Maree *et al.*, 2016). In this study, buffalo were challenged directly (via intradermolingual injection) with three FMDV isolates (KNP/196/91/1 [SAT 1]; KNP/19/89/2 [SAT 2]; KNP/1/08/3 [SAT 3]). OP fluid and serum were collected (samples received from the day of challenge up to 109 days post-challenge) and stored as previously published (Kitching and Donaldson, 1987). For both sets of samples, total nucleic acid was extracted using the MagNA Pure LC / Total Nucleic Acid Isolation Kit as above (Chapter 3.3.5) and used as template in rRT-LAMP (wet) and the reference rRT-PCR.

3.3.7 Evaluation of rRT-LAMP and RT-LAMP-LFD in endemic laboratory settings

To evaluate the performance of assays in low-resource laboratory settings (Sokoine University of Agriculture, Morogoro, Tanzania), lyophilised rRT-LAMP/RT-LAMP-LFD (using 1 in 5 dilutions in NFW), rRT-PCR (Engima® FL) and Ag-LFDs were tested using 14 archival epithelial tissue suspensions collected from the field. Samples were provided by The Tanzania Veterinary Laboratory Agency (TVLA, Dar es Salaam, Tanzania) and suspensions were prepared in phosphate-buffered saline (pH 7.2). Samples represented the following serotypes and regions (previously serotyped at TVLA): O (Musoma Rural; Tabora; Mara; Njombe; Kilimanjaro; Mtwara), A (Kagera), SAT 1 (Dar es Salaam; Morogoro), SAT 2 (Morogoro) and three un-typed samples (Figure 3.5).

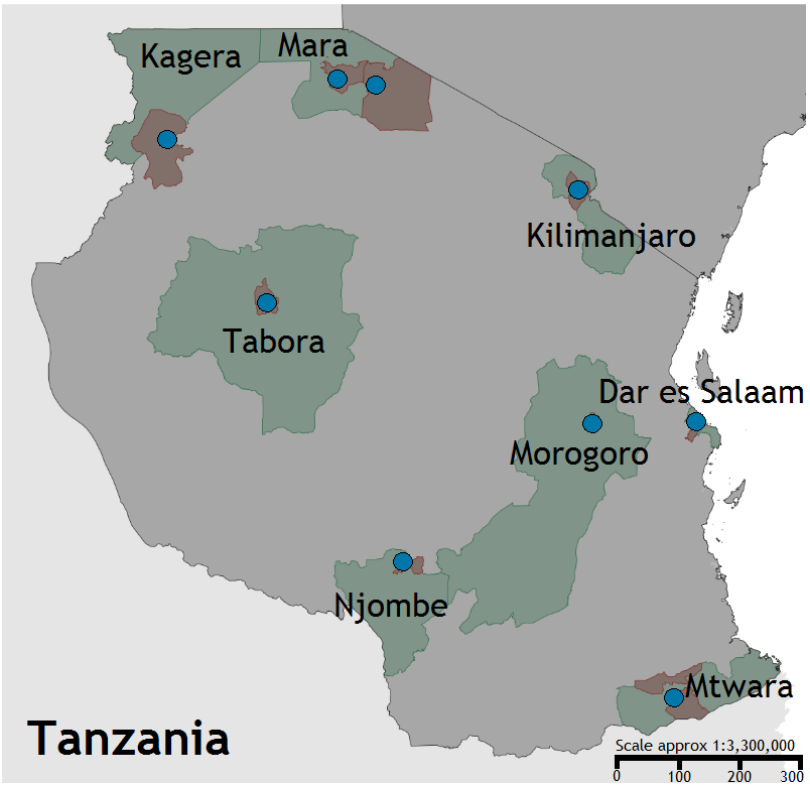

Figure 3.5 Field sampling locations of the archival epithelial tissue suspensions. These suspensions were used for assay evaluation in low-resource laboratory settings. Blue points represent the location of small holder farms; green shading represents national parks; red shading represents regions.

3.3.8 Field evaluation of rRT-LAMP and RT-LAMP-LFD reagents

Field evaluation was carried out in Tanzania and Kenya, with Ankole-cross, Zebu and Zebu-cross cattle. Research locations were selected on the basis of existing collaborations with investigators working on the epidemiology of FMD and individual farms were selected opportunistically following reports of FMDV infection (Figure 3.6). Serum, OP fluid and mouth/foot epithelium (where possible) samples were collected from cattle across different stages of infection (acute, convalescent and recovered). For each animal, the time since the start of clinical signs was estimated based on aging of the oldest lesion (using FAO guidelines [DEFRA, 2005]) and known clinical history (provided by livestock owners and veterinary officers). Samples were also collected from cattle in the affected herds which were asymptomatic at the time of sampling (with no recent clinical history of FMD). The number of samples collected was the maximum amount that could be logistically collected in terms of livestock availability and the working time frame.

In total, samples from 60 individual cattle from 10 farms across East Africa were analysed *in situ* (Appendix 3b). This work comprised eight cattle from two Maasai small holdings from the Mvomero and Morogoro Rural Districts (Morogoro Region, Tanzania, June 2014), 41 cattle from seven small holdings located in the Serengeti District (Mara Region, Tanzania, October 2014) and 11 cattle from three farms in Nakuru County, Kenya (October 2013 and December 2014). Five of the cattle from Serengeti District (Tanzania) were sampled on two separate occasions, six days apart. For analysis, cattle were grouped into one of four categories: 1-7 days post onset of clinical signs (n = 16), 8-14 days post onset of clinical signs (n = 10), 15+ days post onset of clinical signs (n = 27) and clinically normal with no recent clinical history of FMDV infection (n = 12).

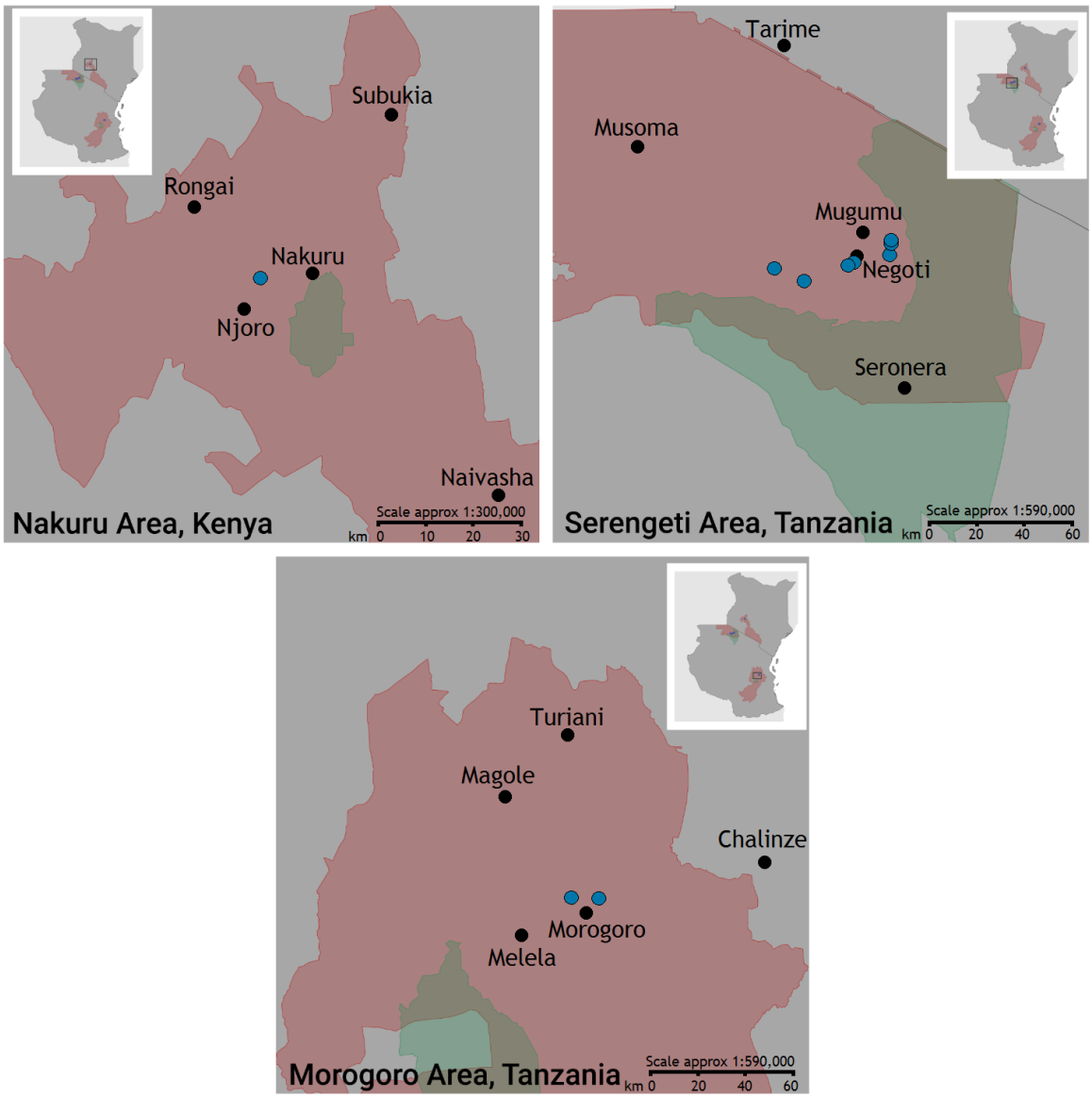

Figure 3.6 Sampling locations in East Africa. Blue points represent the location of small holder farms; green shading represents national parks; red shading represents regions/counties.

Samples were collected and processed as follows:

Loose epithelial tissue: Epithelial tissue surrounding ruptured vesicular lesions was collected from either the mouth or the feet using sterile forceps and was prepared using the SVANODIP® FMDV-Ag Extraction Kit, according to the manufacturer's instructions. In brief, approximately 0.2 g of epithelial tissue was homogenised using the sample extraction vial in 1 ml of sample buffer from the SVANODIP® FMDV-Ag LFD kit. The homogenate was left to settle for 1 minute and the supernatant added neat to the mobile rRT-PCR and Ag-LFD platforms, and processed as previously described prior to rRT-LAMP and RT-LAMP-LFD by 1 in 5 dilution in NFW (Waters *et al.*, 2014).

Serum: Cattle blood (10 ml) was collected (by veterinarians) from the jugular vein using Vacutainer Plus Plastic Serum Tubes (BD, Plymouth, UK). An aliquot was centrifuged using an E8 field-based centrifuge (LW Scientific) at 1400 x g for 3 minutes at room temperature. Serum was added neat to the mobile rRT-PCR platform and diluted 1 in 5 in NFW prior to rRT-LAMP and RT-LAMP-LFD.

OP fluid: OP fluid was collected using a suitably sized probang cup following OIE guidelines (OIE, 2012) (Figure 3.7). OP fluid was added neat to the mobile rRT-PCR platform and diluted 1 in 10 in NFW prior to analysis using rRT-LAMP and RT-LAMP-LFD.

Figure 3.7 Oesophageal-pharyngeal fluid sampling of a Zebu-cross cow in a Maasai herd (Morogoro, Tanzania).

3.3.9 Biosafety procedures in the field

During field work, a biosafety boundary was established outside of each farm premises, to separate livestock-containing areas (considered contaminated) from livestock-free areas (considered uncontaminated). This was established to ensure that field work did not contribute to the further spread of FMDV. Sample preparation and assay assembly was performed inside the contaminated area. Assembled reactions (rRT-LAMP master mix plus sample) were surface disinfected with either citric acid (0.2% w/v) or FAM® 30 (1:240) prior to transfer to the uncontaminated area where rRT-LAMP on the Genie® II was performed. Different personnel were present in these areas to facilitate the transfer of samples. Personnel entering the contaminated area donned disposable over-suits, two

pairs of gloves and over-shoes, which were surface disinfected as above prior to disposal within a bag (along with disposable consumables) for incineration at local laboratory facilities. Any non-disposable equipment which was used in the contaminated area (e.g. probang cup, forceps, scissors and boots) was suitably surface disinfected prior to transfer back to the uncontaminated area. Following each test, and between farms, the Genie® II was surface disinfected (as above) and sprayed with DNAZap™ DNA Degradation Solutions (Thermo Fisher Scientific). At the end of field work the Genie® II and non-disposable equipment was surface disinfected (as above) prior to fumigation with formaldehyde (10 mg/m^3) at 30°C (relative humidity > 70 for > 60 minutes).

3.3.10 Statistical analysis

Cohen's Kappa statistic (κ) and the proportion of observed agreement (A_{obs}) were used to measure the agreement between diagnostic tests. All statistical tests were performed in the statistical package R (R Core Team, 2014). Cohen's Kappa statistic (κ) was interpreted as published in Landis and Koch (1977).

3.4 Results

3.4.1 Laboratory evaluation of rRT-LAMP and RT-LAMP-LFD

Initial determination of assay performance:

During initial optimisation of rRT-LAMP/RT-LAMP-LFD, it was evident that lyophilisation of ISO-004 reactions resulted in a reduction of analytical sensitivity (Appendix 1b). Consequently, all further evaluation was carried out using the ISO-001 master mix. Lyophilised rRT-LAMP/RT-LAMP-LFD ISO-001 reagents maintained equivalent analytical sensitivity to their wet counterparts (Chapter 2.4.1) using both an artificial RNA standard (10^1 copies/µl) and RNA extracted from FMDV a cell culture isolate, with the time to positivity (T_P) consistently reduced for lyophilised reagents (Figure 3.8).

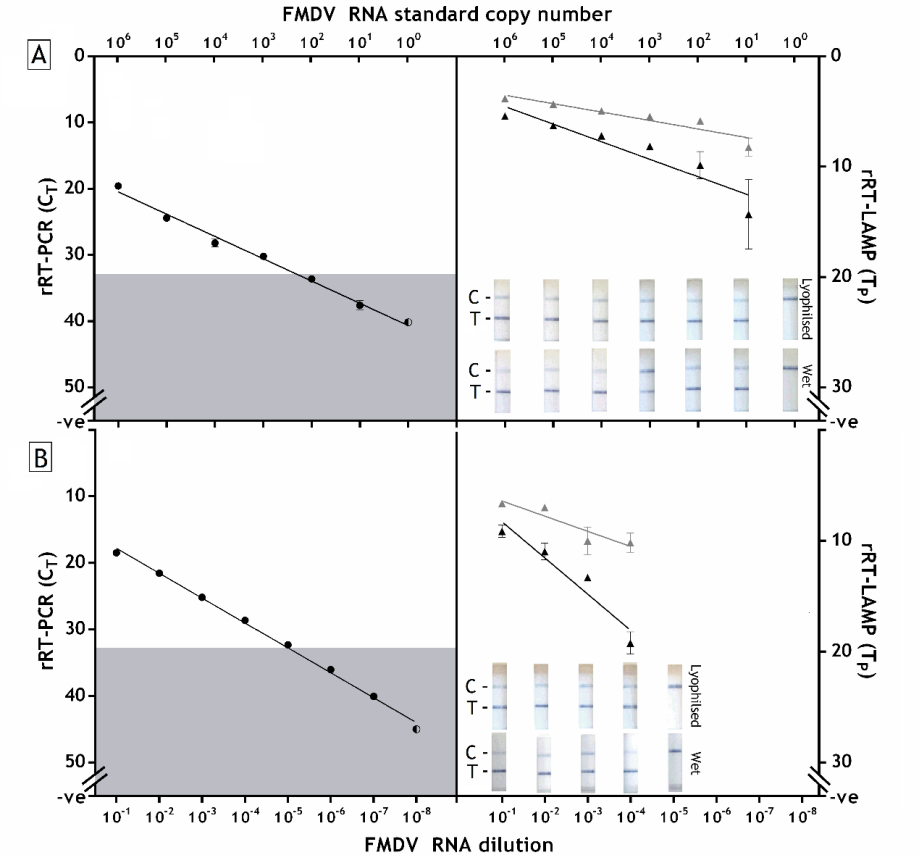

Figure 3.8 Analytical sensitivity of molecular assay formats using (A) an RNA standard and (B) dilution series of FMDV RNA. (●) reference real-time reverse transcription (rRT)-PCR; (▲) real-time reverse transcription loop-mediated isothermal amplification (rRT-LAMP) (wet); (▲) rRT-LAMP (lyophilised). The grey shaded area for the reference rRT-PCR, represents cycle threshold values over the diagnostic cut-off threshold of C_T < 32 (Shaw *et al.*, 2007). Points represent the mean of two replicates (with error bars representing range). Half-shading: one replicate positive and the other negative. For RT-LAMP combined with lateral-flow detection (RT-LAMP-LFD), the presence of two blue lines signifies a positive result (control and test line); the presence of a single band signifies a negative result (control line).

Determination of the diagnostic window of detection:

For determination of the rRT-LAMP clinical detection window, FMDV RNA was detected in serum from one to four days post-challenge. For OP fluid, FMDV RNA was detected from one day post-challenge onwards, until cattle reached their experimental end-point (lesions present on three feet) (Figure 3.9). These clinical samples were also used to determine how the use of simple sample preparation procedures developed in Chapter 2 affected the diagnostic window for detection. By diluting sera 1 in 5 in NFW, high agreement with the reference rRT-PCR (using extracted RNA) was achieved, with FMDV RNA detected in 17/19 FMDV-positive samples ($\kappa = 0.791$, $p < 0.001$, $A_{obs} = 0.895$). For OP fluid, 1 in 10 dilutions enabled FMDV RNA to be detected in 19/21 FMDV-positive samples, again displaying high agreement with the reference rRT-PCR (using extracted RNA) ($\kappa = 0.741$, $p = 0.005$, $A_{obs} = 0.905$). The four discordant samples (serum = 2; OP fluid = 2) displayed high reference rRT-PCR C_T values (Figure 3.9). For both sample types, the T_P values were consistently higher when using simple sample preparation methods comparatively to extracted nucleic acid (Figure 3.9).

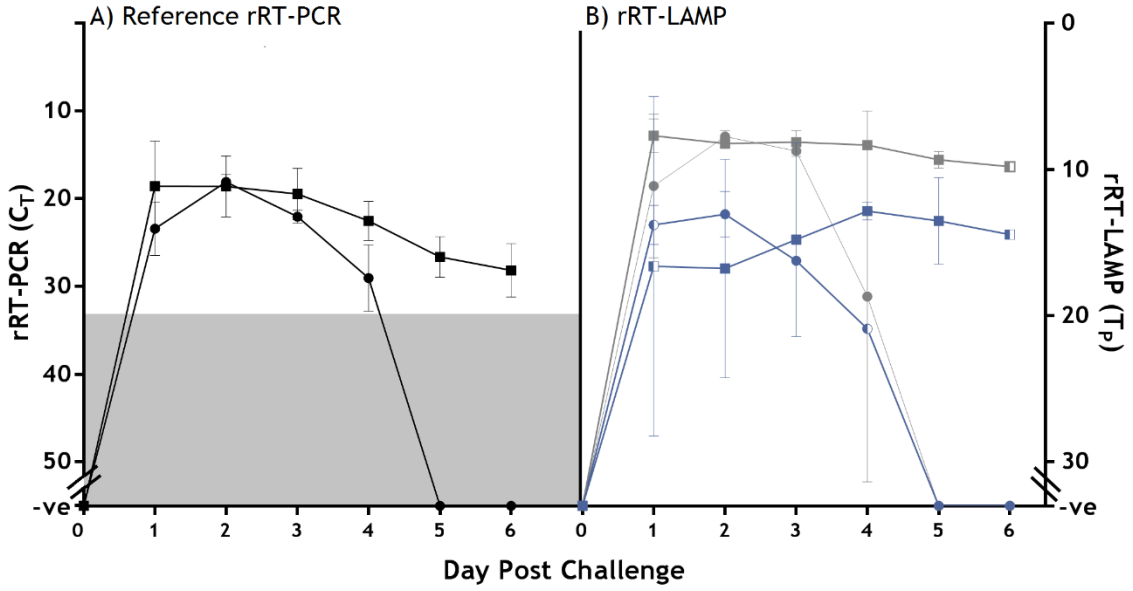

Figure 3.9 Determination of the clinical detection window for real-time reverse transcription loop-mediated isothermal amplification (rRT-LAMP) (wet) (B), compared against the reference real-time reverse transcription PCR (rRT-PCR) (A). Samples were either used following RNA extraction or dilution in nuclease-free water (NFW). For serum samples: (●) rRT-PCR (extracted nucleic acid); (●) rRT-LAMP (extracted nucleic acid); (●) rRT-LAMP (1 in 5 NFW dilution). For OP fluid samples: (■) rRT-PCR (extracted nucleic acid); (■) rRT-LAMP (extracted nucleic acid); (■) rRT-LAMP (1 in 10 NFW dilution). Points represent the mean of the four animals (each with two replicates); half-shaded points represent points with a mix of positive and negative results. Bars represent standard deviation. Samples were collected up to day six days post challenge, when cattle reached their experimental end-point (lesions present on three feet). A diagnostic cut-off of $C_T < 32$ (Shaw *et al.*, 2007) was used to distinguish between rRT-PCR positive and negatives.

FMDV RNA was detected in all archival epithelial samples following nucleic acid extraction (data not shown). When epithelial suspensions were added to lyophilised ISO-001 rRT-LAMP and RT-LAMP-LFD following 1 in 5 dilution in NFW, positive amplification was observed for all FMDV serotypes evaluated (Table 3.2).

Table 3.2 Results for lyophilised rRT-LAMP evaluation on clinical epithelial samples

Serotype	Sample	rRT-LAMP*	(T_a)	RT-LAMP-LFD
Asia 1	TUR/2/2013	10.24	(88.86)	Positive
A	IRN/24/2012	8.54	(88.84)	Positive
	TUR/7/2013	11.09	(88.89)	Positive
	TUR/4/2013	10.54	(89.21)	Positive
SAT 1	TAN/50/2012	16.54	(89.41)	Positive
SAT 2	TAN/14/2012	11.24	(89.29)	Positive
	BOT/15/2012	12.39	(89.11)	Positive
N/A	Negative epithelium	No T_p	(No T_a)	Negative

(SAT) Southern African Territories; (NWZ) North West Zimbabwe; (rRT-PCR) real-time reverse transcription PCR; (rRT-LAMP): real-time reverse transcription loop-mediated isothermal amplification; (T_a) Anneal temperature; (T_P) time to positivity; (RT-LAMP-LFD) RT-LAMP combined with lateral-flow detection; (Ag-LFD) Antigen-detection lateral-flow device. *Reagents used in a lyophilised format. All samples were epithelial suspensions.

3.4.2 Evaluation of rRT-LAMP on FMDV surveillance samples

For evaluation of rRT-LAMP on archival FMDV surveillance samples, RNA was extracted from 246 longitudinal field study samples (cattle = 158; buffalo = 88). High agreement was present between rRT-LAMP (wet) and the reference rRT-PCR ($\kappa = 0.900$, $p < 0.001$, $A_{obs} = 0.956$) in the presence of an rRT-PCR diagnostic cut-off ($C_T < 32$; Shaw *et al.*, 2007). However, when comparing rRT-LAMP to rRT-PCR (no diagnostic cut-off), agreement was reduced ($\kappa = 0.714$, $p < 0.001$, $A_{obs} = 0.861$), with false negative rRT-LAMP samples consistently having high rRT-PCR C_T values (between 30.68 and 49.55) (Figure 3.10). Similar results were evident for buffalo OP fluid and serum samples, with high agreement present when a diagnostic cut-off was employed in rRT-PCR ($\kappa = 0.930$, $p < 0.001$, $A_{obs} = 0.966$) comparatively to rRT-PCR with no diagnostic cut-off ($\kappa = 0.697$, $p < 0.001$, $A_{obs} = 0.863$) (Figure 3.11). As such, rRT-LAMP assay performance was comparable to rRT-PCR considering a diagnostic cut-off, however was subject to reductions in analytical sensitivity if used in its absence.

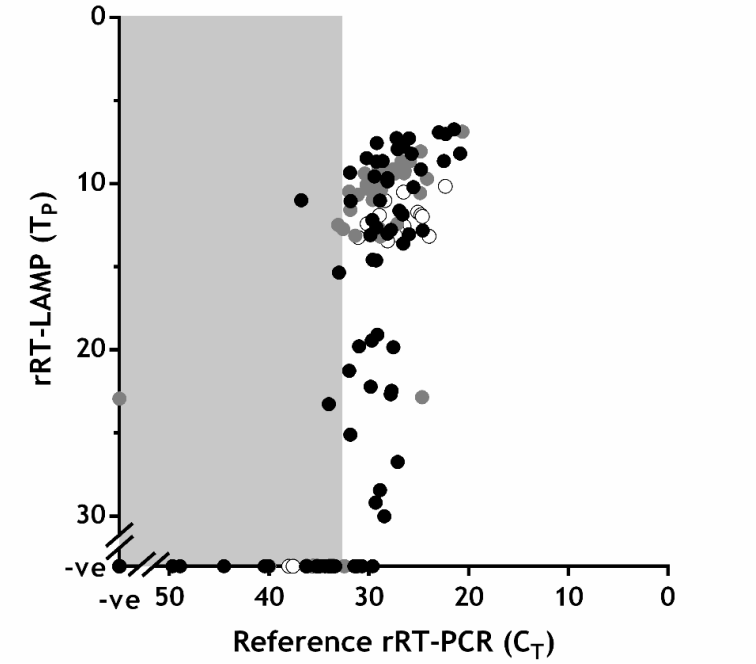

Figure 3.10 Evaluation of rRT-LAMP as a laboratory surveillance tool. Graph shows the concordance between the reference real-time reverse transcription PCR (rRT-PCR) and real-time reverse transcription loop-mediated isothermal amplification (rRT-LAMP). Evaluation was performed over: (●) 158 oesophageal-pharyngeal fluid samples collected from cattle; (●) 50 oesophageal-pharyngeal fluid samples collected from buffalo; (○) 38 serum samples collected from buffalo. The grey shaded area highlights reference rRT-PCR C_T values over the FMDV diagnostic threshold of C_T < 32 (Shaw *et al.*, 2007). Each point represents the mean of two replicates.

3.4.3 Evaluation of rRT-LAMP and RT-LAMP-LFD in endemic laboratory settings

Fourteen archival epithelial tissue suspensions, representing four FMDV serotypes and nine locations across Tanzania, were used to compare the performance of POCTs on clinical samples within a local laboratory setting in an FMD endemic region (Table 3.3). Complete agreement was evident between RT-LAMP and RT-LAMP-LFD assay results (using 1 in 5 dilutions of epithelial tissue suspensions in NFW), which were both in high agreement with rRT-PCR results using the Enigma® FL ($\kappa = 0.759$, $p = 0.033$, $A_{obs} = 0.929$) (Table 3.3). Ag-LFDs showed reduced sensitivity, with three rRT-LAMP/RT-LAMP-LFD FMDV-positive samples called as negative (Table 3.3).

Table 3.3 Epithelial tissue suspensions used for low-resource laboratory evaluation

Serotype	Location (region)	Enigma® FL* rRT-PCR	rRT-LAMP*	(T_a)	RT-LAMP-LFD	Ag-LFD
O	Musoma Rural	25.00	8.50	(88.81)	Positive	Weak positive
	Tabora	32.00	12.75	(88.82)	Positive	Negative
	Tabora	25.00	8.75	(88.60)	Positive	Negative
	Mara	26.00	8.75	(89.00)	Positive	Strong positive
	Njombe	23.00	9.00	(89.20)	Positive	Weak positive
	Kilimanjaro	31.00	10.00	(88.76)	Positive	Weak positive
	Mtwara	23.00	14.00	(88.86)	Positive	Weak positive
A	Kagera	No C_T	9.00	(89.12)	Positive	Weak positive
SAT 1	Dar es Salaam	28.00	15.00	(89.31)	Positive	Negative
	Morogoro	27.00	8.50	(89.10)	Positive	Weak positive
SAT 2	Morogoro	27.00	10.00	(88.90)	Positive	Weak positive
Un-typed	Unknown	32.00	10.00	(88.46)	Positive	Positive
	Kilimanjaro	No C_T	No T_p	(No T_a)	Negative	Negative
	Kilimanjaro	No C_T	No T_p	(No T_a)	Negative	Negative

(Enigma® FL) Enigma® Field Laboratory; (rRT-PCR) real-time reverse transcription PCR; (rRT-LAMP) real-time reverse transcription loop-mediated isothermal amplification; (T_a) Anneal temperature; (RT-LAMP-LFD) RT-LAMP combined with lateral-flow detection; (Ag-LFD) Antigen-detection lateral-flow device. *Reagents used in a lyophilised format.

3.4.4 Field evaluation of rRT-LAMP and RT-LAMP-LFD reagents

The Genie® II and rRT-LAMP / RT-LAMP-LFD protocols devised in the laboratory were trialled on 145 samples, from 60 cattle, across 10 farms in East Africa (five cattle sampled on two occasions) (Figure 3.11 and 3.12).

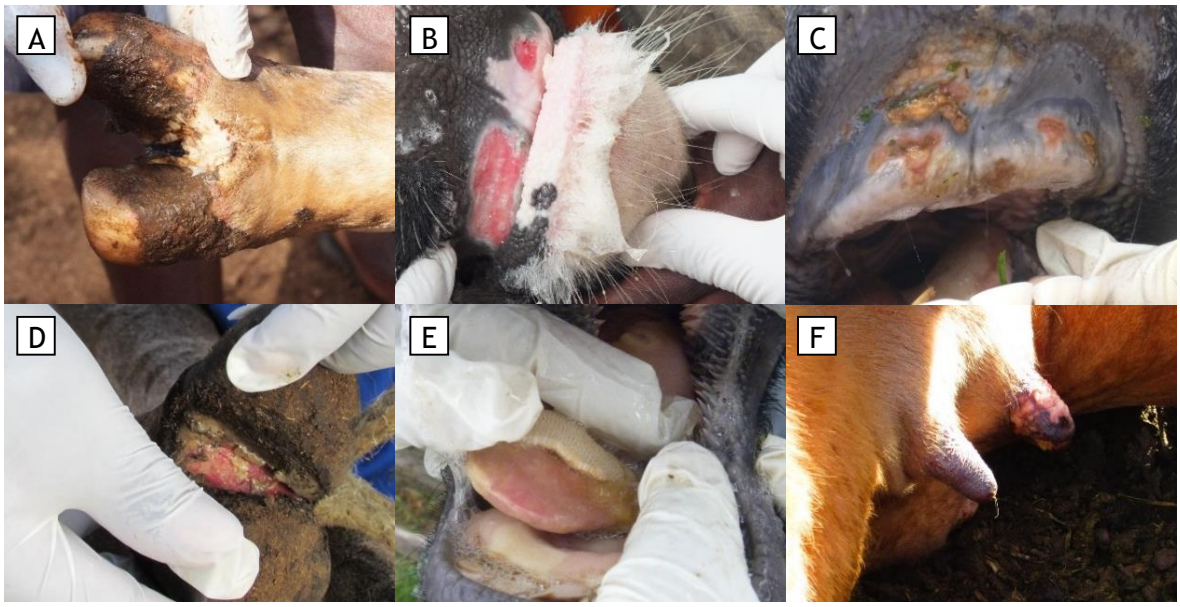

Figure 3.11 Characteristic foot-and-mouth disease lesions observed in cattle. (A) un-ruptured lesion in the interdigital cleft with vesicular fluid present in the blister (1 day post onset of clinical signs); (B) ruptured dental pad lesions (2 days post onset of clinical signs); (C) healing lesions on the dental pad and gum (8-10 days post onset of clinical signs); (D) ruptured lesion in the interdigital cleft (2 days post onset of clinical signs); (E) tongue epithelium sloughed off after lesion rupture (3 days post onset of clinical signs); (F) un-ruptured lesions on the teats (1 day post onset of clinical signs).

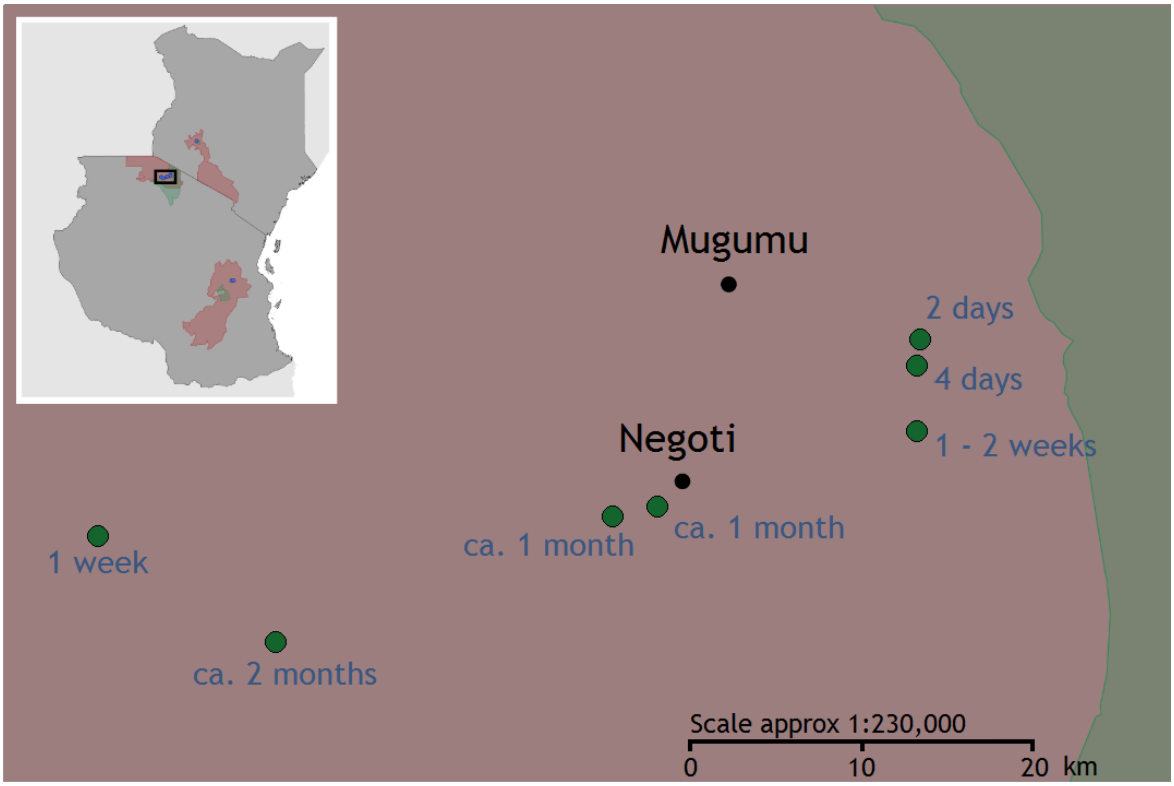

Figure 3.12 Epidemiological tracing of foot-and-mouth disease (FMD) outbreaks in Serengeti Area, Tanzania (October 2014). Points represent the location of the small-holder farms; green shading represents national parks; red shading represents regions/counties. The dates given indicate the estimated time since the start of clinical signs.

For the 16 cattle 1-7 days since onset of clinical signs, rRT-LAMP identified FMDV RNA in 13/14 epithelial, 11/14 OP fluid and 11/16 sera samples. Of the ten cattle 8-14 days post onset of clinical signs, rRT-LAMP identified FMDV RNA in 8/9 epithelial, 6/9 OP fluid and 1/10 sera samples. Of the 27 clinically recovered cattle (15+ days post onset of clinical signs, based on recent clinical history and aging of the oldest lesion), rRT-LAMP identified FMDV RNA in 14/27 OP fluid samples, while all serum samples were negative. Of the 12 clinically normal cattle sampled (with no recent clinical history of FMDV infection), all OP fluid and sera samples were negative in rRT-LAMP (Appendix 3b). High agreement was evident between rRT-LAMP and RT-LAMP-LFD for all sample types: sera ($\kappa = 0.837$, $p < 0.001$, $A_{obs} = 0.947$), OP fluid ($\kappa = 0.852$, $p < 0.001$, $A_{obs} = 0.926$) and epithelial samples ($\kappa = 0.646$, $p = 0.123$, $A_{obs} = 0.957$). All test results were consistent with clinical observations (Figure 3.13 and 3.14).

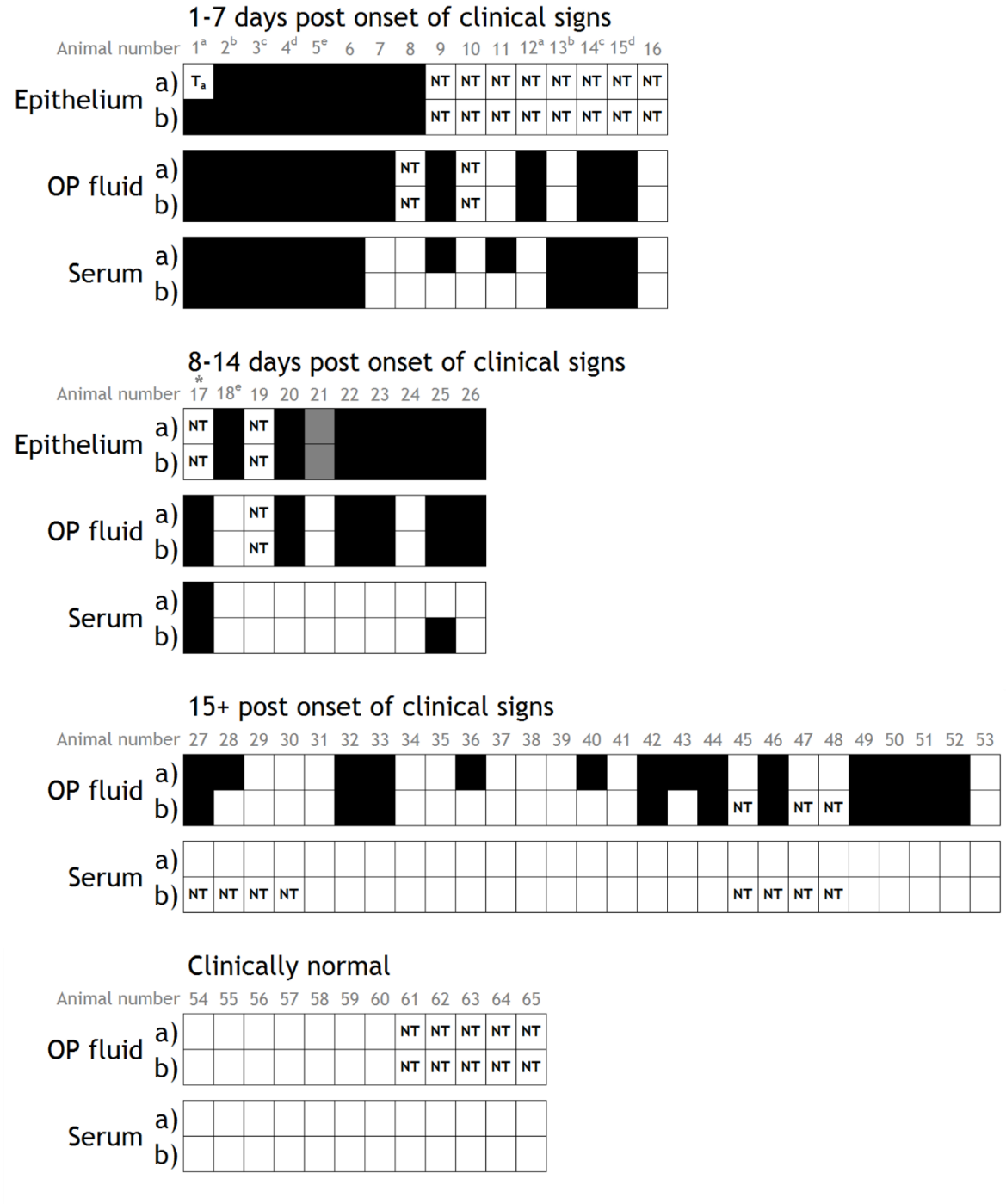

Figure 3.13 *In situ* (a) real-time reverse transcription loop-mediated isothermal amplification (rRT-LAMP) and (b) RT-LAMP combined with lateral-flow detection (RT-LAMP-LFD) results for 145 samples. Cattle were either acutely infected with foot-and-mouth disease (FMD), displayed healing FMD lesions, were clinically recovered from FMD or were clinically asymptomatic (with no recent clinical history of FMD). (OP fluid) oesophageal-pharyngeal fluid; (black) positive result; (white) negative result; (NT) not tested; (T_a) anneal temperature, amplification was evident however the T_a was outside of the accepted range. Each column represents one animal; rows represent sample type. For some animals more than one epithelial sample was tested, grey squares represent a mix of positive and negative results. [a-e]The five animals sampled on two occasions, six days apart. The onset of clinical signs was estimated based on the aging of the oldest lesion and known clinical history of FMD. *Potential mis-aged lesion.

Samples that were positive in rRT-LAMP but negative in RT-LAMP-LFD (n = 6) were consistently associated with high rRT-LAMP T_P values (Appendix 3b). Two samples were positive in RT-LAMP-LFD but negative in rRT-LAMP. One of these (an epithelial sample) displayed rRT-LAMP amplification, however the T_a value was out of the accepted temperature range by 0.18°C (T_a = 89.68).

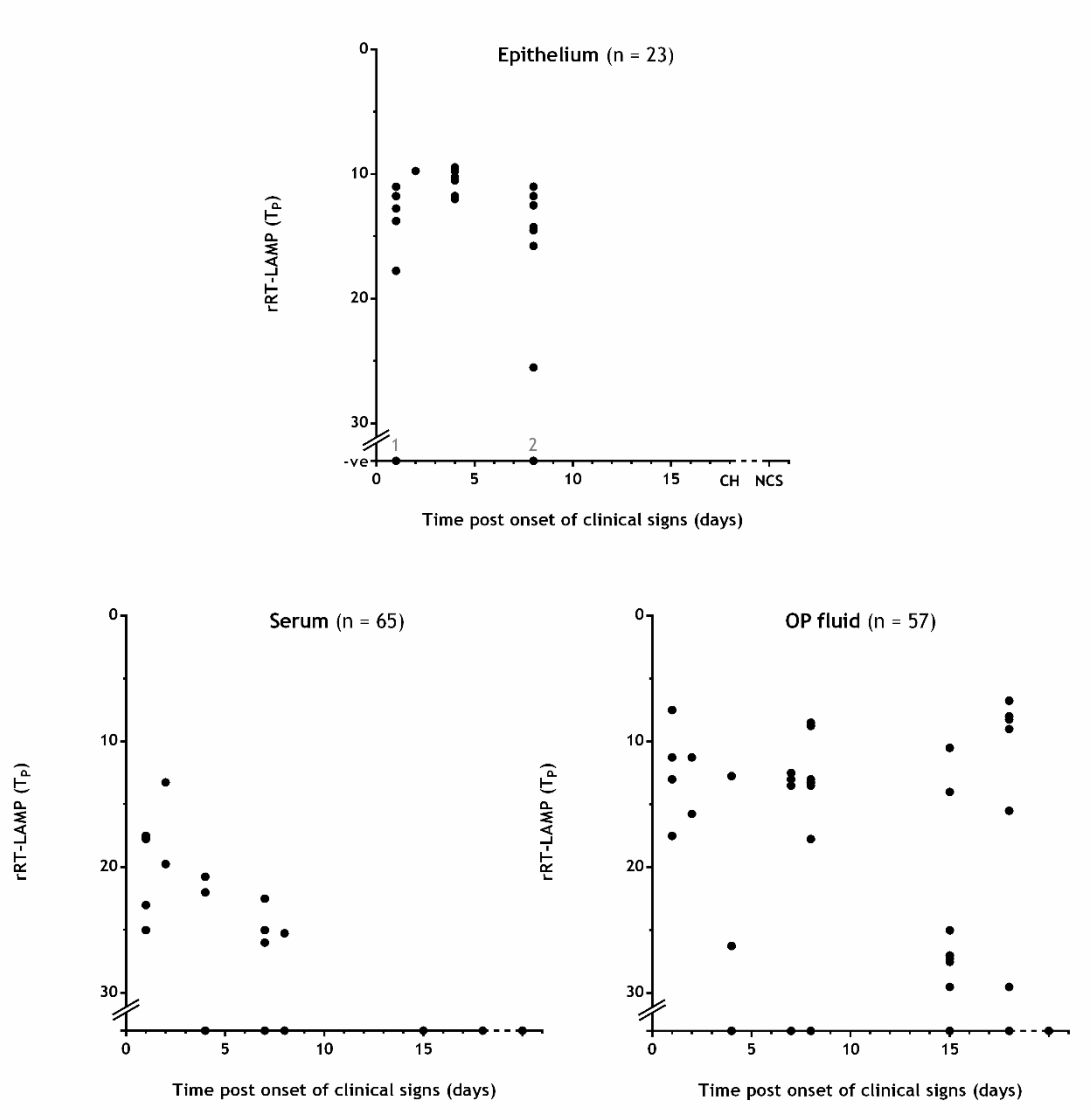

Figure 3.14 *In situ* real-time reverse transcription loop-mediated isothermal amplification (rRT-LAMP) results for the 145 East African samples. Time to positivity (T_P) is indicated for rRT-LAMP (average across two replicates). (CH) clinical history of FMD, for instance animal had signs of FMD one to two months previously; (NCS) no clinical signs or recent history of FMD. [1]Amplification was evident however the anneal temperature (T_a) was outside of the accepted range (T_a = 89.68); [2]Lesion material collected was not a sufficient amount for processing.

During POCT evaluation, there was no access to a laboratory-based rRT-PCR machine, therefore comparison against the reference rRT-PCR was performed as previously described (Madi *et al.*, 2012) using an Enigma® FL. High agreement was present between molecular tests across the 34 samples tested (13 epithelium; 17 OP fluid; 4 sera): rRT-LAMP and rRT-PCR ($\kappa = 0.635$, $p < 0.001$, $A_{obs} = 0.853$) and RT-LAMP-LFD and rRT-PCR ($\kappa = 0.781$, $p < 0.001$, $A_{obs} = 0.912$) (Figure 3.15).

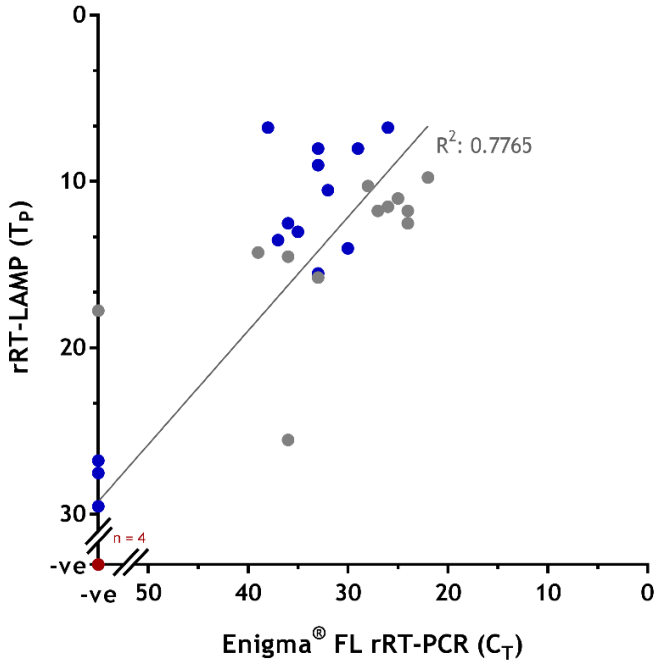

Figure 3.15 Comparison between real-time reverse transcription PCR (rRT-PCR) (Enigma® Field Laboratory [FL]) and real-time reverse transcription loop-mediated isothermal amplification (rRT-LAMP) (Genie® II) on field samples tested *in situ* within Tanzania (Serengeti District and Morogoro). Colour is representative of sample type, (grey) epithelial tissue suspensions [n = 13]; (blue) oesophageal-pharyngeal fluid [n = 17]; (red) serum [n = 4]. Samples were added neat to the rRT-PCR and diluted in nuclease-free water prior to analysis in rRT-LAMP.

In addition, 23 epithelial samples were assayed using Ag-LFDs, with only slight agreement evident between both rRT-LAMP and Ag-LFD ($\kappa = 0.008$, $p = 0.486$, $A_{obs} = 0.522$) and RT-LAMP-LFD and Ag-LFD results ($\kappa = 0.095$, $p = 0.332$, $A_{obs} = 0.565$). Out of 12 rRT-PCR positive epithelial samples, five were negative by Ag-LFD (Appendix 3b).

Four clinical samples (two epithelial and two vesicular fluid) from two of the acutely infected cattle tested (tag numbers 7804 and 7805) in the Serengeti District, Tanzania were also shipped to WRLFMD for characterisation. All four samples were confirmed positive for FMDV using rRT-PCR and were typed as serotype SAT 1 by antigen capture enzyme-linked immunosorbent assays (Ferris and Dawson, 1988) (data not shown).

3.5 Discussion

Robust and rapid diagnosis of FMD is essential for the effective implementation of disease monitoring, control and eradication strategies. However, previous publications which highlight RT-LAMP as a potential POCT-ready solution describe reagents and protocols unsuitable for field deployment or use in low-resource laboratories (Dukes *et al.*, 2006; Shao *et al.*, 2010; Yamazaki *et al.*, 2013; Waters *et al.*, 2014). This chapter describes the development, evaluation and deployment of lyophilised RT-LAMP reagents and protocols suitable for decentralised detection of FMDV.

Lyophilisation of ISO-001 RT-LAMP reagents had no negative impact on assay performance, with both rRT-LAMP and RT-LAMP-LFD maintaining comparable analytical sensitivity to their equivalent wet formats. Furthermore, the T_P for lyophilised reagents was consistently lower (improved) than the wet formats, providing further support for the 30 minute incubation time as discussed in Chapter 2. When assessing RT-LAMP as a laboratory surveillance tool, and consistent with the results detailed in Chapter 2, RT-LAMP assay performance was comparable to the diagnostic rRT-PCR when considering a diagnostic cut-off value (Shaw *et al.*, 2007) and using extracted nucleic acid, however was subject to reductions in analytical sensitivity if used in the absence of these. Although cut-off values are often implemented in rRT-PCR to mitigate against false-positive results, duplicate samples that fall within the borderline range (C_T: 32-50) also require careful monitoring for low levels of FMDV (Shaw *et al.*, 2007).

Validation of RT-LAMP assays in low-resource laboratory settings highlighted their potential to improve the diagnostic capacity within low-to-middle income countries (LMICs). At present, laboratories within these settings are often constrained by limited laboratory capacity (skilled personnel and availability of technologies/consumables), cumbersome procurement systems and poor transport links (that affect maintenance of the cold chain). The provision of lyophilised reagents within disposable consumables, in addition to simple reporting procedures, helps to address these issues by (i) negating the need to order reagents and consumables from multiple suppliers, (ii) simplifying reagent storage requirements and (iii) minimising user intervention, thus opening up sensitive molecular technologies to unskilled staff. Furthermore, the use of portable platforms, such as the Genie® II, can be mains or battery powered, thereby removing the requirement for a continual mains power supply which can be unreliable in low-resource laboratories. As such, since the evaluation of rRT-LAMP in SUA, a Genie® II and lyophilised

reagents have been purchased by the laboratory, who are trialling rRT-LAMP for routine FMDV detection.

When deployed for field validation, both RT-LAMP assay formats generated results consistent with clinical observations and Enigma FL® rRT-PCR results, enabling FMDV RNA to be detected across the FMD clinical window from acute infection, to delayed viral clearance (after field evaluation, Enigma Diagnostics Ltd. liquidated, therefore the Enigma® FL was not taken forward for further evaluation). In total, samples from 66 cattle across 12 endemic field settings within East Africa were tested. The early detection of FMDV was further substantiated by data obtained from samples collected from experimentally infected animals, where FMD-positive results were generated from serum and OP fluid collected at the onset of clinical signs.

During both laboratory and field trials (and consistent with data in Chapter 2), the use of simple sample preparation procedures led to small reductions in sensitivity. Consequently, although simple sample preparation procedures enable rapid confirmation of FMD-positives in the field using RT-LAMP, confirmation of FMD-negatives remains a challenge and is an area where future improvements could be targeted. However, in all these studies, molecular assays consistently outperformed Ag-LFDs, displaying higher analytical sensitivity (even in the absence of RNA extraction) and compatibility with a greater number of clinical sample types. Ag-LFDs do however remain useful for confirmation of FMD positive animals during the acute stage of clinical infection (using epithelial samples) and were consistent with molecular assay results under these circumstances.

Field validation highlighted a number of important factors to consider for future protocol design specific to the use of rRT-LAMP and RT-LAMP-LFD *in situ*. For example, appropriate sample collection is required to ensure (i) sufficient amount of material is available for processing and (ii) samples collected are not contaminated with soil (e.g. foot epithelium) or blood/bolus/rumen fluid (OP fluid) and (iii) serum samples are collected and processed appropriately to prevent haemolysis. Although LAMP is consistently reported to show increased tolerance to inhibitors compared to PCR (Poon *et al.*, 2006; Waters *et al.*, 2014), high levels of contaminants in samples may increase false-negative (reaction inhibition) or false-positive results (non-specific amplification). Furthermore, although this chapter demonstrated the suitability of epithelium, serum and OP fluid for FMD diagnosis, in order to extend the diagnostic window of detection (increase the time points in which FMD can be detected), alternative sample types (e.g. mouth / nasal swabs) may be suitable when epithelial material cannot be collected.

The requirement to open RT-LAMP-LFD reactions post-amplification for end-point analysis leads to a high cross-contamination risk, especially where unskilled hands are involved. A prototype closed RT-LAMP-LFD system, the AMPlite®, has recently been developed by The Animal and Plant Health Agency (APHA, Addlestone, UK) to minimise this risk (see Appendix 4 for preliminary validation). However further validation and commercialisation of this prototype device is required.

In conclusion, this chapter presents the development and evaluation of lyophilised FMDV-specific rRT-LAMP and RT-LAMP-LFD assays. Both assays were highly compatible with field use, with robust chemistry conditions negating the requirement for RNA extraction. Furthermore, rRT-LAMP and RT-LAMP-LFD offer advantages over current POCT assays by being applicable for use with a larger number of sample types (Ag-LFDs are compatible with epithelium and vesicular fluid only), thus increasing the temporal diagnostic window of detection Consequently, RT-LAMP provides a realistic solution for simple and rapid confirmation of FMD in field settings and pan-FMDV surveillance in LMICs. Therefore, this chapter demonstrates an important transition for FMDV-specific molecular assays into formats suitable for decentralised deployment.

CHAPTER 4

Direct detection and characterisation of foot-and-mouth disease viruses in East Africa using a portable real-time PCR platform

Elements of this chapter have been published in the following peer-reviewed article:

Howson, E.L.A., Armson, B., Lyons, N.A., Chepkwony, E., Kasanga, C.J., Kandusi, S., Ndusilo, N., Yamazaki, W., Gizaw, D., Cleaveland, S., Lembo, T., Rauh, R., Nelson, W.M., Wood, B.A., Mioulet, V., King, D.P and Fowler, V.L. (2017d). Direct detection and characterisation of foot-and-mouth disease virus in East Africa using a field-ready real-time PCR platform. *Transbound Emerg Dis*. doi: 10.1111/tbed.12684 [Epub ahead of print].

Results from this chapter have been presented at:

North American PRRS Symposium 2016, Chicago, USA. Poster presentation: Progress in the development of real-time RT-PCR assays for detection of foot-and-mouth disease.

North American PRRS Symposium 2015, Chicago, USA. Poster presentation: Mobile rRT-PCR for rapid diagnosis of foot-and-mouth disease.

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ORIGINAL ARTICLE

WILEY Transboundary and Emerging Diseases

Direct detection and characterization of foot-and-mouth disease virus in East Africa using a field-ready real-time PCR platform

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Summary

Effective control and monitoring of foot-and-mouth disease (FMD) relies upon rapid and accurate disease confirmation. Currently, clinical samples are usually tested in reference laboratories using standardized assays recommended by The World Organisation for Animal Health (OIE). However, the requirements for prompt and serotype-specific diagnosis during FMD outbreaks, and the need to establish robust laboratory testing capacity in FMD-endemic countries have motivated the development of simple diagnostic platforms to support local decision-making. Using a portable thermocycler, the T-COR™ 8, this study describes the laboratory and field evaluation of a commercially available, lyophilized pan-serotype-specific real-time RT-PCR (rRT-PCR) assay and a newly available FMD virus (FMDV) typing assay (East Africa-specific for serotypes: O, A, Southern African Territories [SAT] 1 and 2). Analytical sensitivity, diagnostic sensitivity and specificity of the pan-serotype-specific lyophilized assay were comparable to that of an OIE-recommended laboratory-based rRT-PCR (determined using a panel of 57 FMDV-positive samples and six non-FMDV vesicular disease samples for differential diagnosis). The FMDV-typing assay was able to correctly identify the serotype of 33/36 FMDV-positive samples (no cross-reactivity between serotypes was evident). Furthermore, the assays were able to accurately detect and type FMDV RNA in multiple sample types, including epithelial tissue suspensions, serum, oesophageal–pharyngeal (OP) fluid and oral swabs, both with and without the use of nucleic acid extraction. When deployed in laboratory and field settings in Tanzania, Kenya and Ethiopia, both assays reliably detected and serotyped FMDV RNA in samples (n = 144) collected from pre-clinical, clinical and clinically recovered cattle. These data support the use of field-ready rRT-PCR platforms in endemic settings for simple, highly sensitive and rapid detection and/or characterization of FMDV.

4.1 Summary

The requirement for accurate and rapid diagnosis during foot-and-mouth disease (FMD) outbreaks, and the need to establish laboratory testing capacity in FMD-endemic countries for the detection and characterisation of FMD virus (FMDV), has motivated the development of simple diagnostic platforms and assays to support local decision making. Despite this, these platforms and assays largely remain in the research and development phase (with the exception of lateral-flow devices for antigen detection) and are largely restricted to pan-FMDV diagnosis. Using a commercially available portable thermocycler, the T-COR™ 8, this chapter describes the laboratory and field evaluation of a commercially available, lyophilised pan-FMDV real-time RT-PCR (rRT-PCR) assay and a recently developed lyophilised FMDV-typing assay (East Africa-specific for serotypes: O, A, Southern African Territories [SAT] 1 and 2). Laboratory evaluation showed that the analytical sensitivity of the lyophilised pan-FMDV and typing rRT-PCR assays were comparable to their laboratory-based equivalents. Furthermore, the FMDV-typing rRT-PCR produced serotyping data for 33 of 36 FMDV reference samples, with no cross-reactivity between serotypes detected. Both lyophilised rRT-PCR assays were able to accurately detect FMDV RNA across a large diagnostic clinical window of detection by being compatible with a range of clinical sample types, including epithelial tissue suspensions, serum, oesophageal-pharyngeal (OP) fluid and oral swabs, both with and without the use of nucleic acid extraction. When deployed in low-resource laboratory and field settings in Tanzania, Kenya and Ethiopia, the lyophilised pan-FMDV rRT-PCR was used to diagnose 144 samples collected from 78 cattle either clinical, convalescent or FMDV-negative). Field results were consistent with clinical observations and a reference laboratory-based rRT-PCR. During field evaluation, the FMDV-typing assay identified the serotype of all 24 FMDV-positive samples with a pan-FMDV C_T value of less than 29, rapidly confirming active outbreaks of both serotypes O and A. Throughout field evaluation, no false-positives were evident in either lyophilised assay for clinically normal animals. These data support the use of portable rRT-PCR platforms in non-specialised, resource-limited settings for simple, highly sensitive and rapid detection and/or characterisation of FMDV.

4.2 Introduction

The high sample throughput and analytical sensitivity associated with real-time reverse transcription PCR (rRT-PCR) have resulted in the technique being adopted by numerous diagnostic laboratories as a principal tool for the detection of veterinary pathogens (OIE, 2012). For instance, during the UK 2007 foot-and-mouth disease (FMD) outbreak, of 3246 clinical samples submitted to the UK National Reference Laboratory for FMD (The Pirbright Institute [TPI]), 99.1% were assayed using rRT-PCR, while only 21.8% were analysed by the "gold-standard" virus isolation assay (Reid *et al.*, 2009). However, the requirement for dedicated laboratory facilities, extensive equipment (nucleic acid extraction methods and thermocyclers) and highly trained personnel, impacts on the feasibility of using rRT-PCR in resource-limited settings and non-specialised facilities. The development of simple assays for FMD virus (FMDV) detection, monitoring and characterisation therefore remains an ongoing research effort.

As discussed in Chapter 1, there has been progress to transition rRT-PCR into formats suitable for use in decentralised settings (Belák *et al.*, 2010), with numerous publications detailing portable rRT-PCR platforms for FMDV detection (Donaldson *et al.*, 2001; Callahan *et* al., 2002; Hearps *et al.*, 2002; King *et al.*, 2008; Paixão *et al.*, 2008; Madi *et al.*, 2012; Ambagala *et al.*, 2016; Goller *et al.*, 2017). Furthermore, lyophilised pan-FMDV rRT-PCR assay kits are now available for a number of these platforms, increasing the compatibility with low-resource laboratory and field settings (Boyle *et al.*, 2004). However, current assay formats and platforms are limited by low sample throughput, the requirement for RNA extraction (and thus methods and equipment to perform this) or are commercially unavailable. Chapter 2 introduced a newly developed, commercially available, lyophilised pan-FMDV rRT-PCR assay (TC-9092-064; Tetracore Inc., MD, USA) which showed compatibility with crude sample preparation procedures, such as dilution of sample in nuclease-fee water (NFW). Additionally, this assay displayed diagnostic performance above that of alternative isothermal solutions. When combined with the T-COR™ 8 (Tetracore Inc.), a commercially available, portable, battery-powered thermocycler (Almassian *et al.*, 2013), this rRT-PCR assay offer a realistic possibility for decentralised FMD diagnosis, however further development and evaluation is required.

To date, evaluation of portable rRT-PCR platforms has only been performed using pan-FMDV assay formats. However, in order for effective FMD control mechanisms to be implemented (for instance selection of appropriate vaccine strains) it is important to fully understand the epidemiological context of the disease, including accurate identification

of the particular serotypes present (Sumption *et al.*, 2012). As such, there have been efforts to design serotype-specific FMDV rRT-PCR assays. By targeting variable capsid-coding regions of the FMDV genome, which display high sequence variability even within FMDV serotypes, assays can be regionally tailored to detect specific topotypes/strains (Giridharan *et al.*, 2005; Ahmed *et al.*, 2012; Reid *et al.*, 2014; Jamal and Belsham, 2015; Bachanek-Bankowska *et al.*, 2016; Knowles *et al.*, 2016). The transfer of these FMDV-typing rRT-PCR assays onto portable platforms could further strengthen the diagnostic capacity of resource-limited laboratories by offering a simple solution for rapid and improved FMDV characterisation.

This chapter evaluates the performance of a commercially available lyophilised pan-FMDV rRT-PCR assay, and a recently lyophilised East Africa-specific typing assay (Bachanek-Bankowska *et al.*, 2016), both performed on a commercially available portable thermocycler. In order to maximise the diagnostic clinical window of detection (points at which FMDV RNA can be detected following infection), it was important to evaluate these assays on a number of different sample types (epithelial tissue suspensions, serum, oesophageal-pharyngeal [OP] fluid). This chapter also evaluated the use of swabs in scenarios where it was not possible to collect epithelial material from ruptured lesions. Evaluation was performed within laboratory (UK, Kenya and Tanzania) and East African field settings (Kenya, Tanzania and Ethiopia), providing an approach that can both detect and type FMDV *in situ* using molecular methods.

4.3 Materials and Methods

4.3.1 Ethics statement

Clinical samples used in this chapter for laboratory evaluation were either archival samples generated in previous *in vivo* studies approved by The Pirbright Institute (TPI) Ethical Review Committee under the Animal Scientific Procedures Act (ASPA), or comprised of samples submitted by endemic countries to the to the Food and Agricultural Organization (FAO) World Reference Laboratory for FMD (WRLFMD) at TPI. Negative bovine epithelium was obtained from a UK abattoir as previously described (Chapter 2.3.1).

Field sampling in Tanzania was conducted as part of an ongoing research project under the Wellcome Trust Intermediate Fellowship (WT104017MA), which aligned with the standards set in the ASPA guidelines. Sampling in Kenya was carried out as part of a training programme run by The European Commission for the Control of Foot-and-Mouth Disease (EuFMD), with permission from the Kenyan Director of Veterinary Services. In Ethiopia, field sampling was carried out as part of The World Organisation for Animal Health (OIE) twinning project "Strengthening the capacity of foot-and-mouth disease diagnosis and surveillance in Ethiopia and East Africa".

4.3.2 Laboratory-based reference real-time reverse transcription PCRs

Reference pan-FMDV assay: The one-step rRT-PCR, used as the reference test, was performed as stated previously (Chapter 2.3.2). At TPI, rRT-PCR was performed on a bench top real-time PCR machine (Stratagene Mx3005P™: Agilent Technologies, CA, USA) using nucleic acid extracted using the MagMAX™-96 Viral RNA Isolation Kit (Applied Biosystems®, Thermo Fisher Scientific, MA, USA) following an automated procedure on a KingFisher™ Flex (Thermo Fisher Scientific). RNA was extracted from 50 µl sample and eluted in a final volume of 90 µl MagMAX™ elution buffer (Thermo Fisher Scientific).

In East African laboratories, RNA was extracted using the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany). RNA was extracted from 140 µl of sample and eluted in a final volume of 60 µl QIAamp® buffer AVE (Qiagen). rRT-PCR reactions were performed on either a PikoReal™ Real-Time PCR System (Thermo Fisher Scientific) (Foot-and-Mouth Disease Laboratory, Kenya) or Applied Biosystems® 7500 fast thermal cycler (Applied Biosystems®) (Sokoine University of Agriculture, Tanzania). It was not possible to benchmark in Ethiopia as there was no access to a laboratory-based rRT-PCR machine.

For all rRT-PCR assays, positive reactions were defined as those which gave a detectable cycle threshold (C_T) value.

Reference typing assay: The East-Africa specific typing rRT-PCR assay was performed as previously described (Bachanek-Bankowska *et al.*, 2016). Reactions were performed in duplicate using extracted nucleic acid (MagMAX™-96 Viral RNA Isolation Kit / KingFisher™ Flex) on a bench-top thermocycler (Stratagene Mx3005P™). This assay was selected as the primers designed by Bachanek-Bankowska *et al.* (2016) were tailored to detect FMD viruses currently circulating in East Africa, the setting for field evaluation in this chapter.

4.3.3 Lyophilised real-time reverse transcription PCRs

Pan-FMDV assay (lyophilised): Pan-FMDV rRT-PCR was performed in duplicate using lyophilised T-COR™ 8 (#2) reagents with inhibition control (TC-9092-064, Tetracore Inc., MD, USA) as stated previously (T-COR™ 8 [#2]: Chapter 2.3.3). Reactions were performed in duplicate on the T-COR™ 8 (Tetracore Inc.).

Typing assay (lyophilised): The East Africa-specific typing rRT-PCR (Tetracore Inc.) was performed using the same chemistry, thermal cycling conditions and parameters as the lyophilised pan-FMDV rRT-PCR (T-COR™ 8 [#2] reagents), with primers and probes as previously published in Bachanek-Bankowska *et al.* (2016). Probes for each serotype were modified for multiplex detection using the following fluorescence channels: O (Dragonfly Orange™ [DFO]), A (6-fluorescein amidite [FAM]), Southern African Territories (SAT) 1 (Cy®5) and SAT 2 (Texas Red® [TxR]). Reactions were performed in duplicate using the T-COR™ 8.

4.3.4 Laboratory evaluation of lyophilised rRT-PCR reagents

Initial determination of assay performance:

Analytical sensitivity (limit of detection [LOD]) was determined using FMDV RNA extracted from four separate decimal dilution series (10^{-1} to 10^{-8}), each consisting of an FMDV cell culture isolate diluted in negative bovine epithelial tissue suspensions (10% [w/v] in M25 phosphate buffer: 35 mM Na_2HPO_4, 5.7 mM KH_2PO_4, pH 7.6). The four cell culture isolates selected represented the serotypes currently circulating in East Africa and those targeted by the FMDV-typing assay: O/TAN/39/2012 (topotype East Africa-2); A/TAN/6/2013 (topotype AFRICA, lineage G-I); SAT 1/KEN/72/2010 (topotype I [North West Zimbabwe]);

SAT 2/KEN/2/2008 (topotype IV). RNA extraction was performed using the MagMAX™-96 Viral RNA Isolation Kit / KingFisher™ Flex (as described above in 4.3.2)

Diagnostic sensitivity of the pan-FMDV reagents had been previously evaluated and is described in Chapter 2.4.2. For the typing assay, diagnostic sensitivity was assessed using RNA extracted from 36 samples (serotypes O, A, SAT 1 and SAT 2) originating from East Africa (Samples from Tanzania and Kenya included in Appendix 5). These samples had been previously characterised by WRLFMD using antigen capture enzyme-linked immunosorbent assays (Ferris and Dawson, 1988) and sequencing of the VP1 region (Knowles and Samuel, 2003). The diagnostic specificity of the typing assay was determined using RNA extracted from original epithelial tissue suspensions of the following vesicular disease viruses: swine vesicular disease virus (UKG/24/1972; UKG/50/1972; UKG/51/1972; UKG/68/1972), vesicular stomatitis Indiana virus (VSIV) and vesicular stomatitis New Jersey virus (VSNJV).

Comparison of thermocyclers:

To ensure that neither the reagents nor the thermocycler used affected the performance of assays, RNA was extracted from a dilution series of cell culture isolate FMDV O/UAE/2/2003 in negative bovine epithelium (as previously described in Chapter 2.3.6). The extracted RNA was used as template in the reference and lyophilised pan-FMDV rRT-PCRs, performed both on a benchtop (Stratagene Mx3005P™) and portable (T-COR™ 8) thermocycler.

Determination of the diagnostic window for detection:

The diagnostic window of detection (time points post-infection when FMDV RNA can be detected within clinical samples) was determined using RNA extracted from unvaccinated intradermolingual needle inoculated cattle, challenged with FMDV isolate A/TAI/17/2016 (topotype ASIA, lineage Sea-97). Archival samples comprised of serum (n = 11) and mouth swabs (n = 11) taken daily from two animals from the day of challenge, and epithelium (n = 4) and OP fluid (n = 2) collected from the same animals post-mortem (carried out on the day of culling). These samples were also used to further evaluate the simple sample preparation procedures (dilution of samples in NFW) developed in Chapter 2 (Chapter 2.3.7) on clinical samples.

4.3.5 Field evaluation of lyophilised rRT-PCR reagents

Field work was carried out in FMD endemic settings, which were selected on the basis of existing collaborations with investigators working on the epidemiology of FMD. Samples were collected opportunistically from locations where a local animal health worker or farmer reported the presence (or recent presence) of clinical signs consistent with FMDV infection. Samples (n = 144) from 78 individual cattle over 13 farms across East Africa were analysed *in situ*. The number of samples collected was the maximum amount that could be logistically collected in terms of livestock availability and the working time frame. This comprised of 13 cattle from two small holdings in Kericho County (Kenya, June 2016), 16 cattle from two small holdings in Nakuru County (Kenya, June 2016), 43 cattle from seven small holdings in Morogoro Rural and Mvomero Districts (Morogoro Region, Tanzania, September 2016) and six cattle from two small holdings in Adama (Ethiopia, October, 2016) (Figure 4.1, Appendix 6). The samples collected (n = 9) and rRT-PCR reactions performed in Ethiopia were by carried out by Dr Veronica Fowler (TPI).

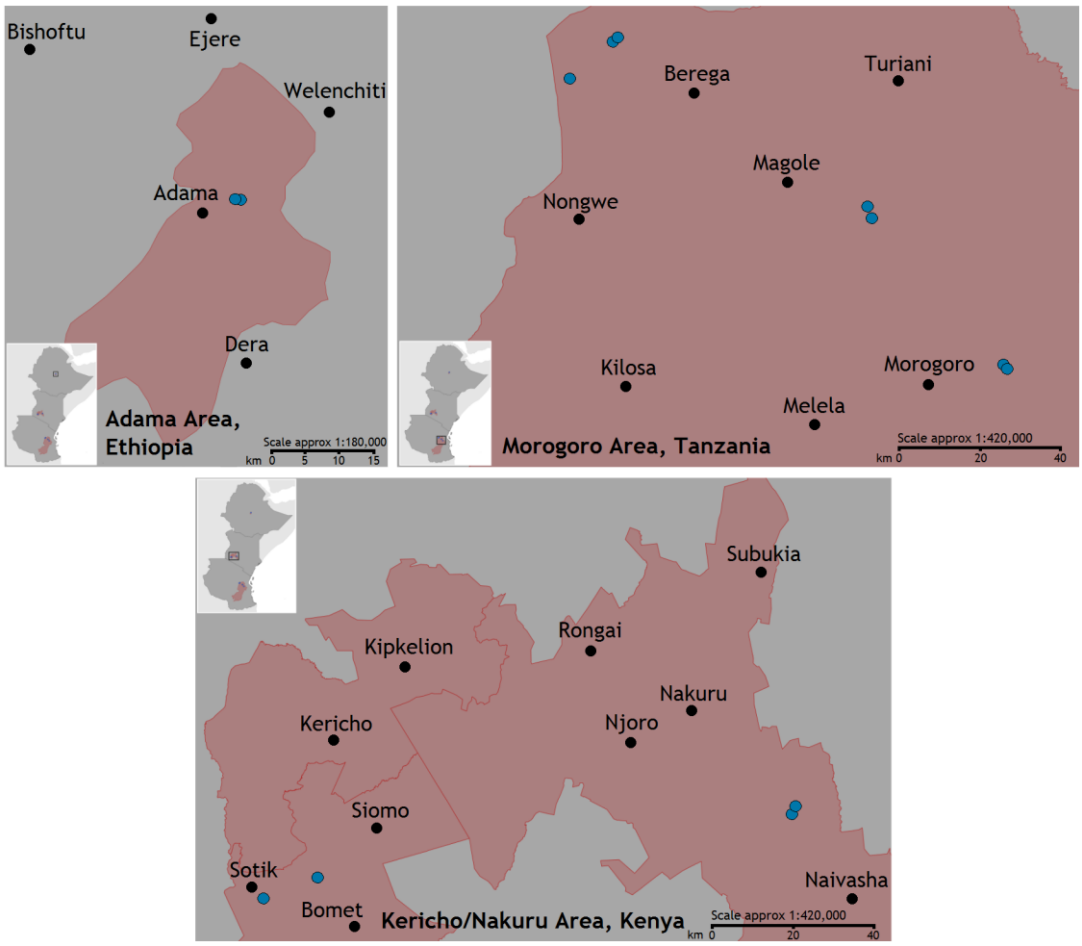


Figure 4.1 Sampling locations in East Africa. Blue points represent the location of each small holder; green shading represents national parks; red shading represents counties. Field sampling locations are representative of FMDV pool 4.

Samples were collected from cattle at different stages of infection (acute, convalescent and recovered) and included one or more of the following sample types per animal: serum, swab, OP fluid and mouth/foot epithelium (Appendix 6). For each animal, the time since the start of clinical signs was estimated based on aging of the oldest lesion (using FAO guidelines [DEFRA, 2005]) and known clinical history (provided by livestock owners and veterinary officers). Cattle from the same herds that had no history or clinical signs of FMD at the time of the visit were also opportunistically sampled (n = 12). Samples were collected and processed as follows:

Loose epithelial tissue: Epithelial tissue of ruptured vesicular lesions from either the mouth or the feet was prepared using the SVANODIP® FMDV-Ag Extraction Kit and SVANODIP® FMDV-Ag LFD kit (Boehringer Ingelheim, Bracknell, UK) as previously described (Chapter 3.3.8). Epithelial samples from the feet (interdigital space/coronary band) were briefly washed in sterile water prior to processing to remove soil contaminants. The homogenate was left to settle for one minute, and then the supernatant was removed and diluted 1 in 10 in NFW prior to analysis using lyophilised rRT-PCR.

Swabs: When collection of epithelial material was not possible (e.g. no loose epithelium present), the surface of ruptured lesions in the mouth or on the feet (interdigital space/coronary band) were swabbed (GenoTube Livestock: Thermo Fisher Scientific) (Figure 4.2). The feet were cleaned in sterile water (as above) prior to swabbing to remove soil contaminants. Swabs were agitated by hand in 1 ml NFW, which was then used directly in analysis using lyophilised rRT-PCR.

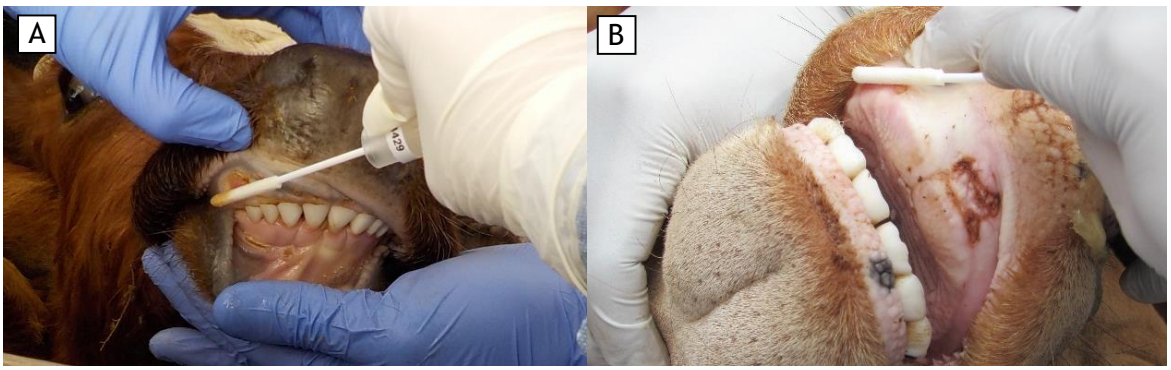


Figure 4.2 Lesion swab sampling of two cattle displaying clinical signs characteristic of foot-and-mouth disease (Nakuru, Kenya). Swabs were taken in these instances due to the absence of any loose epithelial material on the lesion sites: (A) ruptured lesion on the gum; (B) ruptured and healing lesion on the dental pad.

OP fluid: OP fluid was collected using a suitably sized probang cup following the guidelines within the diagnostic manuals of the OIE (2012) and diluted 1 in 10 in NFW prior to analysis using lyophilised rRT-PCR.

Serum: Blood (10 ml) was collected (by veterinarians) from either the jugular or tail vein using Vacutainer® Plus Plastic Serum Tubes (BD, Plymouth, UK), or similar. Samples were transported back to a local laboratory and centrifuged at 3000 x g for 10 minutes. Serum was removed and diluted 1 in 10 in NFW prior to analysis.

4.3.6 Biosafety procedures in the field

During field work, a biosafety boundary was established and biosafety procedures followed as previously stated in Chapter 3 (3.3.9). Between runs and farms the T-COR™ 8 was surface disinfected (with either citric acid [0.2% w/v] of FAM® 30 [1:240]) and sprayed with DNAZap™ PCR DNA Degradation Solutions (Thermo Fisher Scientific). At the end of field work the T-COR™ 8 was surface disinfected (as above) prior to fumigation with formaldehyde (10 mg/m^3) at 30°C (relative humidity > 70 for > 60 minutes). Samples for concordance testing were added to MagMAX™-96 Viral RNA lysis buffer within the contaminated area, surface disinfected (as above) for transfer to the uncontaminated area and transported (double-contained) to appropriate local laboratory facilities on ice.

4.3.7 Statistical analysis

Cohen's Kappa statistic (κ) and the proportion of observed agreement (A_{obs}) were used to measure the agreement between diagnostic tests. Statistical analyses were performed using R (R Core Team, 2014).

4.4 Results

4.4.1 Laboratory evaluation of lyophilised rRT-PCR reagents

Initial determination of assay performance:

The analytical sensitivity of the lyophilised pan-FMDV reagents, performed on the T-COR™ 8, was equivalent to the reference rRT-PCR across the four serotypes tested (both consistently detected down to 10^{-6} for each serotype) (Figure 4.3). The internal control was positive in all lyophilised pan-FMDV assays. For the typing assay, analytical sensitivity was reduced in comparison to the pan-FMDV reagents, with detection of serotypes A and SAT 1 showing a one $\log_{10}$ reduction, and detection of O and SAT 2 displaying a two $\log_{10}$ reduction in LOD (Figure 4.3).

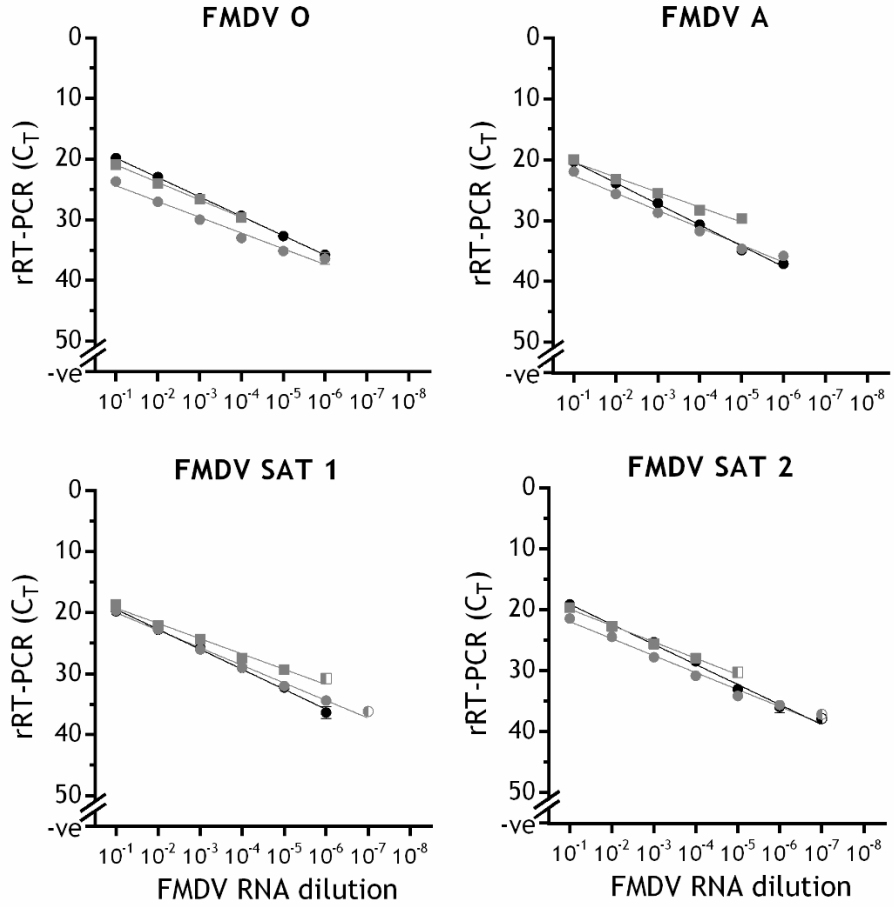


Figure 4.3 Analytical sensitivity of lyophilised reagents in comparison to the reference real-time reverse transcription PCR (rRT-PCR). RNA was extracted from four separate dilution series of foot-and-mouth disease virus (FMDV) cell culture isolates: O/TAN/39/2012; A/TAN/6/2013; Southern African Territories [SAT] 1/KEN/72/2010; SAT 2/KEN/2/2008. (●) reference rRT-PCR performed on a benchtop thermocycler; (◐) pan-FMDV lyophilised reagents performed on the T-COR™ 8; (■) typing lyophilised reagents performed on the T-COR™ 8. Points represent the mean of two replicates; half-shaded points represent that of the replicates, one was positive and the other negative; error bars indicate the range.

Using the same four FMDV isolates, lyophilised typing reagents (multiplex format) were compared to their wet equivalents (singleplex format) (Bachanek-Bankowska *et al.*, 2016) (Figure 4.4). The analytical sensitivity was comparable across all four serotypes, with all disagreements limited to one of the two replicates (Figure 4.4).

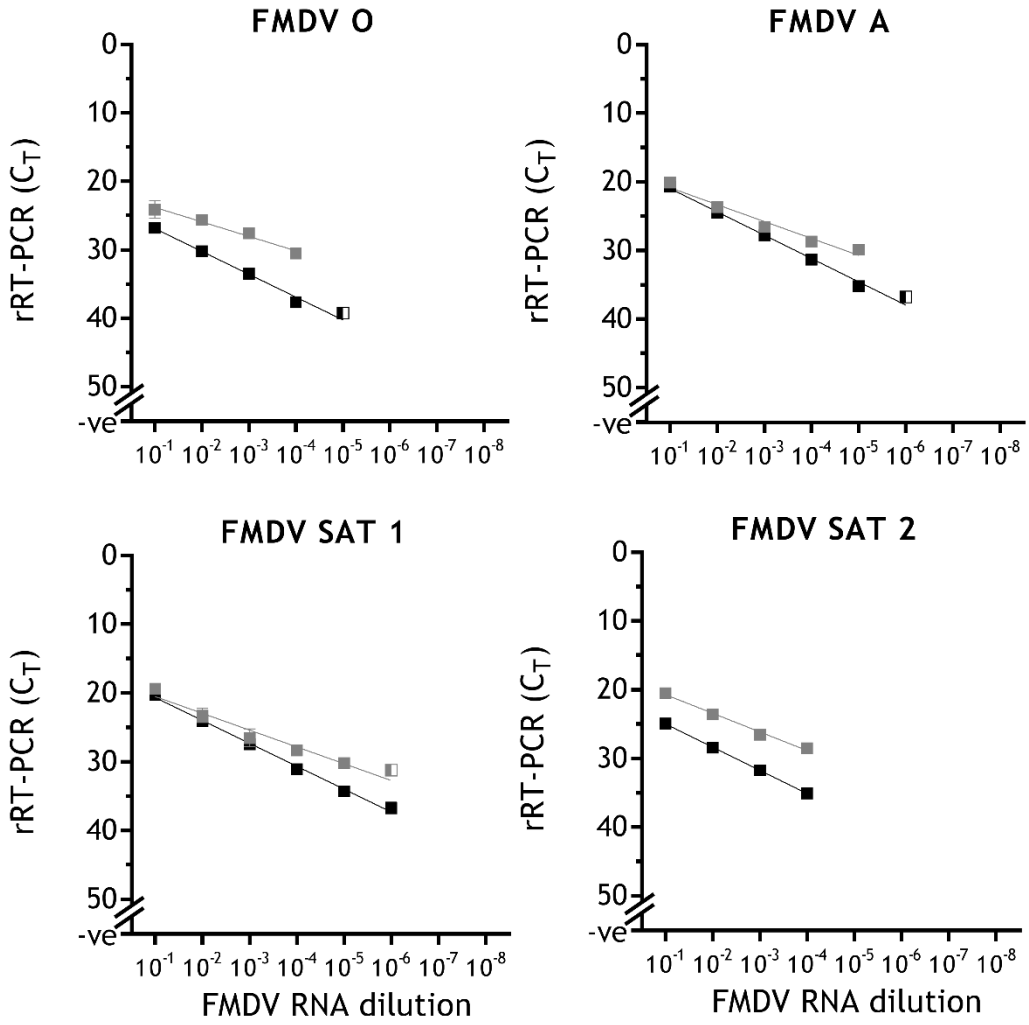


Figure 4.4 Analytical sensitivity of lyophilised East Africa-specific typing reagents in comparison to the reference typing real-time reverse transcription PCR (rRT-PCR). Assays were performed on RNA extracted from four dilution series of foot-and-mouth disease virus (FMDV) cell culture isolates: O/TAN/39/2012; A/TAN/6/2013; Southern African Territories [SAT] 1/KEN 72/2010; SAT 2/KEN/2/2008). (■) reference typing rRT-PCR performed on a benchtop thermocycler (Bachanek-Bankowska *et al.*, 2016); (■) lyophilised typing reagents performed on the T-COR™ 8. Points represent the mean of two replicates; half-shaded points represent that of the identical replicates, one was positive and the other negative. The error bars indicate the range.

Diagnostic sensitivity of the lyophilised pan-FMDV assay was assessed in Chapter 2 across 43 FMDV-positive samples (previously diagnosed by the WRLFMD) (Chapter 2.4.1), with 100% concordance evident with the reference rRT-PCR. Diagnostic sensitivity of the lyophilised serotype-specific assay was assessed using 36 FMDV-positive clinical field samples (previously diagnosed by the WRLFMD), originating from East Africa and six epithelial tissue suspension samples representing viruses that cause similar characteristic lesions to FMDV. These included swine vesicular disease virus (SVDV: UKG/24/1972; UKG/50/1972; UKG/51/1972; UKG/68/1972) and vesicular stomatitis virus (VSV: Indiana 1 [VSIV]; New Jersey [VSNJV]). Of the FMDV-positive samples, the serotype-specific typing assay detected 7/8 serotype A, 9/10 serotype O, 7/7 serotype SAT 1 and 10/11 serotype SAT 2 samples (Appendix 5). The three samples for which no serotype was detected generated high C_T values on the reference rRT-PCR (values of 35.64, 27.18 and 30.65) (Figure 4.5) and lyophilised pan-FMDV rRT-PCR (values of 35.50, 29.85 and 32.15). No cross-reactivity among serotypes was observed for any of the clinical samples tested, and the serotype-specific assay yielded negative results against SVDV, VSNJV and VSIV isolates (Appendix 5).

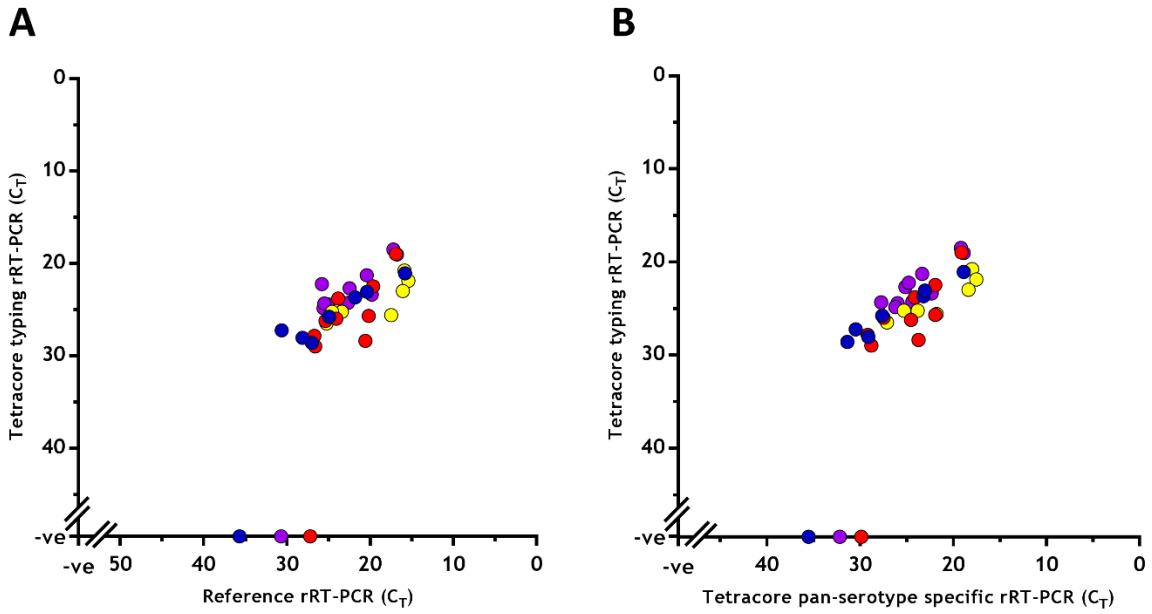


Figure 4.5 Comparison between lyophilised typing reagents and pan-FMDV (foot-and-mouth disease virus) real-time reverse transcription PCR (rRT-PCR). Diagnostic sensitivity was assessed using RNA extracted from a panel of 36 FMDV-positive clinical field samples from East Africa. (A) Comparison against the reference rRT-PCR; (B) Comparison against lyophilised rRT-PCR. The colour of points indicates serotype: (blue) A; (red) O; (yellow) Southern African Territories (SAT) 1; (purple) SAT 2. For both graphs, points represent the mean of two replicates.

Comparison of thermocyclers:

Neither the pan-FMDV reagents (reference rRT-PCR or lyophilised rRT-PCR) nor the platform used (Stratagene Mx3005P™ or T-COR™ 8) affected analytical sensitivity. Both reagents consistently detected down to 10^{-5} of an FMDV RNA dilution series using both platforms (Figure 4.6). The internal control was detected in all lyophilised pan-FMDV assays, on both thermocyclers.

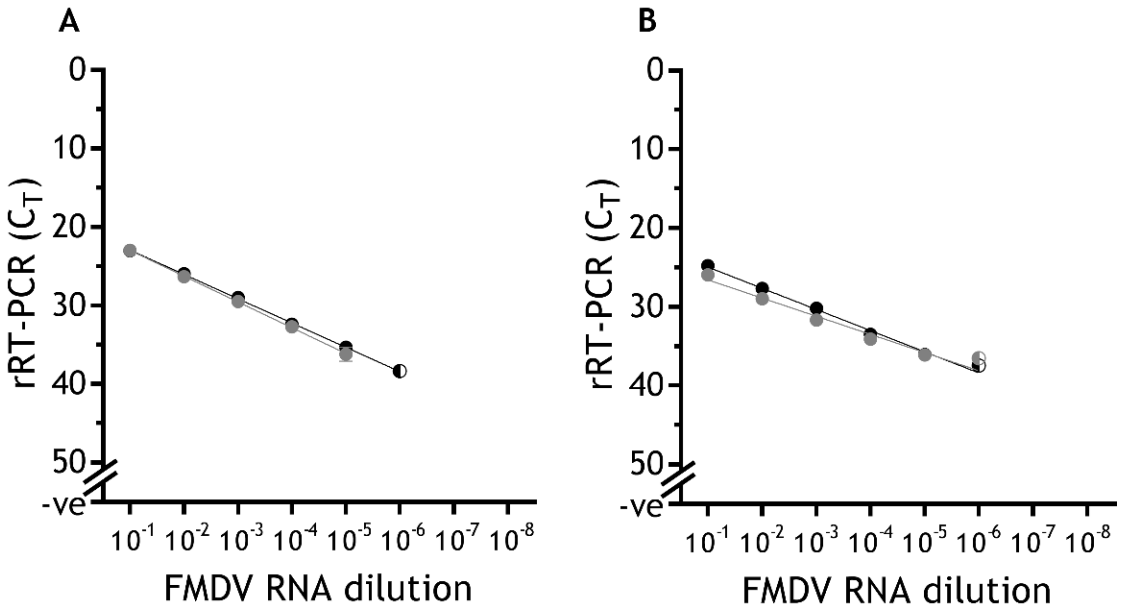


Figure 4.6 Comparison between real-time reverse transcription PCR (rRT-PCR) reagents and thermocycling platforms. The comparison was performed using a dilution series of foot-and-mouth disease virus (FMDV) RNA (isolate O/UAE/2/2003). (A) Reactions performed on a bench-top thermocycler (Stratagene Mx3005P™); (B) Reactions performed on the T-COR™ 8. (●) reference rRT-PCR; (◐) pan-FMDV lyophilised rRT-PCR. Points represent the mean of two replicates (bars indicate range); half-shaded points represent that of the identical replicates, one was positive and the other negative.

Determination of the diagnostic window for detection:

For determination of the clinical detection window (for the lyophilised pan-FMDV reagents), FMDV RNA was detected in serum from one to four days post-challenge, mouth swabs from two days post-challenge onwards and in all epithelium and OP fluid samples collected post-mortem when RNA extraction was performed (Figure 4.7). Using the same reagents, the use of simple sample preparation procedures (using 1 in 10 dilutions of un-extracted clinical samples in NFW) enabled FMDV RNA to be detected in 26/28 FMDV-positive samples (defined using the reference rRT-PCR with extracted RNA). The two discordant samples (false-negatives) had C_T values of 35.3 (serum four days post-challenge) and 29.0 (OP fluid taken post-mortem) when using extracted RNA in the

lyophilised pan-FMDV rRT-PCR (Figure 4.7). In comparison, when using simple sample preparation procedures (1 in 10 dilution in NFW) for analysis with the reference rRT-PCR, FMDV RNA was detected in only 19/28 FMDV-positive samples (data not shown).

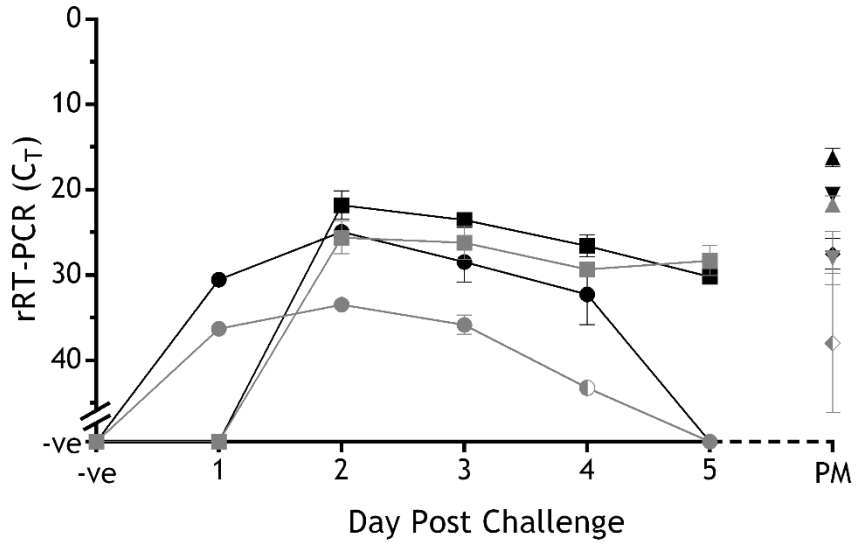


Figure 4.7 Determination of the clinical detection window for lyophilised pan-FMDV (foot-and-mouth disease virus) real-time reverse transcription PCR reagents. Assays performed on extracted RNA: (●) serum; (■) mouth swabs; (▲) foot epithelium; (▼) mouth epithelium; (♦) oesophageal-pharyngeal (OP) fluid. Assays performed on samples diluted 1 in 10 in nuclease-free water: (●) serum; (■) mouth swabs; (▲) foot epithelium; (▼) mouth epithelium; (♦) OP fluid. Points represent the mean of two animals, each with two replicates; half-shaded points represent that of the identical replicates, one was positive and the other negative; error bars indicate the standard deviation. (PM) samples collected post-mortem (animals were culled at either 5 or 6 days post challenge).

4.4.2 Field evaluation of lyophilised rRT-PCR reagents

An initial comparison of T-COR™ 8 reagents was performed to ascertain whether the T-COR™ 8 (#1) reagents were also outperformed by the newer T-COR™ 8 (#2) in field settings (evident in laboratory settings in Chapter 2.4.2). This evaluation was performed in Kenya, across 41 samples (epithelium [n = 6]; serum [n = 24]; lesion swab [n = 10]; OP fluid [n= 1]) (Figure 4.8). For FMDV detection, 40/41 samples were in agreement between the two sets of reagents (T-COR™ 8 [#1] reagents did not detect FMDV RNA in one serum sample from a cow with acute FMD infection). For T-COR™ 8 (#2) reagents, the internal control was amplified in all samples. For T-COR™ 8 (#1) reagents, five FMDV-positive epithelial samples had negative internal control results (in these samples FMDV RNA detection displayed low C_T values between 16.0 and 19.7). As such, all further analysis in the field was performed using T-COR™ 8 (#2).

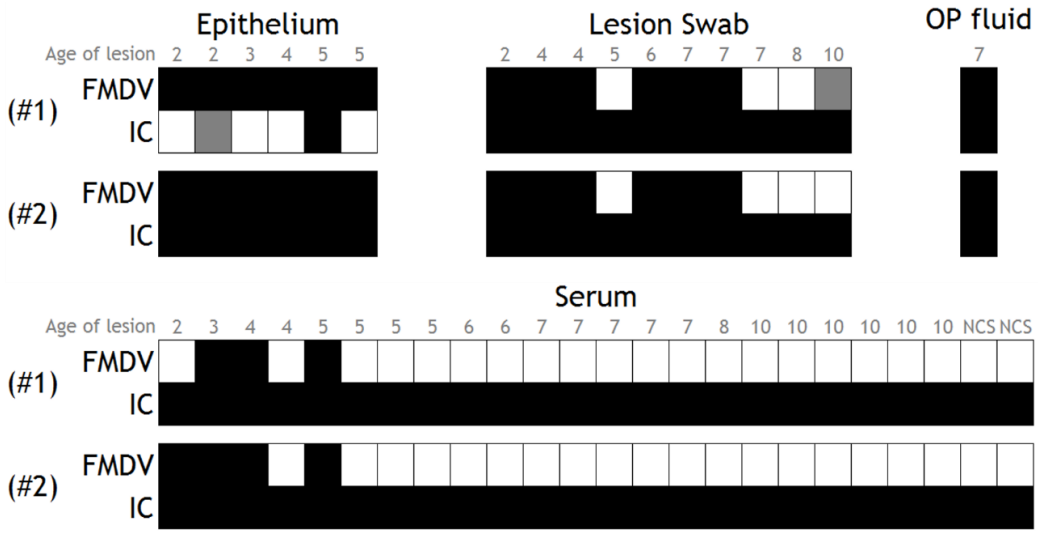


Figure 4.8 Comparison between T-COR™ 8 (#1) and (#2) real-time reverse transcription PCR (rRT-PCR) reagents in field settings using simple sample preparation methods (1 in 10 dilution in nuclease-free water). Squares represent the mean of two replicates: (black) both positive; (grey) one replicate was positive; (white) both negative. rRT-PCR reactions were multiplexed for detection of foot-and-mouth disease virus (FMDV) RNA and an exogenous internal control (IC). Each column represents one sample, numbers in grey indicate the approximate age of the oldest lesion; (NCS) no clinical signs.

The T-COR™ 8 and the rRT-PCR protocols (using T-COR™ 8 [#2] reagents) developed in the laboratory were then taken forward for subsequent field testing, over 144 samples from 78 cattle across 13 farms in East Africa (Figure 4.9).

Figure 4.9 Setting up the T-COR™ 8 real-time reverse transcription PCR in field settings (two small-holdings in Kenya).

Using the pan-FMDV lyophilised rRT-PCR reagents in combination with the T-COR™ 8, FMDV RNA was identified in 5/5 epithelial, 1/1 swab and 3/3 serum samples collected

from cattle 1-3 days post initial FMDV infection. Of the cattle 4-7 days post FMDV infection, FMDV RNA was identified in 13/14 epithelial, 15/19 swab, 3/4 OP fluid and 3/29 serum samples collected. Of the cattle where initial FMDV infection occurred 8+ days previously (based on recent clinical history and aging of the oldest lesion), FMDV RNA was identified in 3/12 OP fluid samples, while all sera (n = 25) and swab (n = 10) samples were negative. FMDV was not detected in any OP fluid (n = 8), mouth swab (n = 2) or sera (n = 12) samples collected from the 12 clinically normal cattle (Figure 4.10, Appendix 6). The internal control was amplified in 143/144 samples (The negative result was derived from a FMDV-positive lesion swab).

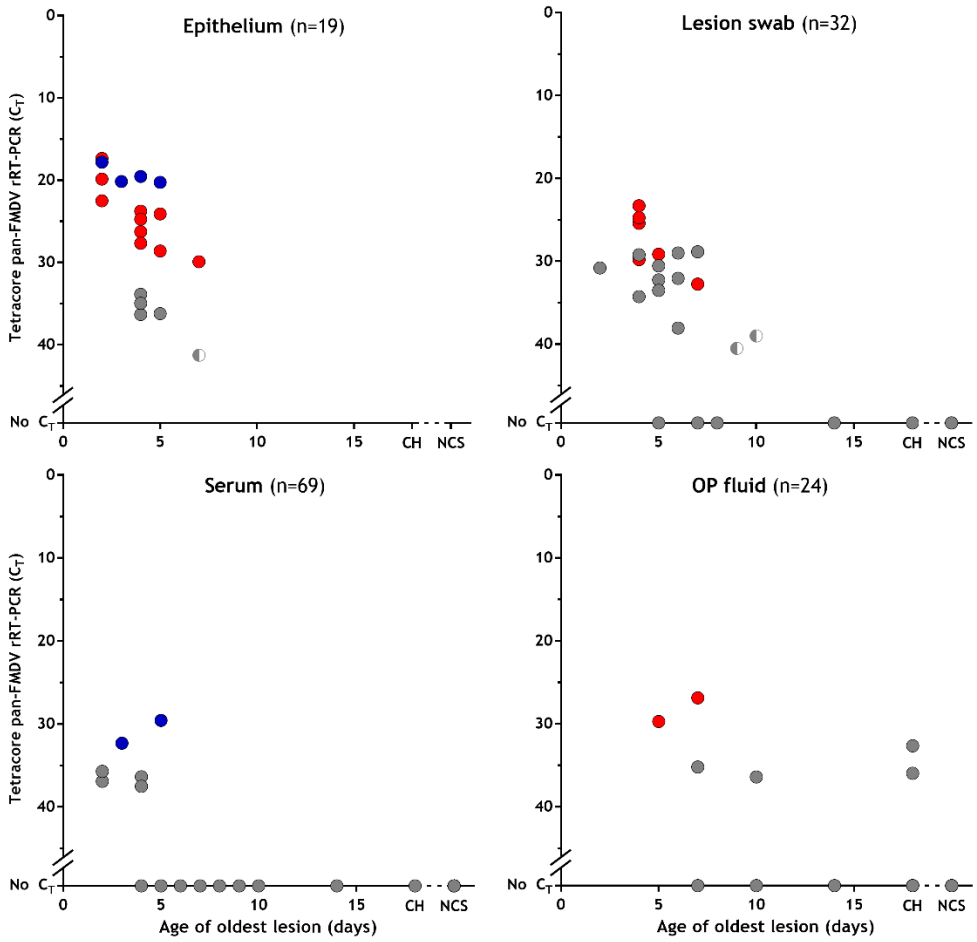


Figure 4.10 *In situ* real-time reverse transcription PCR (rRT-PCR) results for 144 East African samples using lyophilised rRT-PCR reagents (T-COR™ 8). Samples were collected from cattle displaying clinical signs of foot-and-mouth disease (FMD), from cattle with a clinical history (CH) of FMD in the last two months, and cattle with no clinical signs or recent clinical history within the last two months (NCS). Each point represents the mean C_T for a single sample (tested in duplicate) on the pan-FMD virus rRT-PCR using simple sample preparation procedures (1 in 10 dilution in nuclease-free water); half-shaded points indicate that out of the replicates, one was positive and the other negative (samples that share the same C_T will only appear as a single point with individual C_T values presented within Appendix 6). Positive samples were then tested using the lyophilised typing assay, the colour represents the serotype detected: (blue) A; (red) O; (grey) serotype not detected.

Samples considered positive by the lyophilised pan-FMDV assay (n = 46), in addition to a selection of samples from cattle considered clinically normal (n = 7), were subsequently characterised using the lyophilised typing assay (using 1 in 10 dilutions in NFW for sample preparation), which has been developed to detect all known FMDV strains relevant to this region (Bachanek-Bankowska *et al.*, 2016). Of these, 24 were identified as either A or O (Figure 4.11). No amplification was present for samples collected from clinically normal cattle (Figure 4.10, Appendix 6). FMDV-positive samples where the serotype was not detected (n = 22) had pan-FMDV C_T values of > 29 (using lyophilised reagents) (Figure 4.10, Appendix 6).

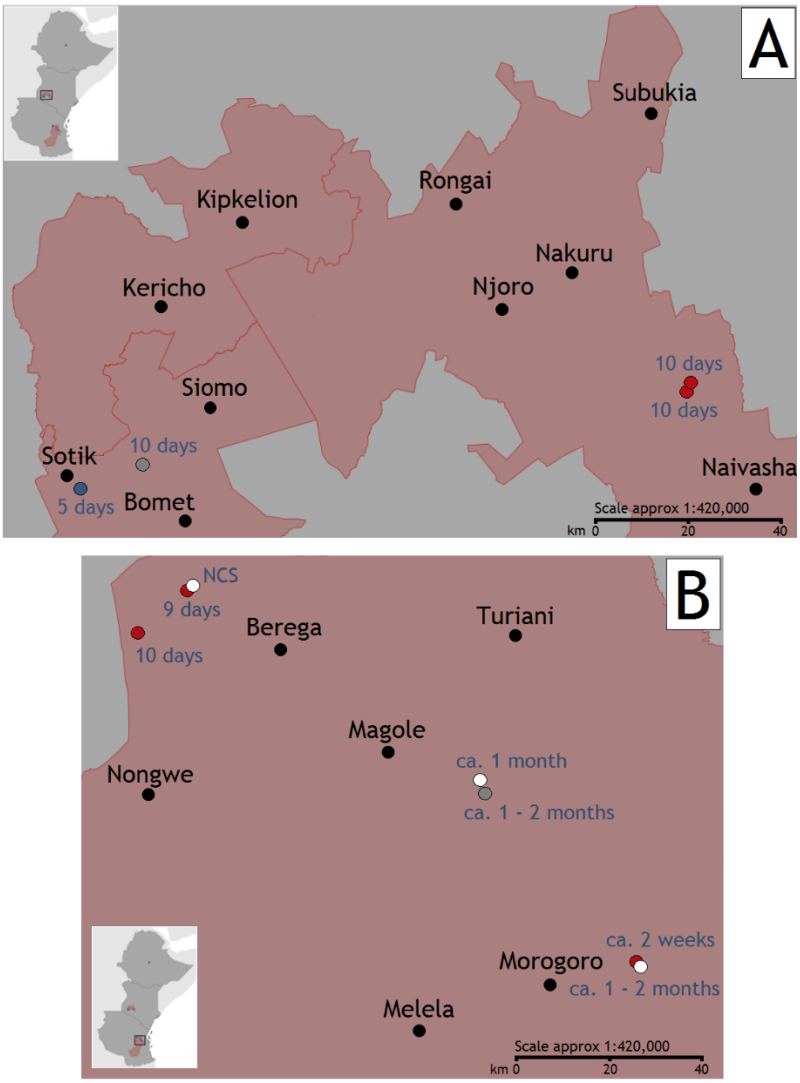


Figure 4.11 Epidemiological tracing of foot-and-mouth disease (FMD) outbreaks in East Africa. Red shading represents regions/counties. (A) Kericho/Nakuru Area, Kenya (June 2016); (B) Morogoro Area, Tanzania (September 2016). Points represent the small-holders sampled: (white) samples negative for FMD; (grey) positive for FMD but serotype could not be detected; (red) positive for FMD, serotype O; (blue) positive for FMD, serotype A. The dates given indicate the estimated time since the start of clinical signs; (NCS) no clinical signs.

For comparison, 99 of the samples tested using the lyophilised pan-FMDV rRT-PCR (using the T-COR™ 8 with dilution in NFW for sample preparation) were also tested using the reference rRT-PCR (using extracted RNA) within local laboratory settings in East Africa. High agreement was apparent (κ = 0.864, p < 0.001, A_{obs} = 0.936) between pan-FMDV assays in terms of positive and negative test results (Figure 4.12), with all discordant results between tests having C_T values of > 36 (Appendix 6), which is at the threshold of the analytical sensitivity for both tests.

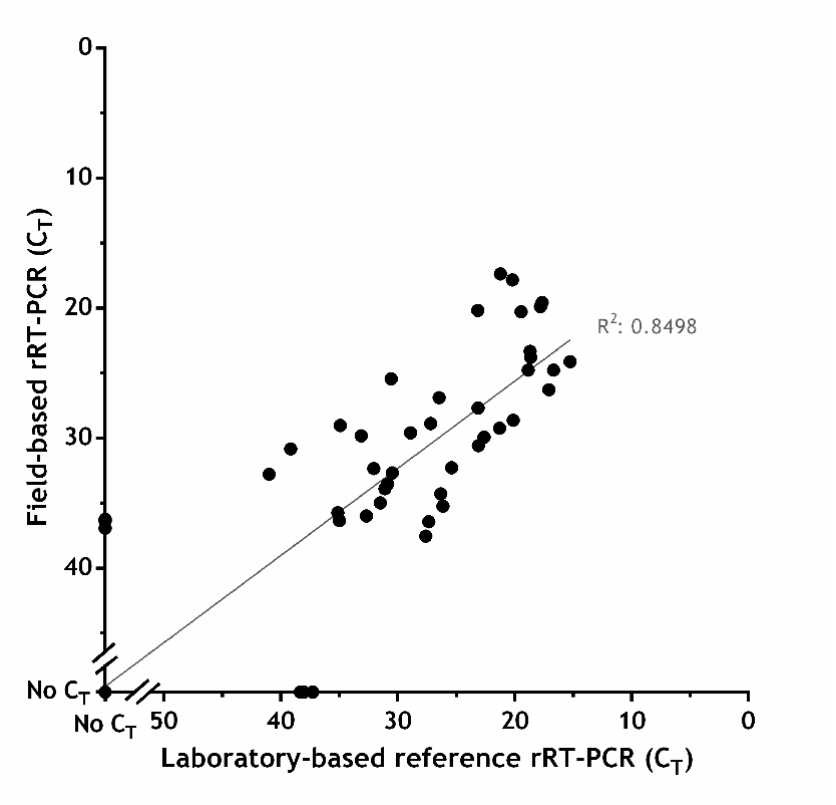


Figure 4.12 Comparison between real-time reverse transcription PCR (rRT-PCR) field and laboratory results. Field assays were performed using lyophilised pan-FMDV (foot-and-mouth disease virus) rRT-PCR (using dilution in nuclease-free water for sample preparation) and laboratory assays were performed using the reference rRT-PCR performed in local laboratories (using extracted RNA). Points represent the mean of two replicates.

4.5 Discussion

Throughout the ongoing research effort to develop point-of-care tests for the diagnosis of FMD, a number of publications have detailed the transfer of FMDV-specific rRT-PCR assays onto portable detection platforms (Hearps *et al.*, 2002; Paixão *et al.*, 2008; Madi *et al.*, 2012; Howson *et al.*, 2015;). However, current assays are either not commercially available or are suitable for research purposes only (i.e. not diagnostic use). This chapter evaluates the performance of commercially available, lyophilised FMDV-specific assays, in combination with a commercially available portable thermocycler, in laboratory and decentralised settings within East Africa (low-resource laboratory and field settings). Such tests have the potential to contribute valuable epidemiological information to support country-level and regional control programs, such as the Progressive Control Pathway for FMD (Sumption *et al.*, 2012).

As previously discussed in Chapter 3, the provision of reagents in a lyophilised kit format simplifies reagent storage by negating the requirement for a cold chain, whilst minimising the requirement for skilled personnel and multiple pipetting stages by streamlining assay set up (assays only require the addition of a re-suspension buffer and the test sample). The lyophilised pan-FMDV reagents, in combination with the T-COR^{TM} 8, maintained comparable diagnostic performance to the reference rRT-PCR when evaluated with extracted RNA. Furthermore, these reagents were able to detect FMDV RNA from multiple clinical sample types, in the presence or absence of nucleic acid extraction, as early as one day post-infection. Consequently, they offer a potential solution for molecular-based diagnosis in field settings.

Although T-COR^{TM} 8 (#1) and (#2) reagents displayed comparable sensitivity for FMDV detection in the field, in concordance with Chapter 2, amplification of the internal control was consistently improved using T-COR^{TM} 8 (#2) reagents. Due to the requirement to confirm that FMDV-negative reactions contain an undetectable amount of FMDV RNA as opposed to being a result of amplification inhibition, T-COR^{TM} 8 (#2) reagents were selected for further evaluation. Throughout field validation of the T-COR^{TM} 8 in East Africa, these pan-FMDV lyophilised reagents generated results consistent with clinical observations, with FMDV detected in samples from the early onset of infection through to delayed viral clearance. Results were gained in less than 1.5 hours from sample collection. Furthermore, high concordance was evident between results gained in the field (T-COR^{TM} 8 rRT-PCR using dilution of samples in NFW) and local East African laboratories (reference rRT-PCR using RNA extraction), with any disagreements

associated with samples that had C_T values above the diagnostic threshold of $C_T < 32$ (Shaw *et al.*, 2007).

Results in this chapter demonstrated that when epithelial material could not be collected from ruptured lesions, swabbing the surface of the lesions was sufficient for detecting the presence of FMDV, extending the diagnostic window of detection from that shown in Chapter 3 (increasing the time points in which FMD can be detected). This is important as during outbreaks of FMD and disease surveillance, it is unlikely that all animals would be examined during the acute clinical phase (where epithelial samples are most appropriate).

Although the provision of simple FMDV positive/negative results is sufficient for the confirmation of FMD during outbreaks, the value of this information is limited in countries where FMD is endemic. In these situations, it is beneficial to characterise circulating FMDV outbreaks in order to make tailored control programs, including vaccination-based strategies, a realistic possibility (Namatovu *et al.*, 2013). In support of this, this chapter evaluated a lyophilised version of a published East Africa-typing specific assay (Bachanek-Bankowska *et al.*, 2016). Lyophilisation and multiplexing of the assay had limited effect on the analytical sensitivity of the FMDV typing assay, however the sensitivity of these reagents was reduced in comparison to the pan-FMDV rRT-PCR assays.

In field settings, the lyophilised typing assay was able to characterise FMDV present in four different sample types, collected from seven small holdings (three in Kenya, three in Tanzania and one in Ethiopia), where cattle presented two to seven day old FMD lesions. Samples which were unable to be typed (n = 22) were all considered as weak positives by the lyophilised pan-FMDV rRT-PCR ($C_T > 29$) and therefore at the threshold at which characterisation information can be routinely obtained by sequencing. The typing assay performance in field settings is consistent with the ability to obtain characterisation data within laboratory settings. Two serotypes were detected during the period of testing (O and A), leading to the first reported characterisation of FMDV in field-based settings using molecular methods. Furthermore, the preliminary FMD surveillance data collected revealed interesting epidemiological patterns, for example in June 2016, two separate FMD outbreaks were evident in the Kericho and Nakuru areas of Kenya, with FMDV O and A serotypes detected 120 km apart.

In conclusion, this chapter describes the laboratory and field evaluation of lyophilised FMDV-specific rRT-PCR assays in combination with a portable thermocycler. In addition to providing a means to rapidly confirm cases of FMD on or close to the farm during

outbreaks, these technologies could potentially provide a route for low-resource laboratories to establish robust field/laboratory testing capacity, thereby providing support to programmes that monitor and control FMD. The simplicity of the T-COR™ 8 to operate, combined with robust lyophilised reagents, high sensitivity and compatibility with simple sample preparation methods, demonstrate an important transition for FMDV-specific rRT-PCR assays into formats suitable for use in decentralised, resource-limited settings.

CHAPTER 5

Using sequence data to predict the performance of real-time PCR assays

Results from this chapter have been presented at:

EuFMD Research Group Open Session 2016, Cascais, Portugal. Oral presentation: GoPrime: *In silico* evaluation of real-time PCR primers and probes for detection of foot-and-mouth disease virus.

5.1 Summary

Real-time PCR (rPCR) has become a widely accepted diagnostic tool for the detection and quantification of nucleic acid. In order for these assays to achieve high sensitivity and specificity, primer/probe-template complementarity is essential, however mismatches are often unavoidable and can result in false-negative results and errors in quantifying target sequences. Consequently, primer and probe sequences require continual evaluation to ensure they remain fit for purpose (especially when considering RNA viruses with a high mutation rate). The effect of mismatches on rPCR has been widely studied, however data has only currently been used to advise on primer design. Following further investigation into primer/probe-template mismatches, this chapter describes the use of experimental data for predicting the performance of rPCR assays. Empirical data generated to investigate the effects of different primer/probe-template mismatches, using foot-and-mouth disease virus (FMDV) as a model, demonstrated that their effects on rPCR cycle threshold (C_T) and limit of detection (LOD) followed a consistent pattern in terms of mismatch type and position. For example, the most deleterious mismatches occurred at the 3'-end of primers (a single G-A mismatch in the final nucleotide resulted in a change in C_T of 11.57), with a maximum of two mismatches being tolerated in this region before amplification was completely inhibited. For the probe, single mismatches at the 5'-end had almost no effect on C_T, with single mismatches throughout the rest of the probe impacting rPCR C_T by 1.63 - 3.38 (four or more mismatches across the probe inhibited rPCR). A linear model was used to calculate the effect of different mismatches on the C_T and LOD of reactions, results of which became the parameters of GoPrime: a mathematical model designed to predict the performance of rPCR primers and probes across multiple sequence data. Evaluation of GoPrime was performed using a set of experimental rPCR data, using DNA oligonucleotides designed to vary in the primer/probe target regions as template (n = 97). GoPrime on average predicted the impact of mismatches on C_T to be 3.15 (SD 2.23) away from the observed value, and impact of mismatches on LOD to be 1.58 (SD 0.76) away from the observed value. GoPrime was also applicable to other areas of the FMDV genome, with predictions for the likely targets of a FMDV-typing assay consistent with published experimental data. These data support the use of mathematical models for rapidly predicting the performance of rPCR primers and probes *in silico*.

5.2 Introduction

Real-time PCR (rPCR) has become an essential tool in molecular biology and is routinely used for detection, quantification and differentiation of nucleic acids in both research and diagnostic settings (Mackay *et al.*, 2002; Espy *et al.*, 2006). Central to the specificity and sensitivity of rPCR assays are the primers and probes, with amplification affected by factors such as primer/probe-template complementarity and the presence of secondary structures (e.g. primer dimers) (Cha and Thilly, 1992). However, designing primers and probes with full sequence complementarity to all of the required targets can be problematic. For instance, when considering RNA viruses such as foot-and-mouth disease virus (FMDV), the high mutation rate (in the range of 10^{-3} to 10^{-5} per nucleotide site, per genome replication [Domingo *et al.*, 1985; Drake and Holland, 1999]), can result in fully conserved regions being too short to accommodate primer/probe sets. This is especially true when designing assays to target the more varied genomic regions for serotype/strain differentiation (Bachanek-Bankowska *et al.*, 2016). Consequently, primer/probe-template mismatches are often unavoidable and a compromise approach that accommodates sequence mismatches is often adopted to design diagnostic tests.

The effects of mismatches on PCR amplification have been well studied and quantified for both primers (Kwok *et al.*, 1990; Huang *et al.*, 1992; Christopherson *et al.*, 1997; Klein *et al.*, 2001; Smith *et al.*, 2002; Whiley and Sloots, 2005; Yao *et al.*, 2006; Bru *et al.*, 2008; Boyle *et al.*, 2009; Süß *et al.*, 2009; Klungthong *et al.*, 2010; Stadhouders *et al.*, 2010) and probes (Süß *et al.*, 2009; Klungthong *et al.*, 2010). For instance, primer-template mismatches in the 3'-end region of the primer (defined in this chapter as the last 4 nucleotides) have been shown to have a larger effect on PCR amplification than those located towards the 5'-end, due to disruption of the DNA polymerase active site (Kwok *et al.*, 1990; Whiley and Sloots, 2005; Bru *et al.*, 2008; Süß *et al.*, 2009; Stadhouders *et al.*, 2010). Furthermore for rPCR probes, mismatches in the centre have been shown to destabilise probe annealing, thereby having a larger impact on amplicon detection than mismatches located at the 5'-end (Süß *et al.*, 2009).

Primer/probe-template mismatches can be especially problematic when considering the use of rPCR for diagnostic purposes. By impacting rPCR amplification, mismatches can alter the cycle threshold (C_T) at which targets are detected, leading to errors in nucleic acid quantification. For instance, a single internally-located mismatch can result in up to a 1,000-fold underestimation of initial copy number (Bru *et al.*, 2008). Notably, mismatches at the 3'-end of primers have been shown to produce effects ranging from a

two-fold underestimation of initial copy number to complete prevention of amplification, thus leading to false-negative results (Stadhouders *et al.*, 2010).

These effects of mismatches result in the requirement for continual evaluation of primer and probe sequences. In addition to laborious manual laboratory-based screening, primer/probe validation traditionally occurs though Basic Local Alignment Search Tool (BLAST) searches against publically available sequences (Altschul *et al.*, 1990), with tools now developed to automate the process (Dyer *et al.*, 2008; Lemmon and Gardner, 2008). However, despite numerous studies into the effects of mismatches, no primer evaluation programs to date have been developed using experimental data, with target sequences only reported as putative hits or misses. With rPCR assays requiring different performance criteria depending upon their use, the provision of binary predictions is limited. For example, high specificity is paramount for assays used to differentiate between diseases, high sensitivity is required for assays used to confirm negative results, and an awareness of cross-reactivity is important for assays that distinguish between closely related sequences, such as FMDV serotypes (Bachanek-Bankowska *et al.*, 2016). As such, the availability of a quantitative primer/probe validation program could support rPCR evaluation by giving researchers and diagnosticians the ability to rapidly predict whether assays are fit for purpose.

This chapter describes the effects of different primer/probe-template mismatches on C_T and limit of detection (LOD), and the presentation of a primer/probe evaluation program (GoPrime), in order to ascertain to what extent the effect of mismatches in rPCR can be predicted.

5.3 Materials and Methods

5.3.1 The effects of primer/probe-template mismatches

To study the effects of primer/probe-template mismatches on C_T and LOD, rPCR was performed using DNA oligonucleotide targets, which had been designed to vary in the primer/probe binding regions.

Linear DNA oligonucleotides templates of 109 bp (Sigma-Aldrich, Munich, Germany) were designed around the target region for a published assay (Callahan *et al*. 2002): a conserved region of the FMDV genome ($3D^{pol}$-coding region). Ninety templates were ordered, each designed to evaluate the effect of different variations in the primer/probe binding regions (Table 5.1). For example, variations across the length of the primer/probe target regions were designed to evaluate the effect of position, with different bases substituted to investigate the effects of mismatch type and mismatch quantity. Sequences were based on O/UKG/35/2001 (accession number KR265074, nucleotides 7862-7970). In addition, a template with full primer/probe-template complementarity was ordered as used as the reference template (R) in all rPCR reactions.

Table 5.1 Linear DNA oligonucleotide templates for real-time PCR targets (5'-3')

	Forward primer target	Probe target	Reverse primer target
R	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
1	ACTGGGTTTTACAAACCTGTG**G**	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
2	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	**C**CCGTGGCAGGACTCGC
3	ACTGGGTTTTACAAACCTGTG**G**	TCCTTTGCACGCCGTGGGAC	**C**CCGTGGCAGGACTCGC
4	ACTGGGTTTTACAAACCTGTG**C**	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
5	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	**G**CCGTGGCAGGACTCGC
6	ACTGGGTTTTACAAACCTGTG**C**	TCCTTTGCACGCCGTGGGAC	**G**CCGTGGCAGGACTCGC
7	ACTGGGTTTTACAAACCT**A**TA**A**	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
8	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	T**T**C**A**TGGCAGGACTCGC
9	ACTGGGTTTTACAAACCT**A**TA**A**	TCCTTTGCACGCCGTGGGAC	T**T**C**A**TGGCAGGACTCGC
10	ACTGGGTTTTACAAACCT**T**T**T**A	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
11	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	T**A**C**T**TGGCAGGACTCGC
12	ACTGGGTTTTACAAACCT**T**T**T**A	TCCTTTGCACGCCGTGGGAC	T**A**C**T**TGGCAGGACTCGC
13	ACTGG**A**TT**C**TAC**G**AAC**T**TGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
14	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TCCGT**A**GC**G**GGAC**T**TGC
15	ACTGG**A**TT**C**TAC**G**AAC**T**TGTGA	TCCTTTGCACGCCGTGGGAC	TCCGT**A**GC**G**GGAC**T**TGC
16	ACTGG**T**TT**G**TAC**C**AAC**A**TGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
17	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TCCGT**T**GC**C**GGAC**T**AGC
18	ACTGG**T**TT**G**TAC**C**AAC**A**TGTGA	TCCTTTGCACGCCGTGGGAC	TCCGT**T**GC**C**GGAC**T**AGC
19	A**T**TA**G**A**T**TC**T**GC**G**AAC**T**TGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
20	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TCCGT**A**GC**G**GG**G**CT**T**GT
21	A**T**TA**G**A**T**TC**T**GC**G**AAC**T**TGTGA	TCCTTTGCACGCCGTGGGAC	TCCGT**A**GC**G**GG**G**CT**T**GT
22	A**A**T**T**GT**T**TG**T**CC**C**AAC**A**TGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
23	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TCCGT**T**GC**C**GG**C**CT**A**G**A**
24	A**A**T**T**GT**T**TG**T**CC**C**AAC**A**TGTGA	TCCTTTGCACGCCGTGGGAC	TCCGT**T**GC**C**GG**C**CT**A**G**A**
25	A**A**TA**G**T**T**TC**T**CC**G**AAC**A**TGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
26	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TCCGT**T**GC**G**GG**C**CT**T**G**A**
27	A**A**TA**G**T**T**TC**T**CC**G**AAC**A**TGTGA	TCCTTTGCACGCCGTGGGAC	TCCGT**T**GC**G**GG**C**CT**T**G**A**

R	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
28	ACTGGTTTCTACGAACATGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
29	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTTGCGGGACTTGC
30	ACTGGTTTCTACGAACATGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTTGCGGGACTTGC
31	ACTGGTTTTTACGAACATGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTTGCGGGACTTGC
32	ACTGGTTTTTACAAACATGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTTGCAGGACTTGC
33	ACTGGTTTTTACGAACATGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTTGCAGGACTCGC
34	ACTGGTTTTTACAAACATGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
35	ACTGGGTTTTACAAACATGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTTGCAGGACTCGC
36	ACTGGTTTCTACGAACATGTGA	TCCTTTGCACGCCGTGGGAC	CCCGTGGCAGGACTCGC
37	ACTGGTTTCTACGAACATGTGA	TCCTTTGCACGCCGTGGGAC	GCCGTGGCAGGACTCGC
38	ACTGGTTTCTACGAACATGTGA	TCCTTTGCACGCCGTGGGAC	TTCGTGGCAGGACTCGC
39	ACTGGTTTCTACGAACATGTGA	TCCTTTGCACGCCGTGGGAC	TACGTGGCAGGACTCGC
40	ACTGGTTTTTACAAACATGTGA	TCCTTTGCACGCCGTGGGAC	CCCGTGGCAGGACTCGC
41	ACTGGTTTTTACAAACATGTGA	TCCTTTGCACGCCGTGGGAC	GCCGTGGCAGGACTCGC
42	ACTGGGTTTTACAAACCTGAGG	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
43	ACTGGGTTTTACAAACCTGCGG	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
44	ACTGGGTTTTACAAACCTGAGC	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
45	ACTGGGTTTTACAAACCTGTTG	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
46	ACTGGGTTTTACAAACCTGTAG	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
47	ACTGGGTTTTACAAACCTGTTC	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
48	ACTGGGTTTTACAAACCTGGAA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
49	ACTGGGTTTTACAAACCTGCAA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
50	ACTGGGTTTTACAAACCTGGTA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
51	ACTGGGTTTTACAAACCTTTGG	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
52	ACTGGGTTTTACAAACCTATGG	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
53	ACTGGGTTTTACAAACCTTTGC	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
54	ACTGGGTTTTACAAACCTGTAA	TCCTTTGCACGCCGTGGGAC	TTCGTGGCAGGACTCGC
55	ACTGGGTTTTACAAACCTGTTA	TCCTTTGCACGCCGTGGGAC	TACGTGGCAGGACTCGC
56	ACTGGGTTTTACAAACCTGTAA	TCCTTTGCACGCCGTGGGAC	TACGTGGCAGGACTCGC
57	ACTGGGTTTTACAAACCTATTA	TCCTTTGCACGCCGTGGGAC	TCTGTGGCAGGACTCGC
58	ACTGGGTTTTACAAACCTATTA	TCCTTTGCACGCCGTGGGAC	TCAGTGGCAGGACTCGC
59	ACTGGGTTTTACAAACCTATTA	TCCTTTGCACGCCGTGGGAC	CCCGTGGCAGGACTCGC
60	ACTGGGTTTTACAAACCTATTA	TCCTTTGCACGCCGTGGGAC	GCCGTGGCAGGACTCGC
61	ACTGGGTTTTACAAACCTGTGA	TCCTTGGCGCACAGCGGTAC	TCCGTGGCAGGACTCGC
62	ACTGGGTTTTACAAACCTGTGA	TCCTTGGCGCACCGCGGTAC	TCCGTGGCAGGACTCGC
63	ACTGGGTTTTACAAACCTGTGA	TCCTTGGCACACCGCGGTAC	TCCGTGGCAGGACTCGC
64	ACTGGGTTTTACAAACCTGTGA	TCCTTGGCACACCGCGGGAC	TCCGTGGCAGGACTCGC
65	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACACCGCGGGAC	TCCGTGGCAGGACTCGC
66	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACACCGTGGGAC	TCCGTGGCAGGACTCGC
67	ACTGGGTTTTACAAACCTGTGA	CCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
68	ACTGGGTTTTACAAACCTGTGA	GCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
69	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAT	TCCGTGGCAGGACTCGC
70	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAA	TCCGTGGCAGGACTCGC
71	ACTGGGTTTTACAAACCTGTGA	GGCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
72	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGCT	TCCGTGGCAGGACTCGC
73	ACTGGGTTTTACAAACCTGTGA	CCATTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
74	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGTAT	TCCGTGGCAGGACTCGC
75	ACTGGGTTTTACAAACCTGTGA	TTCGTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
76	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGTGGC	TCCGTGGCAGGACTCGC
77	ACTGGGTTTTACAAACCTGTGA	CCCTTTGCACACCGCGGGAC	TCCGTGGCAGGACTCGC
78	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACACCGCGGGAT	TCCGTGGCAGGACTCGC
79	ACTGGGTTTTACAAACCTGTGA	CACTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
80	ACTGGGTTTTACAAACCTGTGG	TCCTTGGCACACCGCGGGAC	TCCGTGGCAGGACTCGC
81	ACTGGGTTTTACAAACCTGTGG	TCCTTTGCACACCGCGGGAC	TCCGTGGCAGGACTCGC
82	ACTGGGTTTTACAAACCTGTGC	TCCTTTGCACACCGCGGGAC	TCCGTGGCAGGACTCGC
83	ACTGGGTTTTACAAACCTTTGG	TCCTTTGCACACCGCGGGAC	TCCGTGGCAGGACTCGC
84	ACTGGGTTTTACAAACCTGTGG	CCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
85	ACTGGGTTTTACAAACCTGTGG	TCCTTTGCACGCCGTGGGAT	TCCGTGGCAGGACTCGC
86	ACTGGTTTTTACAAACATGTGA	TCCTTTGCACACCGCGGGAC	TCCGTTGCAGGACTTGC
87	ACTGGTTTTTACAAACATGTGA	TCCTTTGCACACCGCGGGAC	TCCGTGGCAGGACTCGC
88	ACTGGGTTTTACAAACATGTGA	TCCTTTGCACACCGTGGGAC	TCCGTTGCAGGACTCGC
89	ACTGGGTTTTACAAACATGTGA	CCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
90	ACTGGGTTTTACAAACATGTGA	TCCTTTGCACGCCGTGGGAT	TCCGTGGCAGGACTCGC

The primer/probe target sequences of the 90 DNA templates (109 base pairs in length) in 5'-3' orientation. Non-target regions between the primer/probe targets were identical to O/UKG/35/2001 (accession number KR265074: nucleotides 7862-7970). Black sequences represent the reference template (R); grey sequences represent the varying DNA templates, red underlined based depict primer/probe-template mismatch sites.

Real-time PCR:

rPCR reactions were performed using two *Taq*-based rPCR kits:

Excite™ UF 2x Master Mix (Excite™ UF) (Quantig Ltd., Camberley, UK), a *Taq*-based rPCR kit, was selected as it required minimal reaction set-up, increasing the likelihood of assay variation being attributed to target sequence differences rather than human error. Reactions were performed in a total of 20 µl, containing: 5 µl template, 10 µl 2x master mix, 50 nM ROX reference dye, primers and probes as previously described (Callahan *et al*. 2002) and made up to volume with nuclease-free water (NFW). Thermal cycling conditions were 95°C for 3 minutes, followed by 50 cycles of 95°C for 5 seconds and 60°C for 20 seconds.

SuperScript™ III Platinum™ One-Step qRT-PCR Kit (SSIII™) (Thermo Fisher Scientific, MA, USA) was chosen as it is a commonly used *Taq*-based kit (routinely used within the Food and Agricultural Organization (FAO) World Reference Laboratory for Foot-and-Mouth Disease [WRLFMD]), it was used for the reference real-time reverse transcription PCR (rRT-PCR) in Chapters 2 to 4, and it is compatible with both RNA and DNA templates (Appendix 7b). Reagents, parameters and thermal cycling conditions were as reported in Shaw *et al*. (2007), with the addition of 50 nM ROX reference dye per reaction and omission of the reverse transcription (RT) step. Primers and probes were as previously published (Callahan *et al.*, 2002).

All rPCR reactions were performed on an ABI ViiA™ 7 Real-Time PCR system thermocycler (Thermo Fisher Scientific). The C_T was called automatically using ViiA™ 7 Software and positive reactions were defined as those that gave a detectable C_T. Initial rPCR reactions were performed in duplicate using 10^6 copies of template. Where C_T values were detected, further rPCR reactions were performed in duplicate across a $\log_{10}$ dilution series of template (10^6-10^0 copies/reaction) in 0.1 µg/µl carrier RNA (Ambion®, Thermo Fisher Scientific). The effect of mismatches on rPCR were determined by calculating the change in C_T (ΔC_T) and change in LOD (ΔLOD) between the reference and varying oligonucleotide DNA templates.

5.3.2 Development of GoPrime

In order to ascertain the effect of each primer/probe-template mismatch type on ΔC_T and ΔLOD, linear model analysis was performed. Linear model variables were selected based on statistical analysis (to ascertain which primer/probe-template mismatch locations were statistically different from one another) and previously published data (Table 5.2).

Table 5.2 Statistical analyses to determine linear model variables

Comparison	Templates [mismatch]	Tukey test *p*-value	Result
3'-end of primers	A: 45-47 (nt 1 and 2) B: 42-44 (nt 1 and 3) C: 51-53 (nt 1 and 4)	A-B: $p < 0.001$ A-C: $p = 0.083$ B-C: $p = 0.307$	No significant difference present between bases 3 and 4 (grouped together in the linear model). Previously published in Stadhouders *et al*. (2010).
3'-end of probe	A: 72 (nt 1 and 2) B: 74 (nt 1 and 3) C: 76 (nt 2 and 4)	A-B: $p = 0.997$ A-C: $p = 0.569$ B-C: $p = 0.612$	No significant difference present between probe nt 1 – 4 (grouped together in the linear model).
5'-end of probe	A: 71 (nt 20 and 19) B: 73 (nt 20 and 18) C: 75 (nt 19 and 17)	A-B: $p = 0.598$ A-C: $p = 0.862$ B-C: $p = 0.300$	No significant difference present between probe nt 20-17 (grouped together in the linear model).
Regions of probe	A: 65 (middle of probe) B: 72,74,76 (3'-end) C: 71,73,75,79 (5'-end)	A-B: $p = 0.021$ A-C: $p = 0.001$ B-C: $p < 0.001$	Significant differences between mismatches in the middle, 3'-end and 5'-end of the probe (treated separately in the linear model).

One way analysis of variance (ANOVA) tests (with post-hoc Tukey tests), were used to determine which primer/probe-template mismatch positions gave statistically different changes in cycle threshold. (nt): nucleotide. Template numbers refer to the oligonucleotide sequence in Table 5.1.

Linear model analysis (Chambers, 1992) was performed in R (R Core Team, 2014), using the variables stated in Table 5.3 and all quantitative data collected (90 templates, across 10^6 – 10^0 copies of template, using both *Taq*-based kits, to analyse the average effects of mismatches). The results of the linear model were used to parameterise GoPrime: a mathematical model for predicting the effects of mismatches on rPCR. GoPrime was built in collaboration with Dr Richard Orton (The University of Glasgow), who translated the primer/probe mismatch rules and C_T penalties into computer code written in the Java programming language.

Table 5.3 Variables included in linear model analysis

Mismatch type	Variable
Primers (forward or reverse)	Percentage mismatch (forward and reverse combined) Type 1 mismatch at the 3'-end (nucleotide 1) Type 2 mismatch at the 3'-end (nucleotide 1) Type 1 mismatch at the 3'-end (nucleotide 2) Type 2 mismatch at the 3'-end (nucleotide 2) Type 1 mismatch at the 3'-end (nucleotides 3-4) Type 2 mismatch at the 3'-end (nucleotides 3-4)
Probe	Percentage mismatch 3'-end mismatch (final four nucleotides) 5'-end mismatch (final four nucleotides)

Mismatches were grouped as one of two types: (type 1) purine-pyrimidine mismatch (G-T or C-A nucleotide base pairing, leading to a minor conformational change in the primer/probe-template duplex); (type 2) purine-purine or pyrimidine-pyrimidine mismatch (G-A, A-A, G-G, C-T, T-T or C-C nucleotide base pairing, leading to a major conformational change in the primer/probe-template duplex).

5.3.3 Evaluating GoPrime as a predictor of rPCR performance

Artificial variations within the FMDV 3D^{pol}-coding region:

Initial evaluation of GoPrime was performed by comparing the ΔC_T and ΔLOD evident from experimental rPCR results using both the Excite™ UF and SSIII™ protocols (Chapter 5.3.1) (differences between the results for the reference template and each of the 90 varying DNA templates) against the predicted results from GoPrime.

Naturally occurring sequence variations within the FMDV 3D^{pol}-coding region:

A search was performed using the National Centre for Biotechnology Information (NCBI) nucleotide database (NCBI, 2017) in order to find FMDV sequences that had naturally occurring variations in the Callahan *et al.* (2002) target region (Table 5.4). Seven linear DNA oligonucleotides of 109 bp were ordered to include these differences in the primer/probe target regions. These were then used as template in rPCR, using both the Excite™ UF and SSIII™ protocols (Chapter 5.3.1), with results compared to predictions from GoPrime.

Table 5.4 Linear DNA templates representing naturally occurring FMDV field isolates for testing GoPrime predictions (5'-3')

	Forward primer target	Probe target	Reverse primer target
R	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
JX040500	ACTGGGTTTTACAAACCT**A**TGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
KC440884	ACTGG**A**TTTTA**T**AAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
AY593802	ACTGGGTTTTACAAACCTGTGA	TCCTT**C**GCACGCCGTGGGAC	TC**T**GTGGCAGG**G**CTCGC
KC440883	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TC**T**GTGGC**G**GGACTCGC
AY593812	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TC**A**GTGGCAGGACTCGC
KF112882	ACTGGGTTTTACAAACCTGTGA	**C**CCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
HM191257	ACTGGGTTTTACAAACCTGTGA	TCCTT**C**GCACGCCGTGGGAC	TC**T**GTGGCAGGACTCGC

The primer/probe binding regions of the seven DNA oligonucleotides ordered to test the program (109 base pairs in length, with regions between primers consistent with the sequences for each accession number. Black sequences represent the reference template (R); grey sequences represent the varying DNA templates, with red underlined based depicting primer/probe-template mismatch sites. FMDV serotypes were as follows: JX040500 (O); KC440884 (Southern African Territories 2); AY593802 (A); KC440883 (O); AY593812 (O); KF112882 (O); HM191257 (O).

Alternative target sites within the FMDV genome:

In order to ascertain how transferable GoPrime was to other areas of the FMDV genome, GoPrime was used to analyse four FMDV-typing assays designed to target VP1/2A-coding regions (Bachanek-Bankowska *et al.*, 2016). The four sets of primers and probes (specific for either serotype A, O, Southern African Territories [SAT] 1 or SAT 2 field viruses circulating in East Africa) were evaluated against the 66 VP1/2A-coding sequences used

in initial laboratory-based evaluation (A = 15; O = 20; SAT 1 = 19; SAT 2 = 12) (unpublished sequences from The Pirbright Institute). The published experimental results (Bachanek-Bankowska *et al.*, 2016) and GoPrime predictions for the likely targets of each assay were then compared and displayed using GoPrimeTree. The serotype O primers (Bachanek-Bankowska *et al.*, 2016) were also evaluated against 24 viruses from outside of East Africa (sequences from GenBank) (Table 5.5), again with results displayed in GoPrimeTree.

Table 5.5 Foot-and-mouth disease virus isolates used to test GoPrime

Accession Number (NCBI)	Isolate	Location	Year
KJ825809.1	O/IND/250(541)/2013	India	2013
KF694743.1	O/S13KOR/2002	South Korea	2002
JX947858.1	O/UKG/91dpc	UK	2001
KF112880.1	O/MYA/5/2009	Myanmar	2009
JF749851.1	O/IRN/073/2001	Iran	2001
JX040499.1	O/TUR/8/2011	Turkey	2011
JX570650.1	O/BFS/1860/B2.6D.V	UK	1967
JX570643.1	O/BFS/1860/A2.32D.P	UK	1967
HQ632770.1	O/MAY/1/2004	Malaysia	2004
GU384683.1	O/PAK/45/2008	Pakistan	2008
FJ175666.1	O/Israel 07-6387	Israel	2007
AY686687.1	O/ES/2001	China*	2001
FJ542369.1	O/UKG/2000/2001	UK	2001
EF552694.1	O/UKG/4998/2001	UK	2001
EF552688.1	O/UKG/3952/2001	UK	2001
AY593832.1	O/UK2001-FB	UK	2001
AY593826.1	O/o2brescia	Italy	1947
AF511039.1	O/Akesu/58	China	1958
EF175732.1	O/CHA/WFL	China	Unknown
DQ404175.1	O/UKG/173/2001	UK	2001
DQ404159.1	O/UKG/14603/2001	UK	2001
AF026168.2	O/TAW/Chu-Pei	Taiwan	1997
AY317098.1	O/HKN/2002	Hong Kong	2002
AJ539136.1	O/TAW/2/99	Taiwan	1999

5.3.4 Statistical and phylogenetic analysis

All statistical analyses were performed using R (R Core Team, 2014). The root-mean-square deviation (RMSD) was used to measure the differences between the predicted ΔC_T values and the ΔC_T values actually observed. Phylogenetic trees were produced from sequence alignments in Mega (version 7.0.21) (Kumar *et al.*, 2016) using the neighbour-joining method (Saitou and Nei, 1987) and viewed in FigTree (version 1.4.3) (Rambaut, 2016).

5.4 Results

5.4.1 The effects of primer/probe-template mismatches on rPCR

The effects of primer-template mismatches:

Primer-template mismatches instigated a wide variety of effects on rPCR amplification. Single mismatches at the 3'-end of the primer (using both rPCR kits) had the most detrimental effect on C_T (ΔC_T of between 6.44 and 11.57), with the impact of mismatches declining further toward the 5'-end (Figure 5.1).

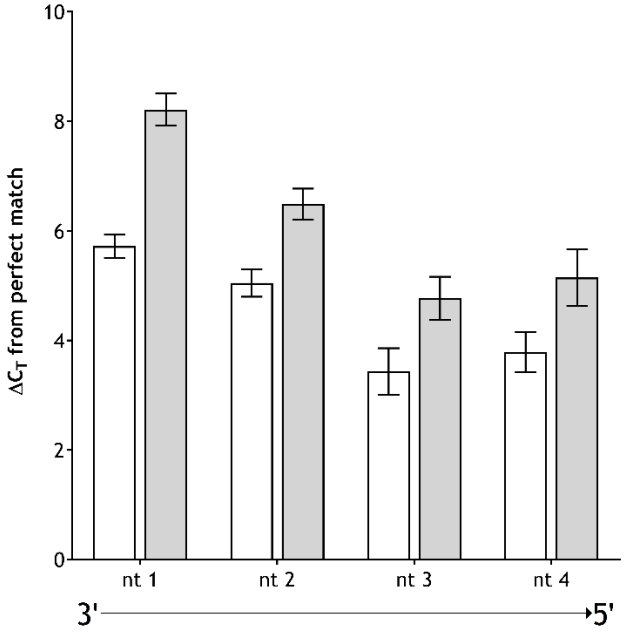


Figure 5.1 The effects of 3'-end primer-template mismatches on real-time PCR (rPCR) cycle threshold (C_T). Results represent the effects of mismatches on cycle threshold (ΔC_T) derived from linear model analysis, across two rPCR kits (Excite™ UF 2x Master and SuperScript™ III Platinum™ One-Step qRT-PCR Kit) and a dilution series of template (10^6 – 10^0 copies/reaction). (nt) nucleotide. (white) type 1 mismatch: purine-pyrimidine mismatch (G-T or C-A nucleotide base pairing, leading to a minor conformational change in the primer/probe-template duplex); (grey) type 2 mismatch: purine-purine or pyrimidine-pyrimidine mismatch (G-A, A-A, G-G, C-T, T-T or C-C nucleotide base pairing, leading to a major conformational change in the primer/probe-template duplex).

Furthermore, the type of mismatch was shown to be important. For instance, at the 3'-end of the reverse primer, a C-A mismatch (type 1) resulted in an average effect of ΔC_T of 8.59, whereas a G-A mismatch (type 2) in the same location resulted in an average effect of ΔC_T of 11.57 (Figure 5.2A). Up to two mismatches in the 3'-end of the primer could be tolerated (Figure 5.2B; Figure 5.2C) before amplification did not occur, however a minimum of 10^4 copies of template per reaction were required in these circumstances.

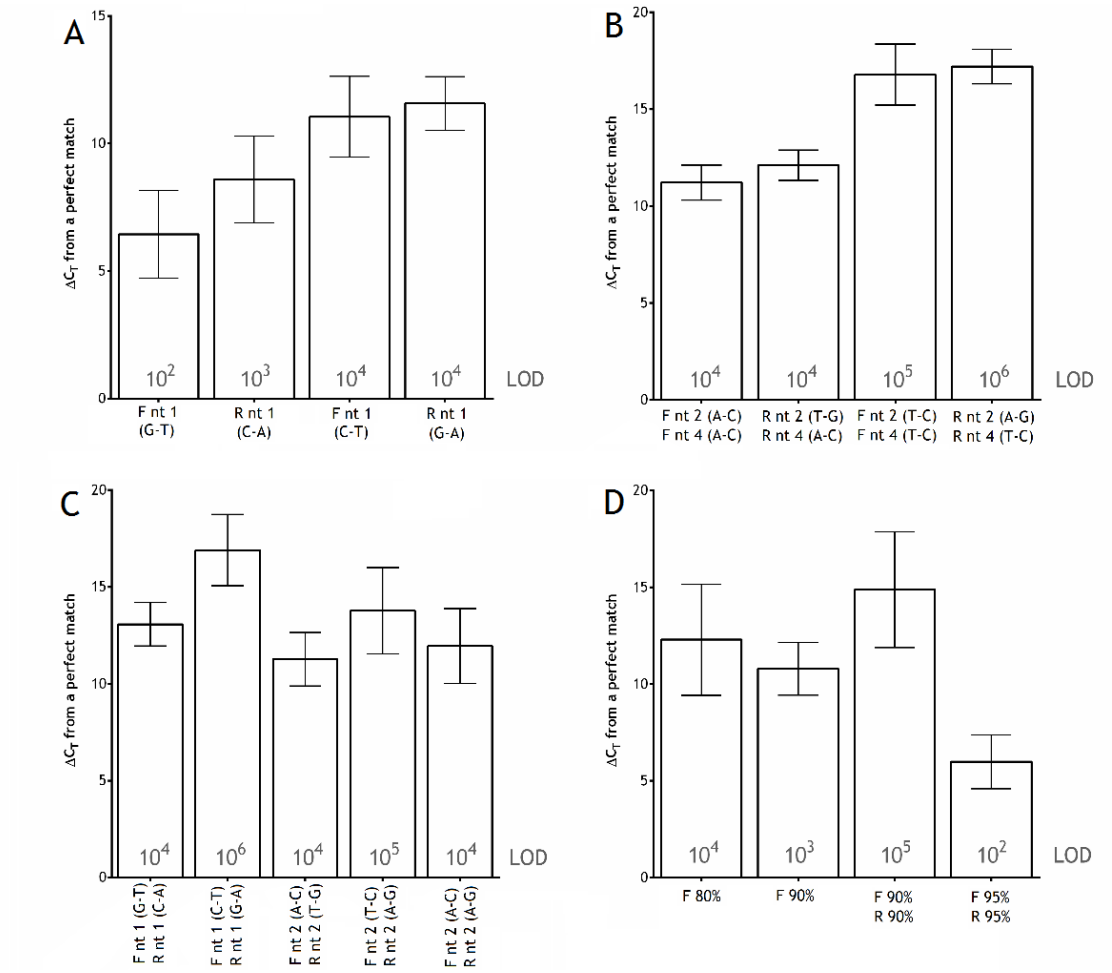


Figure 5.2 The effects of primer-template mismatches on real-time PCR (rPCR) cycle threshold (C_T). (A) single mismatches at the 3'-end; (B) and (C) multiple mismatches at the 3'-end; (D) effect of primer-template percentage complementary. Results represent the average increase in cycle threshold (ΔC_T) from the reference template across two rPCR kits (Excite™ UF 2x Master and SuperScript™ III Platinum™ One-Step qRT-PCR Kit) and a dilution series of template (10^6 – 10^0 copies/reaction). The limit of detection (LOD) for each template is defined as the lowest dilution where all replicates were positive (displayed in grey text). Error bars represent the standard deviation. (F) forward primer; (R) reverse primer; (nt) nucleotide.

When studying the effect of primer/probe-template percentage complementary across the total length of the primers (Figure 5.2D), a minimum of 82.05% primer-template match between the forward and reverse primers (combined) was required for amplification to occur. However, at this percentage, mismatches impacted upon amplicon detection by a ΔC_T of 19.31 with a LOD of 10^6 copies per reaction.

The effects of probe-template mismatches:

Single mismatches at the 5'-end of the site recognised by the TaqMan probe had very little effect on C_T (ΔC_T of 0.06 – 0.15), whereas single mismatches at the 3'-end impacted amplicon detection by a ΔC_T of 2.87 - 3.38 (Figure 5.3A). When studying the effect of the mismatches across the length of the probe, a minimum of 85% probe-template complementarity was required in order for effective detection to occur, impacting upon rPCR ΔC_T by 6.57 with a LOD of 10^4 (Figure 5.3B).

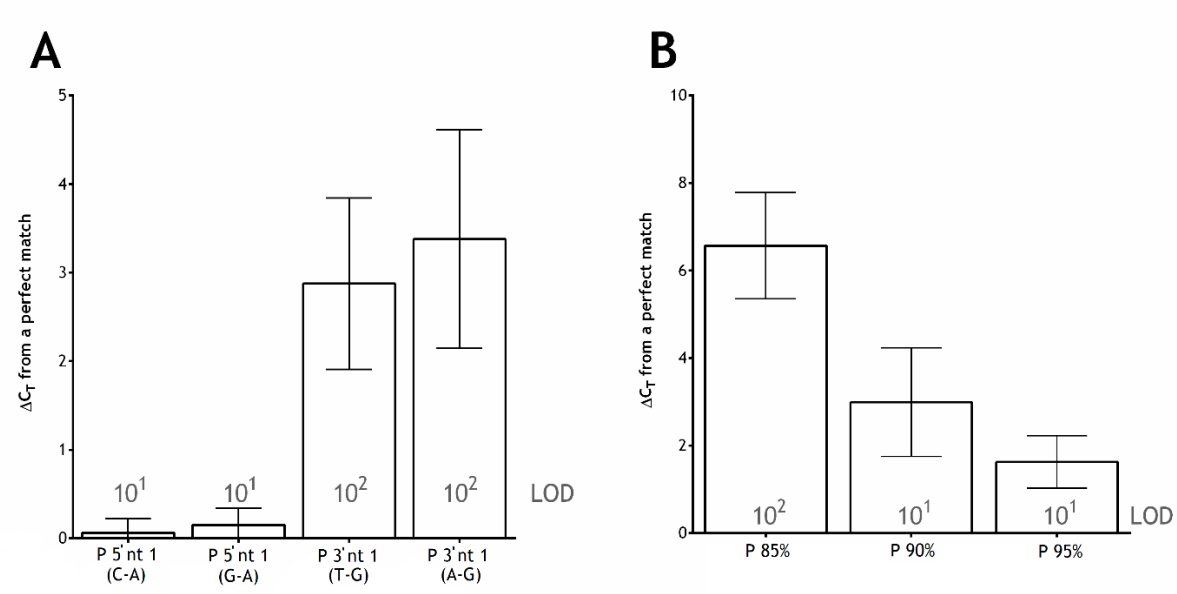


Figure 5.3 The effects of probe-template mismatches on real-time PCR (rPCR) cycle threshold. (A) single mismatches at the 3'-end or 5'-end; (B) effect of probe-template percentage complementary. Results represent the average increase in cycle threshold (C_T) from a perfectly matched template, across two rPCR kits (Excite™ UF 2x Master and SuperScript™ III Platinum™ One-Step qRT-PCR Kit) and a dilution series of template (10^6 – 10^0 copies/reaction). The limit of detection (LOD) for each template is defined as the lowest dilution where all replicates were positive (displayed in grey text). Error bars represent the standard deviation. (P) probe; (nt) nucleotide; percentages represent probe-template complementarity.

The effect of primer/probe-template mismatches across template copy number:

Template titrations were used to evaluate how the initial template copy number affected rPCR amplification in the presence of primer/probe-template mismatches (Figure 5.4). Analysis included all targets with positive C_T values for three or more dilution points (44/90 templates). Linear regression (analysis of the slope) showed that no significant difference was evident between templates (F-value = 1.227, p = 0.162), therefore mismatches instigate a similar ΔC_T and ΔLOD irrespective of the template copy number.

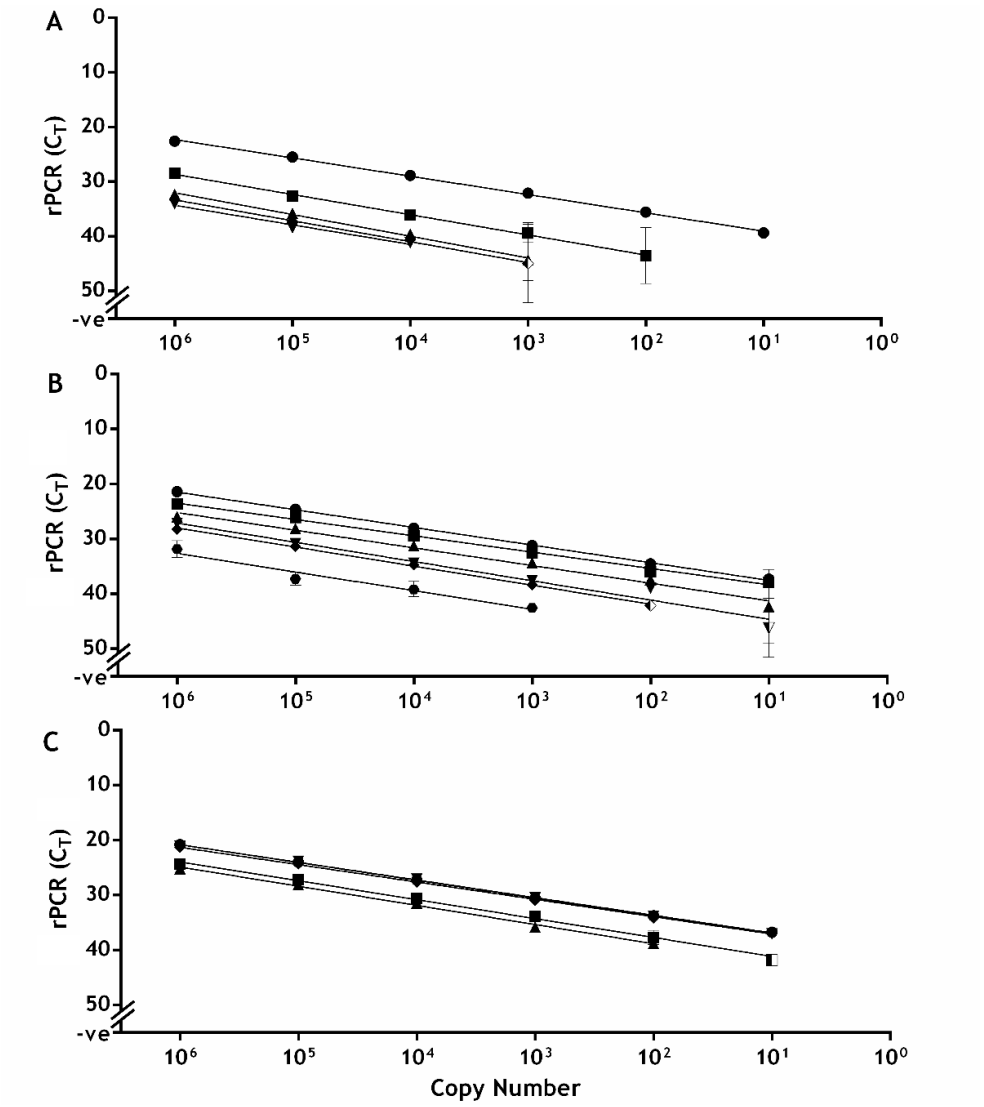


Figure 5.4 The effect of primer/probe-template mismatches across different copy numbers of template. Results are shown for the Excite™ UF 2x Master Mix, and display a subset of the oligonucleotides tested (Table 5.1). (A): (●) perfect match; (■) template 1; (▲) template 2; (◆) template 4; (▼) template 5. (B): (●) perfect match; (■) template 66; (▲) template 65; (◆) template 64; (▼) template 35; (⬣) template 34. (C): (●) perfect match; (■) template 69; (▲) template 70; (◆) template 67; (▼) template 68. Points represent the mean of two replicates; half-shaded points represent when one duplicate was positive and the other negative; error bars represent standard deviation; (ΔC_T) change in cycle threshold.

5.4.2 Development of GoPrime

Using linear model analysis, the average effect of each primer/probe-template mismatch type was determined, accounting for both single and multiple mismatches in the primer and probe binding regions, by implementing additive and dampening effects where necessary (Table 5.6).

Table 5.6 The effect of mismatches calculated from linear model analysis

Factor	Mismatch type	ΔC_T	SE	t value	*p* value
Primer	% mismatch (forward/reverse combined)*	0.86	0.03	31.75	< 0.001
	nt 1 mismatch (type 1)	1.64	0.23	7.15	< 0.001
	2x nt 1 mismatch (type 1)	5.07	0.75	6.76	< 0.001
	nt 1 mismatch (type 2)	4.01	0.33	12.18	< 0.001
	2x nt 1 mismatch (type 2)	8.90	1.14	7.80	< 0.001
	nt 2 mismatch (type 1)	1.19	0.37	3.23	0.002
	2x nt mismatch (type 1)	3.51	0.70	5.03	< 0.001
	nt 2 mismatch (type 2)	3.64	0.36	10.11	< 0.001
	2x nt 2 mismatch (type 2)	6.31	0.97	6.51	< 0.001
	nt 3-4 mismatch (type 1)	1.03	0.33	3.07	0.001
	2x nt 3-4 mismatch (type 2)	1.76	0.86	0.20	0.839
	nt 3-4 mismatch (type 1)	2.91	0.31	9.32	< 0.001
	2x nt 3-4 mismatch (type 2)	4.86	2.69	1.80	0.072
Probe	% mismatch	0.35	0.02	8.51	< 0.001
	3'-end mismatch (nucleotides 1-4)	0.41	0.20	2.01	0.044
	5'-end mismatch (nucleotides 1-4)	-1.05	0.19	-5.55	< 0.001

(nt) nucleotide; (ΔC_T) change in cycle threshold; (SE) standard error. For multiple mismatches, the linear model was able to calculate the effect of having the same type of mutation in both the primers (2x), if two mismatches were present but different the linear model calculated the additive/dampening effect: two 3'-end primer mismatches (ΔC_T: -0.27 [2dp]); two probe 3'-end mismatches (C_T: -0.84 [2dp]); one primer and one probe mismatch (ΔC_T: +0.43 [2dp]). Mismatches were grouped as one of two types: (type 1) purine-pyrimidine mismatch (G-T; C-A: minor conformational change in the primer/probe-template duplex); (type 2) purine-purine or pyrimidine-pyrimidine mismatch (G-A; A-A; G-G; C-T; T-T; C-C: major conformational change in the primer/probe-template duplex). *If a type nt 1 mismatch was present, the percentage mismatch ΔC_T would be calculated and additional nt 1 mismatch ΔC_T penalty added. ΔC_T, SE and t value given to 2 decimal places.

Results from the linear model were used to parameterise GoPrime (freely available through https://github.com/rjorton/GoPrime [Orton, 2017]). To use GoPrime, users input their primer/probe sequences (5' - 3' [fasta format]) and a file of sequences to be analysed (5' - 3' [fasta format]). GoPrime first analyses the template, in both orientations, for possible forward and reverse primer targets (Figure 5.7). This is done based on the minimum requirements for primer-template complementarity, which were defined during data analysis as the lowest dilution of template which produced positive C_T values in all replicates (Table 5.7).

Table 5.7 Minimum primer/probe-template complementarity required for amplification

Factor	Minimum requirements for real-time PCR amplification
Primer 3'-end	Maximum of two mismatches in the 3'-ends (either in one primer, or between the primers).
Primer percentage	A minimum of 82.05% match is required (combined % for the pair)
Probe percentage	A minimum of 85.00% match is required

Once possible forward and reverse primer targets are identified, they are evaluated as possible primer pairs (Figure 5.5). Potential probe targets (optional) are then identified between the primer pairs, searching again in both orientations based on the probe-template mismatch limits determined during data analysis (Table 5.7).

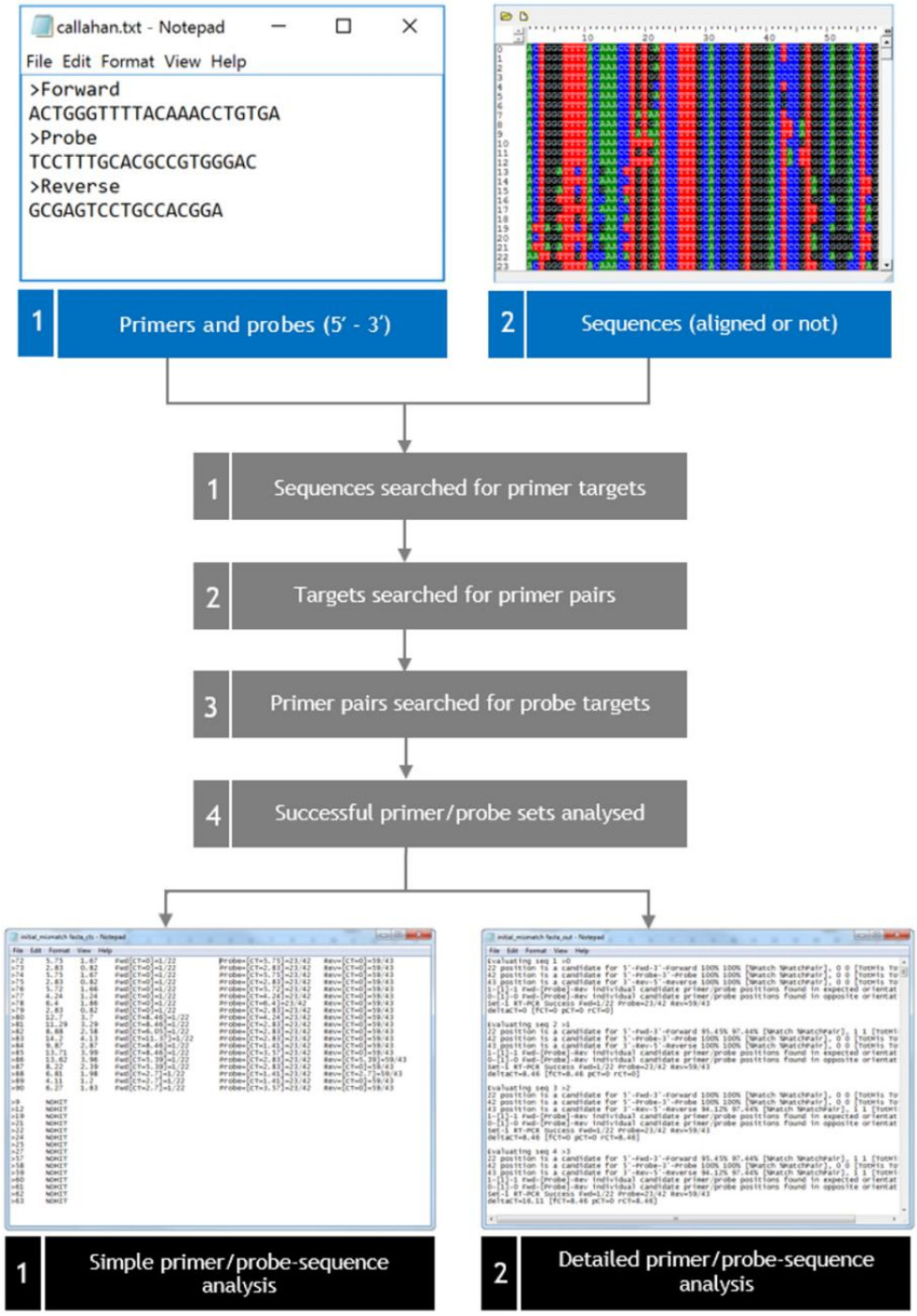


Figure 5.5 GoPrime flow diagram. GoPrime examines sets of primer sequences (optionally including a probe sequence), searches the target genome sequences for potential matches, then predicts the effect of any primers/probe-template mismatches present on real-time PCR cycle threshold and limit of detection.

Once a potentially suitable primer/probe set has been identified, GoPrime uses the results of the linear model to predict whether amplification is likely to occur and the effect of any mismatches present on ΔC_T and ΔLOD, implementing additive/dampening effects of multiple mismatches where necessary. GoPrime provides the outputs as two separate text files. Firstly, a simple analysis, which provides each sequence name against the predicted ΔC_T and ΔLOD, number of mismatches present and the likely amplicon length. The second analysis provides more detail, including the position and orientation of each likely primer/probe target and the type of any mismatches present (Figure 5.5).

As an optional extra, users can display their results using GoPrimeTree. In order to use this, users input the simple output of GoPrime (text file) in addition to a phylogenetic tree (nexus format). GoPrimeTree colour codes the sequences according to the predicted ΔC_T, via annotation of the nexus tree file, so that the predicted targets and effect of the primer/probe-template mismatches can be easily visualised across multiple sequence data (Figure 5.6).

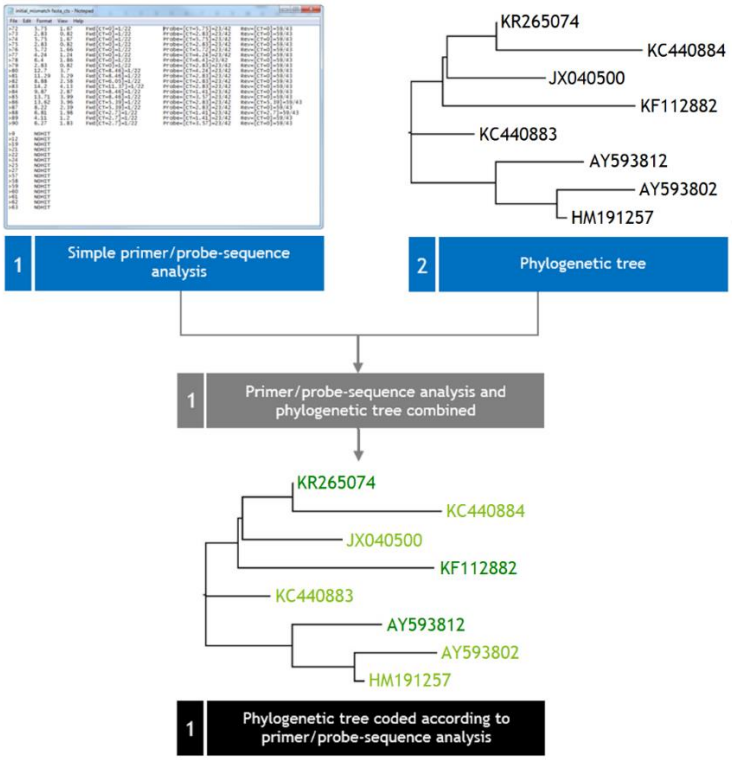


Figure 5.6 GoPrimeTree flow diagram. GoPrimeTree takes the simple output of GoPrime and combines it with a phylogenetic tree, colour coding each sequence according to whether it is likely to be detected and the predicted changes in cycle threshold.

Note, within this chapter GoPrime was parametrised using the experimental results detailed, preliminary analysis of alternative parameters was also carried out by adding published data to the model (Süß *et al.*, 2009; Stadhouders *et al.*, 2010) (Appendix 7a).

5.4.3 Evaluating GoPrime as a predictor of rPCR performance

Artificial variations within the FMDV 3D^{pol}-coding region:

Initial evaluation of GoPrime, comparing the experimental and predicted rPCR results for the 90 varying DNA templates (Table 5.1), showed that on average GoPrime predicted the ΔC_T of reactions 3.32 (SD 2.26; RMSD 4.02) and ΔLOD of reactions 1.65 (SD 0.74) away from the observed result (Figure 5.7). Out of the 90 DNA templates, concordance was evident between GoPrime and the observed rPCR results for 80 templates in terms of whether the target was detected (70 positive; 10 negative). Disagreements were limited to the upper thresholds of rPCR reactions and GoPrime. For example, the five targets which were detected in experimental rPCR but predicted as negative in GoPrime, 10^6 copies were required for detection to occur (average Excite™ UF ΔC_T of 21.00; SSIII™ ΔC_T of 15.14). For the five targets that were predicted to amplify but produced negative experimental results, a large ΔC_T was predicted (average ΔC_T: 13.28; range 11.07-15.49).

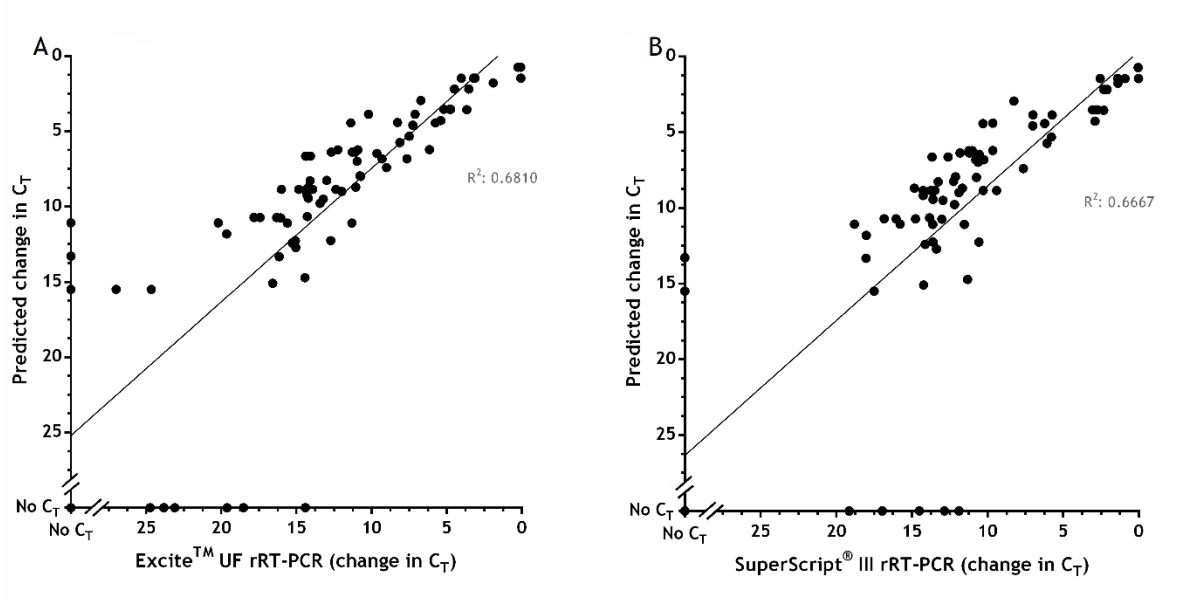


Figure 5.7 Evaluating GoPrime as a predictor of real-time PCR (rPCR) performance using artificial sequence variations. (A) observed change in cycle threshold for Excite™ UF 2x Master Mix; (B) observed change in cycle threshold for SuperScript™ III Platinum™ One-Step qRT-PCR Kit. For the observed change in cycle threshold, points represent the average across all dilutions (10^6-10^0) of starting template.

Naturally occurring sequence variations within the FMDV 3D^{pol}-coding region:

Evaluation of GoPrime using the seven DNA oligonucleotide templates containing sequence variations, observed in naturally occurring FMDV isolates in the Callahan *et al*. (2002) target region (Table 5.9), showed that GoPrime on average predicted the ΔC_T of reactions 1.41 (SD 0.79; range 0.20-2.68; RMSD 1.61) away from the observed result (Figure 5.8A; 5.8B; 5.9). GoPrime on average predicted the ΔLOD of reactions 0.70 (SD 0.39; range 0.06-1.41) away from the observed result (Figure 5.8C; 5.8D; 5.9).

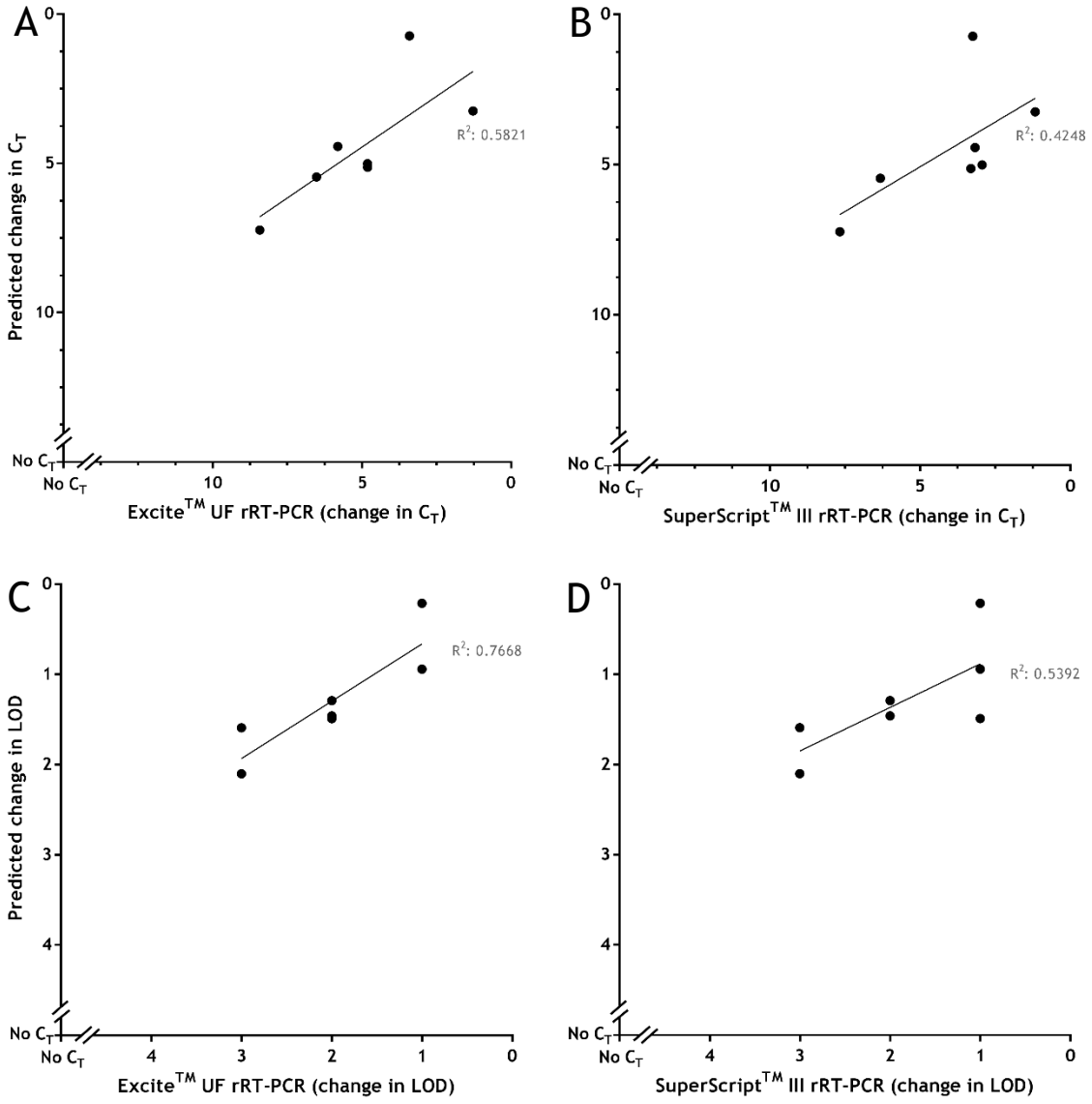


Figure 5.8 Evaluating GoPrime as a predictor of real-time PCR (rPCR) performance using naturally occurring sequence variations. (A) observed change in cycle threshold for Excite™ UF 2x Master Mix; (B) observed change in cycle threshold for SuperScript™ III Platinum™ One-Step qRT-PCR Kit; (C) observed change in limit of detection for Excite™ UF 2x Master Mix; (D) observed change in limit of detection for SuperScript™ III Platinum™ One-Step qRT-PCR Kit. For the observed results, points represent the average change in cycle threshold or limit of detection across all dilutions (10^6-10^0) of starting template.

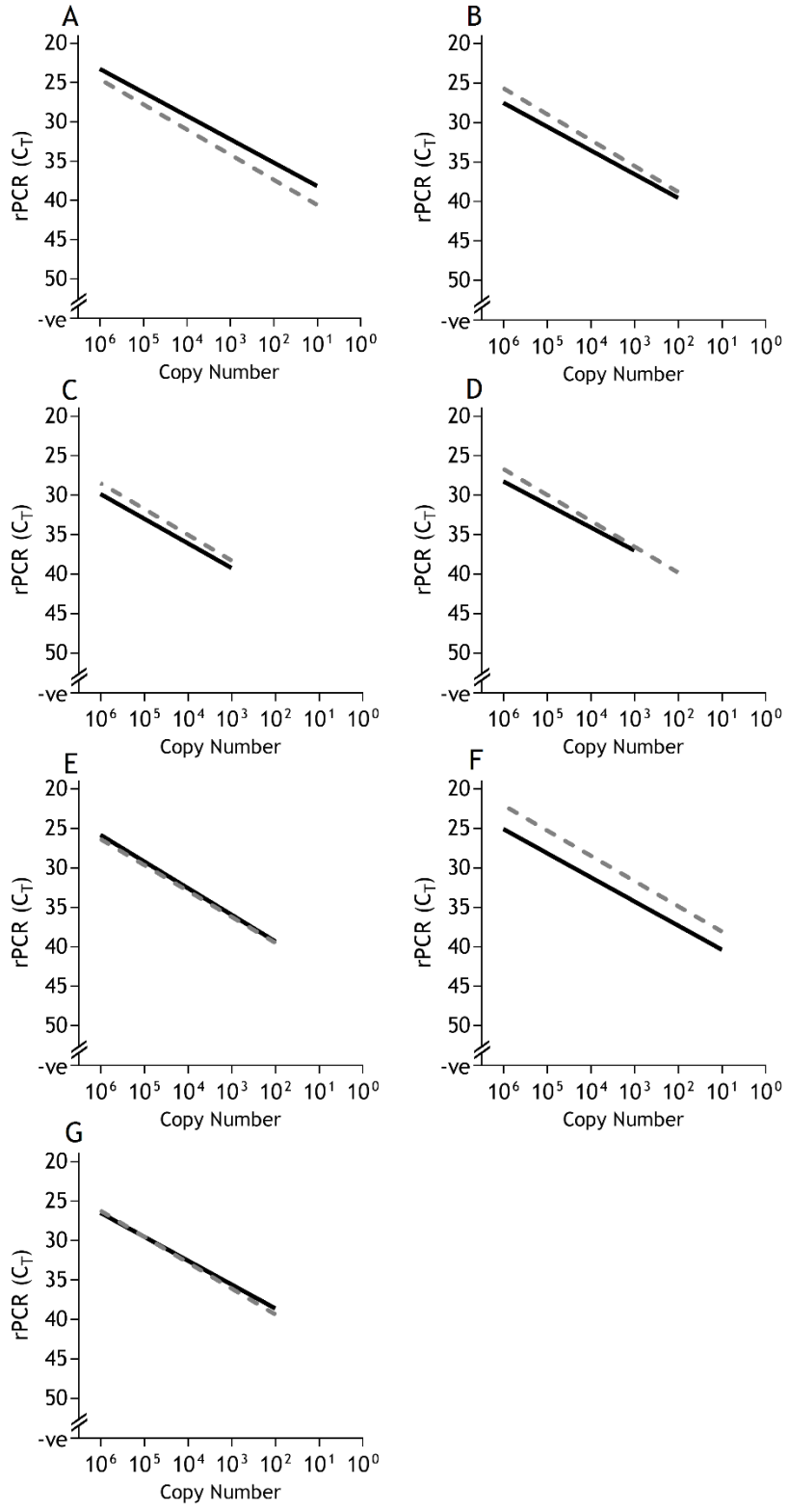


Figure 5.9 Evaluating GoPrime as a predictor of real-time PCR (rPCR) performance using natural sequence variations across different starting quantities of template (10^6-10^0). Each graph represents a different target accession number: (A) JX040500; (B) KC440884; (C) AY593802; (D) KC440883; (E) AY593812; (F) KF112882; (G) HM191257. The black line represents the observed cycle threshold (C_T) values for each template using the Excite™ UF 2x Master Mix. The grey dashed line represents the predicted C_T based on the observed values for the perfect match template plus any ΔC_T predicted.

Alternative target sites within the FMDV genome:

Although GoPrime can only be currently used to evaluate rPCR and two-step rRT-PCR assays (in which RT and PCR stages are separate and mismatches persist through to cDNA), GoPrime was able to identify the likely targets for four FMDV-typing assays (East Africa specific for serotypes A, O, SAT 1 and SAT 2). The targets identified were consistent with previously published results (Bachanek-Bankowska *et al.*, 2016) (Figure 5.10).

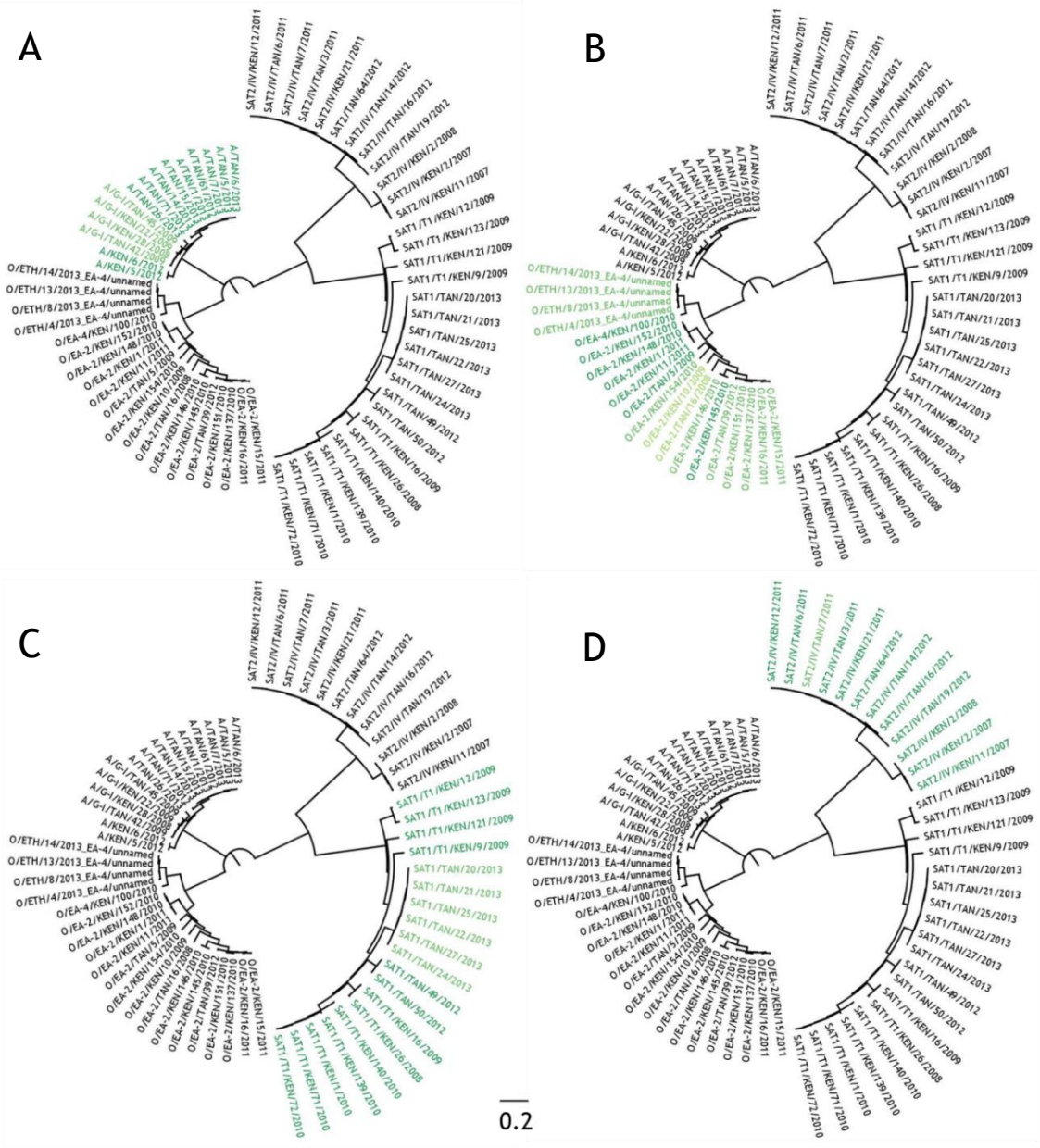


Figure 5.10 Using GoPrime and GoPrimeTree to predict the likely targets of foot-and-mouth disease virus (FMDV)-typing PCR assays (Bachanek-Bankowska *et al.*, 2016) (n = 66). Four primer/probe sets were tested: (A) serotype A; (B) serotype O; (C) serotype Southern African Territories (SAT) 1; (D) serotype SAT 2. For the colour scheme: (●) perfect primer/probe-template match; (●) cycle threshold affected by up to a C_T of 5; (●) cycle threshold affected by up to a C_T of 10; (●) sequence predicted not to amplify.

GoPrime also distinguished between similar targets for the O primer/probe set (East Africa-specific) (Bachanek-Bankowska *et al.*, 2016) when challenged with 24 serotype O viruses from outside of East Africa (Figure 5.11).

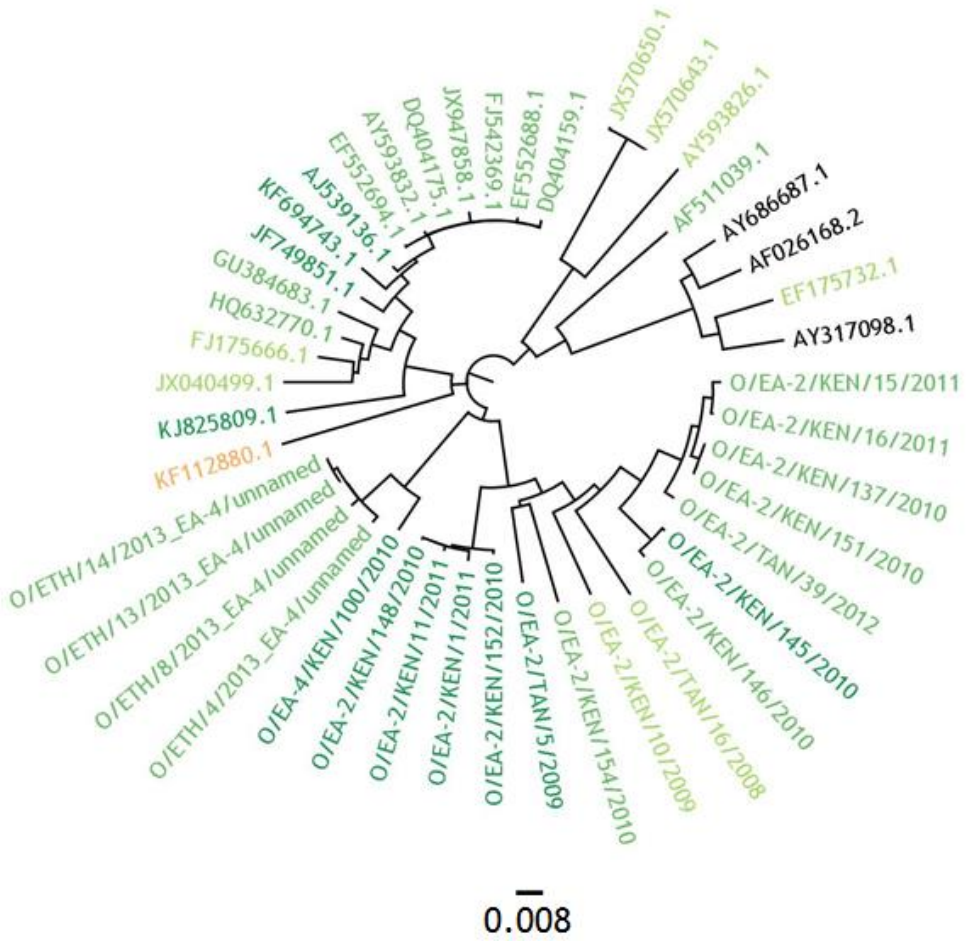


Figure 5.11 Using GoPrime and GoPrimeTree to predict the specificity of foot-and-mouth disease virus (FMDV)-typing PCR assays. Serotype O primers (Bachanek-Bankowska *et al.*, 2016) were evaluated against 20 East African FMDVs (as published in Bachanek-Bankowska *et al.*, 2016) and 24 serotype O sequences from GenBank. For the colour scheme: (●) perfect primer/probe-template match; (●) cycle threshold affected by up to a C_T of 5; (●) cycle threshold affected by up to a C_T of 10; (●) cycle threshold affected by up to a C_T of 15; (●) sequence predicted not to amplify.

5.5 Discussion

Primer/probe-template mismatches are often unavoidable in rPCR, leading to the requirement for continual monitoring of primers/probes against available sequence data, to ensure that assays remain fit for purpose. Consequently, the ability to quantitatively evaluate the performance of rPCR primers and probes *in silico* could aid researchers and diagnosticians by rapidly predicting the effects of mismatches present on rPCR amplification, which is not possible using current methods.

Consistent with previous publications, mismatches in the 3'-end of primers had a more detrimental effect on rPCR amplification than those located towards the 5'-end, due to disruption of the DNA polymerase active site (Kwok *et al.*, 1990; Whiley and Sloots, 2005; Bru *et al.*, 2008; Süß *et al.*, 2009; Stadhouders *et al.*, 2010). The effect of single mismatches within the 3'-end region displayed a consistent pattern, related to both nucleotide position and mismatch type. Furthermore, amplification was evident for reactions with multiple mismatches at the 3'-end, which has not been previously reported (Stadhouders *et al.*, 2010), however a minimum of 10^4 template copies was required for amplification to occur. As previously published by Süß *et al.*, (2009), the amount of starting template did not significantly impact the ΔC_T evident from primer-template mismatches. This is likely due to mismatches having the largest impact upon amplification in the first few rPCR cycles (Nogva and Rudi, 2004), as primers (and therefore mismatches) are incorporated into target sequences for subsequent cycles (Süß *et al.*, 2009).

The effect of probe-template mismatches also displayed a consistent pattern in terms of their position within the probe. For example, mismatches in the centre of the probe were shown to impact rPCR more than those at the 5'-end, due to destabilising probe-template annealing (Süß *et al.*, 2009). Single mismatches at the 5'-end had a minimal effect on the C_T of reactions. This has been previously documented (Süß *et al.*, 2009), and is likely due to these mismatches having minimal impact on the 5' nuclease activity of *Taq* DNA polymerase. Again, the amount of starting template did not significantly alter the ΔC_T evident for probe-template mismatches. This concurs with a previous study which showed the efficiency of mismatched probes to remain constant throughout rPCR reactions (Süß *et al.*, 2009).

Although the experimental data gained in this study was specific to the rPCR conditions and primer/probe sequences evaluated, consistent patterns in the effects of mismatches enabled the creation of GoPrime, which was parameterised using experimental observations. When used to predict the effects of mismatches within the same FMDV

genomic region used to develop the program, GoPrime was on average able to predict the ΔC_T 3.15 away from the observed experimental value (n = 97). However, GoPrime was also applicable over a broader genomic context, and was able to predict the likely targets of four FMDV-typing assays which target alternative regions of the FMDV genome. Furthermore, with rPCR assays requiring different performance criteria depending upon their use, the ability of GoPrime to quantitatively predict the effect of primer/probe-template mismatches on both C_T and LOD could help diagnosticians accurately assess whether rPCR assays are fit for their intended use. For instance, some rPCR assays might give positive results in spite of mismatches when high viral loads are present (such as in acute stages of disease), but generate a false-negative in the presence of lower viral loads (such as in oesophageal-pharyngeal fluid or environmental samples).

In addition to rPCR, GoPrime can be used to predict the ΔC_T and ΔLOD for mismatches present in two-step rRT-PCR as the use of Oligo(dT) or random hexamers for the RT stage results in primer/probe-template mismatches persisting though to cDNA. However, GoPrime's use is limited for one-step rRT-PCR where gene-specific primers are used in both the RT and rPCR stages (one primer is incorporated into cDNA). Preliminary analysis was performed on the effect of primer/probe-template mismatches in one-step rRT-PCR and is detailed in Appendix 7b. However, further analysis of the effects of mismatches on one-step rRT-PCR is required before it can be added as a function for GoPrime. For example, different rRT-PCR kits have previously been shown to differ in their tolerance to mismatches (Stadhouders *et al.*, 2010).

In conclusion, this chapter describes the development of GoPrime: a freely available primer evaluation program which predicts the likely performance of primer/probe sets across multiple sequence data. Experimental data suggested that mismatch impacts follow a consistent pattern, enabling GoPrime to be parametrised from experimental observations. Within this chapter, GoPrime was only validated against primers and probes targeting FMDV, a further research avenue would be to challenge GoPrime with alternative targets, to ascertain the prediction accuracy in these circumstances. By providing a novel quantitative approach to primer/probe evaluation, GoPrime offers increased flexibility to the user by not only predicting the likely targets of primer/probe sets but also estimating the effects of any mismatches present on C_T and LOD *in silico*, thereby enabling selection of the most appropriate primer/probe combination given the research question and diagnostic sample.

CHAPTER 6

Discussion and future directions

6.1 Thesis summary

Accurate and rapid diagnostic tests are an essential component of surveillance programs to control and eradicate disease. As such, efforts have been made in the development and deployment of point-of-care tests (POCTs) in order to accelerate diagnostic confirmation and provide flexibility in disease control. For example, within the veterinary field, POCTs have proven effective during the final stages of rinderpest eradication (Brüning *et al.*, 1999), are planned for use in the first stages of Peste des Petits Ruminants eradication in West Africa (OIE/FAO, 2015) and have been proposed as a way to empower field workers to participate in rabies disease surveillance (Markotter *et al*. 2009; Halliday *et al*. 2012; Cleaveland *et al.*, 2014).

The current diagnostic strategy for foot-and-mouth disease (FMD) is centred almost exclusively around dedicated laboratories. However, the utility and practicality of laboratory-based tests, especially during the period of time when scaling-up activities are required in emergency outbreak situations, and when considering routine diagnostics in resource-poor settings, can be limited by factors including (i) logistically complicated sample transportation to centralised facilities, (ii) requirement for diagnostic infrastructure and (iii) the need for highly trained personnel. POCTs offer a promising solution to this problem as they are developed for use within decentralised settings, such as at the pen-side and in low-resource laboratories.

6.1.1 Drivers for the development of point-or-care tests

The ability to detect FMD virus (FMDV) in challenging environments presents multiple opportunities for FMD diagnostics. For example, the use of POCTs during FMD outbreaks in normally disease free countries could help to improve the readiness of crisis management by increasing the efficiency of local control and surveillance measures. Within these scenarios, POCTs could be used to rapidly confirm the presence of disease, for rapid reporting and decision making, in advance of confirmation and further characterisation within laboratory settings (Figure 6.1). This in turn could enable evidence-based support for culling of livestock, as opposed to using methods such as “slaughter on suspicion” which were implemented during the UK 2001 FMD outbreak (Anderson, 2002). Furthermore, POCT could be useful in outbreak scenarios where the transport of samples to centralised facilities is logistically difficult. As such, the development of simple and portable diagnostic devices has been recommended within

independent reports that have followed recent FMD outbreaks (Anderson, 2002; Anon, 2002a; 2002b).

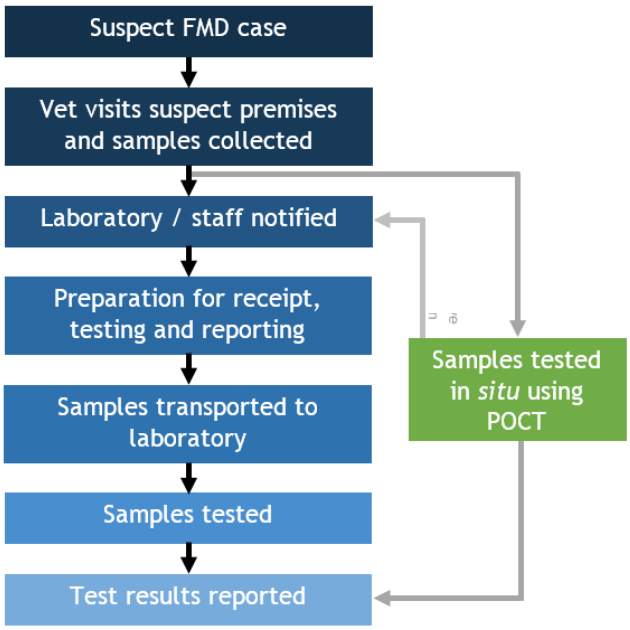


Figure 6.1 World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) contingency plan for UK outbreaks. Black arrows symbolise the current contingency plan (WRLFMD, 2016a), grey arrows symbolise how point-of-care tests could be implemented into current contingency plans, in providing a tool to accelerate diagnostic confirmation and help gain rapid control.

POCTs also have the potential to immediately improve the diagnostic capacity within low-to-middle income countries (LMICs) with endemic FMD infection. Laboratories within these regions are motivated to participate in FMDV surveillance, however are often constrained by poor infrastructure, resulting in an inability to diagnose FMD adequately (Vosloo *et al.*, 2002; Paton *et al.*, 2009). For example, within the Eastern Africa Regional Laboratory Network (EARLN), a network created for the exchange of scientific knowledge related to the control of FMD, only 3 out of 13 FMD diagnostic laboratories routinely use molecular-based assays, with no laboratory established for the systematic characterisation of FMD outbreaks (Namatovu *et al.*, 2013). This, in combination with the logistical challenges associated with transporting samples from the field to appropriate laboratories, results in the majority of FMD outbreaks in East Africa remaining unrecorded (Vosloo *et al.*, 2002). For example, although samples from these countries can be submitted for free characterisation (up to 50 samples per year) at the World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD, The Pirbright Institute, UK), no samples from Tanzania or Kenya have been received in the last two years (WRLFMD, 2016b).

These missed opportunities to detect and characterise circulating FMDV serotypes hinder LMICs advancements along the Progressive Control Pathway for FMD (PCP-FMD), a global strategy led by the World Organisation for Animal Health (OIE) and Food and Agriculture Organization of the United Nations (FAO) to assist and facilitate countries where FMD is still endemic to progressively reduce its impact. With total FMD control unlikely for East Africa in the near future, due to factors such as poor infrastructure, social customs/attitudes and highly complex epidemiology (Vosloo *et al.*, 2002; Ayebazibwe *et al.*, 2010b; Maree *et al.*, 2014), a progressive approach for FMD control is required, with surveillance necessary at all stages (Sumption *et al.*, 2012) (Figure 6.7). Therefore, increasing diagnostic capacity is now considered a priority of the EARLN, especially in regard to molecular capabilities (Namatovu *et al.*, 2013).

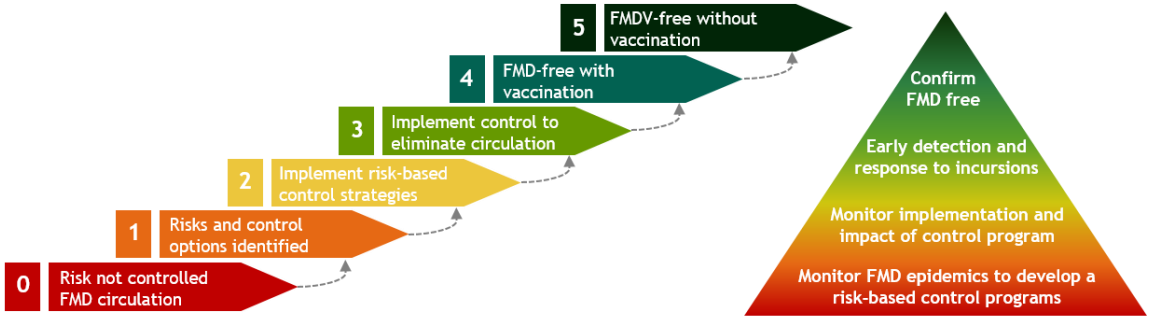


Figure 6.2 Progressive Control Pathway for Foot-and-Mouth Disease (PCP-FMD). Adapted from Sumption *et al.* (2012).

The availability of simple-to-use, portable devices, such as the T-COR™ 8 (Tetracore Inc., MA, USA) and Genie® II (OptiGene Ltd., Horsham, UK), offer a simple solution for molecular diagnostics, regardless of the setting. However, in order to transition such platforms into use, resolutions are required to fill significant gaps that limit the use of POCTs. These include strategies to (i) overcome sample preparation bottlenecks, (ii) ensure confidence in POCT diagnostic performance, (iii) stabilise reagents, (iv) streamline assay protocols, (v) guarantee quality control, (vi) evaluate if assays are fit for purpose and (vii) transition assays into current diagnostic strategies. The overarching aim of this thesis was therefore to create an end-to-end molecular toolbox for the detection and characterisation of FMDV in challenging environments, such in the field or low-resource laboratories (Figure 6.3). Through the development of solutions for the limiting factors listed above, this thesis provides robust evidence that simple POCTs can be successfully deployed for the rapid detection and characterisation of FMDV in decentralised settings.

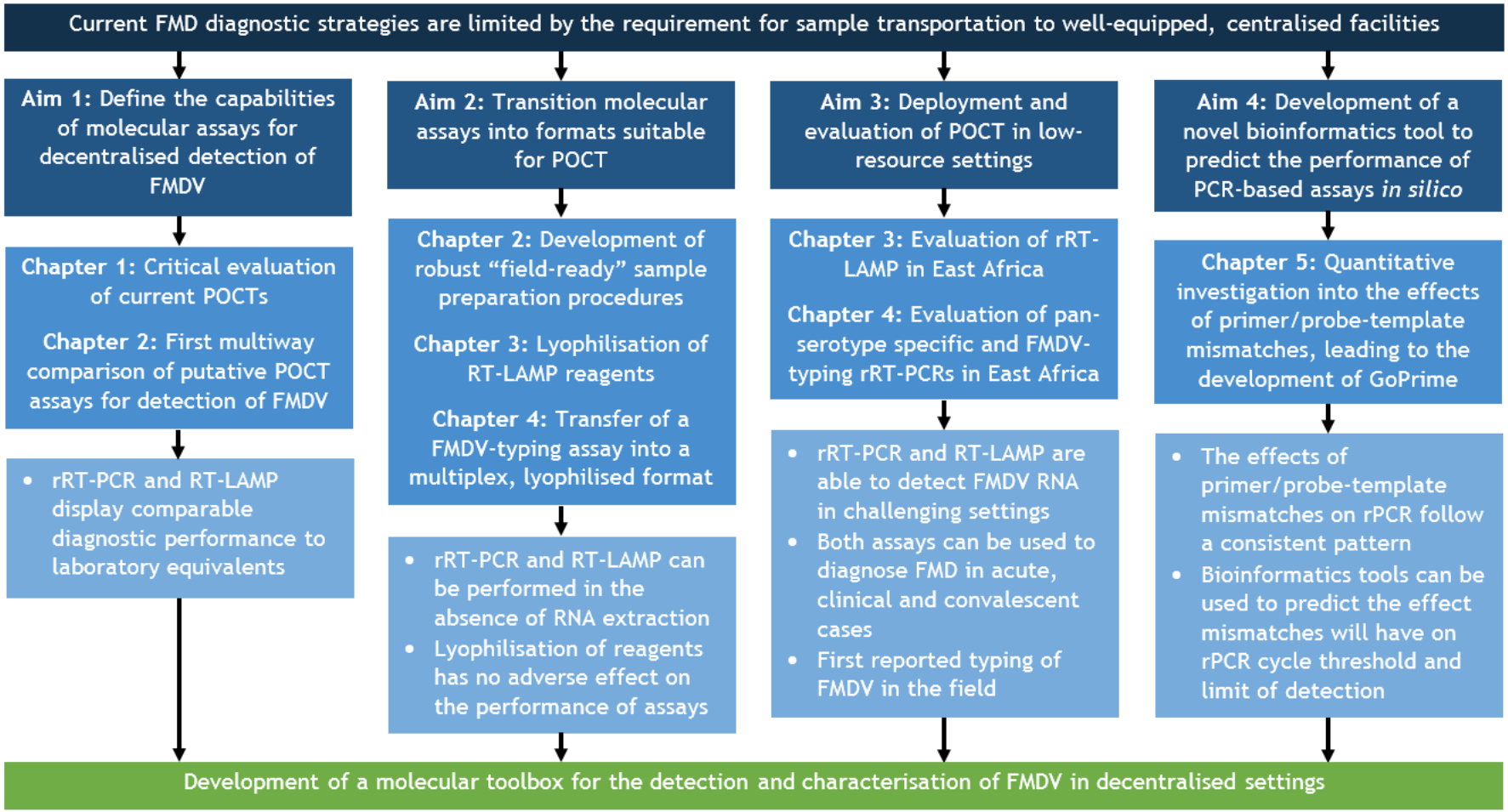


Figure 6.3 Thesis aims, chapters and conclusions.

6.2 Overcoming sample preparation bottlenecks

Technologies for POCTs have existed for a number of years, however one major bottleneck that remained was the development of sample preparation techniques suitable for use in low-resource settings (Dineva *et al.*, 2007). Although a number of simple sample preparation methods have been published (Waters *et al.*, 2014), limited attempts have been made to combine these techniques with FMDV-specific POCTs. This thesis addressed this through the first multiway comparison of 11 sample preparation methods across seven assay formats, concluding that reverse transcription loop-mediated isothermal amplification (RT-LAMP) and real-time reverse transcription PCR (rRT-PCR) can be performed in the absence of RNA extraction, with reliable performance achieved using the dilution of samples in nuclease-free water (NFW) (Howson *et al.*, 2017b).

Importantly, results demonstrate that the dilution of serum, epithelial suspensions, mouth/feet swabs and oesophageal-pharyngeal fluid (OP fluid) in NFW enables FMDV RNA to be detected across a large diagnostic window, with pan-FMDV diagnosis of cattle possible in the clinical, as well as convalescent stages of FMD infection (including the capability to detect FMDV in OP fluid two months after initial infection) (Howson *et al.*, 2017c; 2017d). Furthermore, these methods permit *in situ* characterisation, with FMDV serotype able to be determined from all above sample types, in cattle two to seven days post initial infection (using FMDV-positive samples with a pan-FMDV rRT-PCR cycle threshold [C_T] value of less than 29) (Howson *et al.*, 2017d).

In addition to sample preparation bottlenecks, over the last few years, a large emphasis has been placed on the development of non-invasive sampling procedures, such as the use of rope chews for collection of oral fluids (Mouchantat *et al.*, 2014) and aerosols (Ryan *et al.*, 2009; Waters *et al.*, 2014). Initial investigation into use of FTA® cards for "non-invasive" sampling and low-cost transportation has also been performed (Muthukrishnan *et al.*, 2008), however the technique published still requires wiping of FTA® cards across open mouth lesions. Building upon these studies, this thesis demonstrated that it was possible to detect FMDV RNA in saliva collected non-invasively onto FTA® cards by simply wiping them across the chin and lips of cattle. This promising finding should be investigated further to determine the clinical detection window for this approach.

Combined, these efforts can be used to reduce the sample preparation bottleneck, and future work should be aimed at transitioning these methods to other important susceptible species, especially those in which clinical signs are more discrete and therefore more challenging to detect.

6.3 Ensuring confidence in diagnostic performance

A further inhibitor, with regard to the use of POCTs, is scepticism over whether decentralised tests can produce comparable results to traditional laboratory-based diagnostics. For example, the antigen-detection lateral-flow device (Ag-LFD), which is one of the most recognised POCT platforms, is widely published to have reduced analytical sensitivity comparatively to molecular methods (Metwally *et al.*, 2010). Consequently, misconceptions can arise over the performance of POCTs in general.

Data in this thesis challenges this scepticism, revealing that the transition of laboratory-based assays into POCT-formats does not negatively impact upon assay performance. For instance, pan-FMDV POCT rRT-PCR displays equivalent sensitivity to the reference rRT-PCR, and even in the absence of RNA extraction is comparable to the reference rRT-PCR in the presence of a diagnostic cut-off (Shaw *et al.*, 2007). Although comparable to their laboratory based-equivalents, POCT RT-LAMP and FMDV-typing rRT-PCR assays are comparable to the reference rRT-PCR only when considering a diagnostic cut-off value and using extracted RNA (Howson *et al.*, 2017c; 2017d). Further work is therefore required in order to understand whether comparable diagnostic cut-offs for POCTs are required, or whether cut-off values are useful only for laboratory settings.

In light of this, RT-LAMP and FMDV-typing rRT-PCR POCTs are most likely to be used for confirmation and characterisation of acutely infected animals in cases where rapid diagnosis is required (Figure 6.4). Conversely, pan-FMDV POCT rRT-PCR is able to report a C_T value in weaker samples, and is therefore suitable both for confirmation of negative samples and detection of FMDV across the diagnostic window from pre-clinically infected through to convalescent animals (Figure 6.4).

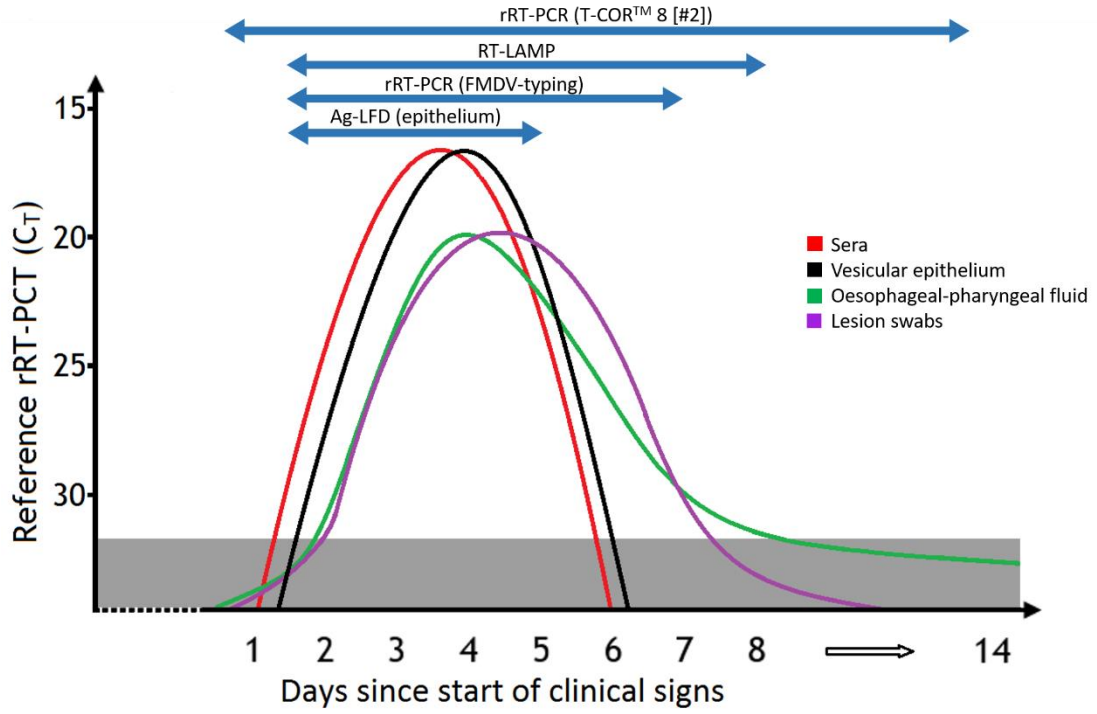


Figure 6.4 The proposed use of point-of-care tests across the foot-and-mouth disease (FMD) diagnostic window. (red) sera; (black) vesicular epithelium; (green) oesophageal-pharyngeal fluid; (purple) mouth/foot swabs. The dotted x-axis line represents the incubation period, which is dependent upon: infectious dose, viral strain, animal species and host determinants. Based on data from Alexandersen *et al.* (2003a) and King *et al.* (2012). (rRT-PCR) real-time reverse transcription PCR; (rRT-LAMP) real-time reverse transcription loop-mediated isothermal amplification (grey shaded area represents cycle threshold [C_T] values over the diagnostic cut-off [Shaw *et al.*, 2007]); (Ag-LFD) antigen-detection lateral-flow device (suitable for use with epithelium and vesicular fluid only).

6.4 Stabilisation of reagents

Assays for use in decentralised settings must be able to incorporate reagents compatible with the environmental conditions found in the countries of deployment. However, the majority of previously published FMDV-specific assays utilise wet reagents, containing temperature sensitive enzymes. These present a challenge for long term storage in disease-free countries (contingency planning) or use in endemically infected countries where electricity supply can be unreliable and erratic (absence of a reliable cold chain).

This thesis addressed this through robust evaluations of lyophilised reagents for (i) pan-FMDV RT-LAMP assays, (ii) a commercially available lyophilised pan-FMDV rRT-PCR assay, and (iii) an East Africa-specific FMDV-typing rRT-PCR assay. Significantly, results indicate that reagent lyophilisation does not negatively impact upon assay performance, even when used in high-ambient temperature settings, with all lyophilised assays displaying

comparable analytical sensitivity to their wet equivalents (Howson *et al.*, 2017c; 2017d).

Importantly, field-work highlighted a number of additional benefits associated with the lyophilisation of reagents. For instance, lyophilisation helps to simplify transportation requirements, as reagents do not require any form of a cold chain. This ultimately reduces shipping costs (Table 6.1), without altering the integrity and performance of the reagents. In light of this, lyophilisation of reagents should not only be considered for other FMD diagnostic methods, but also beyond to other disease surveillance programs.

Table 6.1 Price of shipping molecular kits to East Africa

Origin	Destination	Kit format	Requirements	Shipping time	Shipping cost*
OptiGene Ltd., UK	SUA, Tanzania	RT-LAMP wet	-80°C ice bricks	Three days	GB £ 189
OptiGene Ltd., UK	SUA, Tanzania	RT-LAMP dry	None	Three days	GB £ 114
Tetracore Ltd., USA	SUA, Tanzania	rRT-PCR wet	Dry ice	Four days	GB £ 816
Tetracore Ltd., USA	SUA, Tanzania	rRT-PCR dry	None	Four days	GB £ 248

*Prices quoted from DHL (https://www.dhl.com) on 17/07/2017

6.5 Streamlining assay protocols

In order to successfully utilise POCTs in decentralised settings, assays must be in the format of a "ready-to-use kit" and utilise protocols suitable for use in challenging environments. Previously, the only reported evaluations of FMDV-specific POCTs in field-settings were limited to rRT-RPA, which required an RNA extraction step (Abd El Wahed *et al.*, 2013), and Ag-LFDs during the UK 2007 FMDV outbreak (Ryan *et al.*, 2008).

Building upon these initial studies, molecular assays in this thesis were modified to utilise simple field-ready protocols (outlined in Appendix 8) (Figure 6.5). In addition, while the majority of molecular tests ideally require (i) dedicated spaces for reagent preparation, RNA extraction and template addition, (ii) uninterrupted mains power for cold-storage of reagents and powering of platforms and (iii) skilled laboratory staff for assay assembly and result analysis, the POCTs developed and evaluated in this thesis alleviate these constraints by:

1. Negating complex sample preparation as RNA extraction is not necessary.
2. Streamlining assay set-up by reducing the pipetting stages.
3. Minimising the potential for cross-contamination by using closed systems such as real-time detection or the AMPlite® (Appendix 4).
4. Using battery powered platforms (which can be charged using car batteries), removing the need for reliable mains power.
5. Using platforms that automatically analyse and report results.

Importantly, in these formats, assays were able to (i) rapidly detect FMDV in the field and low-resource laboratories (30 minutes for rRT-LAMP and 80 minutes for rRT-PCR) and (ii) identify FMDV serotype for the first time *in situ* (Howson *et al.*, 2017c; 2017d). Furthermore, preliminary evaluation of the AMPlite® (APHA, Addlestone, UK), concluded RT-LAMP combined with automated lateral-flow detection (RT-LAMP-LFD) can be reduced to a single pipetting stage without compromising assay performance (Appendix 4).

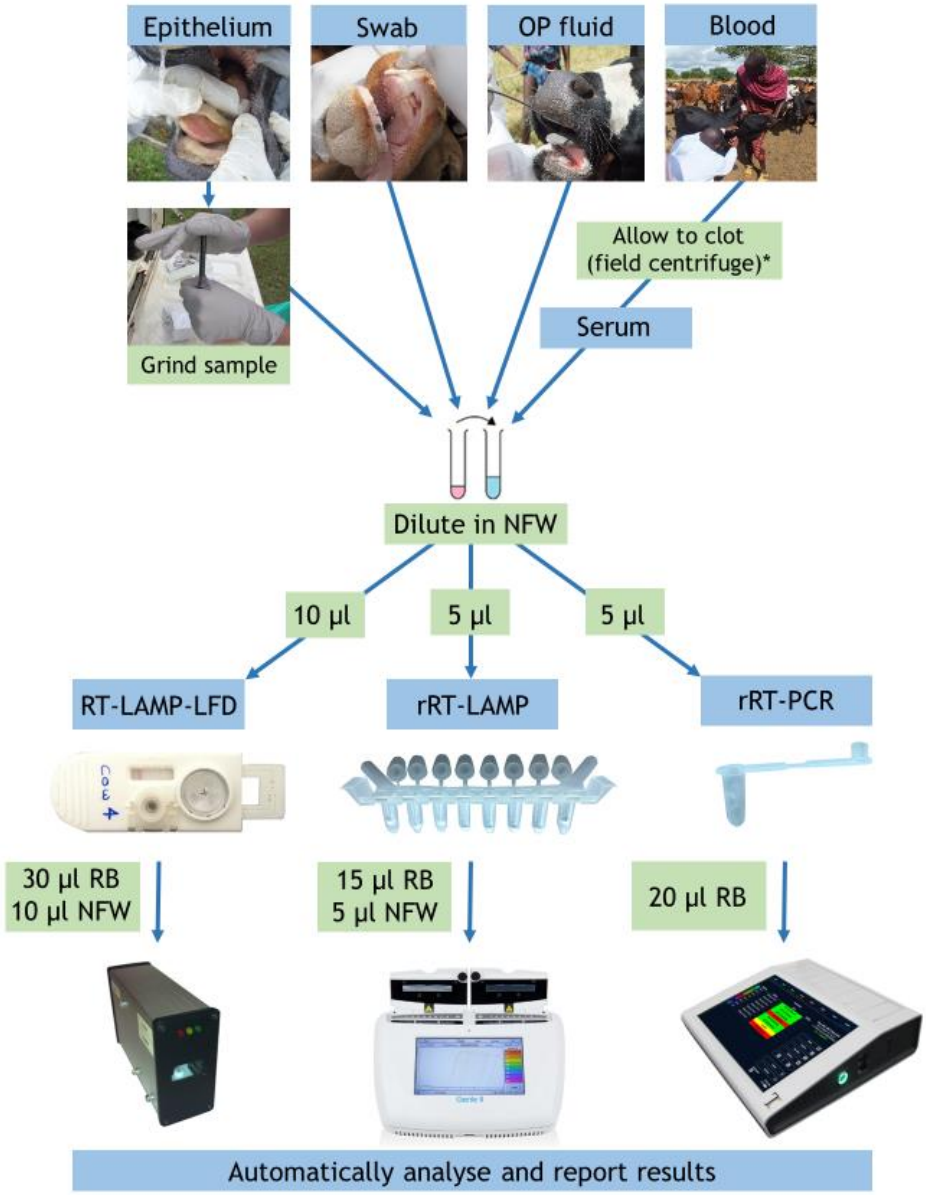


Figure 6.5 Point-of-care test workflow for use in challenging settings. (rRT-PCR) real-time reverse transcription PCR; (rRT-LAMP) real-time reverse transcription loop-mediated isothermal amplification; (RT-LAMP-LFD) reverse transcription loop-mediated isothermal amplification with lateral-flow detection; (RB) resuspension buffer; (NFW) nuclease-free water; (OP fluid) oesophageal-pharyngeal fluid. *optional field centrifuge.

By streamlining protocols, a generic diagnostic approach becomes achievable whereby one method can be used for the detection of multiple diseases. For instance, knowledge gained during the lifespan of project has enabled Tetracore to develop a number of additional POCTs to differentiate between a panel of vesicular diseases (Appendix 9). Opportunity exists for similar expansion of the Genie® II, as this platform is already being used for screening in customs settings (OptiGene Ltd., 2017); supplementation of new assays (including the availability of lyophilised core regents where users add their own primers) would not be logistically challenging should new emerging threats arise.

6.6 Guaranteeing quality control

Quality control of POCTs is often thought of as a complex issue, as performing assays outside of recognised quality assurance systems (such as ISO/IEC 17025), may give rise to concerns regarding test confidence and result acceptance. Consequently, although many FMDV-specific POCTs are published in peer-reviewed journals, none yet appear in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012), with the exception of Ag-LFDs. This thesis has begun to address this through (i) designing molecular assays that contain inbuilt quality checks, and (ii) providing initial validation data for POCT use.

Notably, data presented in this thesis demonstrates that the addition of an exogenous internal control into rRT-PCR (T-COR™ 8 [#2]) and RT-LAMP-LFD (AMPlite®), does not impact upon analytical sensitivity and would reliably report false-negative reactions if subject to inhibition (Howson *et al.*, 2017d). Furthermore, the addition of an anneal curve to rRT-LAMP, which reports an amplicon specific anneal temperature, ensures that non-specific amplification, and consequent false-positive results, are highlighted (Howson *et al.*, 2017c). Importantly, using these inbuilt controls during field work, out of 145 samples (from 60 cattle over 10 farms in 2 countries) analysed using RT-LAMP and 144 samples (from 78 cattle over 13 farms in 3 countries) analysed using rRT-PCR, no false-positives were detected and results were consistent with clinical observations and the reference rRT-PCR. Notably, all assays tested display 100% specificity when challenged with alternative vesicular disease viruses.

As a result, it would be a worthwhile exercise to generate further validation data, in order to produce a dossier for consideration of inclusion of these tests in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012). Indeed, such an exercise

would enhance initial efforts that have already been pursued within this area. For example, as part of an EU funded project (RAPIDIA-FIELD, 2017), small validation exercises have been performed on RT-LAMP and cartridge based rRT-PCR (Enigma® Mini Laboratory: Enigma® Diagnostics, Porton Down, UK), with high consensus in results reported between a number of European and African laboratories (Goller *et al.*, 2017).

6.7 Evaluating if assays are fit for purpose

Although molecular-based assays are well accepted for the diagnosis of FMD, the high mutation rate associated with RNA viruses can result in primer/probe-template mismatches often being unavoidable. Assays therefore require continual evaluation to ensure they remain fit for purpose. Data generated in this thesis revealed that the effects of primer/probe-template mismatches on real-time PCR (rPCR) C_T and limit of detection (LOD) follow a consistent pattern. These experimental results were used to parameterise the mathematical model behind GoPrime, a freely-available (Orton, 2017), novel primer/probe validation program which is able to predict the performance of PCR-based assays *in silico*.

When screening 97 templates against the Callahan *et al.* (2002) primers and probe sequences, GoPrime was on average able to predict the effect of mismatches on C_T to be 3.15 away from the observed experimental value, and accurately predicted the targets of four previously published FMDV-typing assays (Bachanek-Bankowska *et al.*, 2016) across 66 sequences. With an increasing number of rRT-PCR publications for detection of FMDV, in addition to the continual increase in sequence data available online, GoPrime has the potential to rapidly evaluate whether primer and probe sequences are fit for purpose with sufficient coverage to detect circulating FMDV strains.

6.8 Transitioning assays into current diagnostic strategies

Transitioning of POCTs into current diagnostic strategies needs to be considered to determine how these assays could work in parallel with, or replace, existing methodologies, as without proper management, decentralisation of testing could put further stress on veterinary systems. In foreseeing this problem, POCT protocols for human diagnostics have been integrated into contingency plans, such as the World Health Organization (WHO) recommendations on the use of rapid testing for human influenza

diagnosis (WHO, 2005). In order to follow in the footsteps of such strategies, and achieve recognition for the simple molecular POCT protocols developed within this thesis (Appendix 8), it would be an advisable exercise to carry further assay validation by following the "Principles and Methods for Validation of Diagnostic Assays for Infectious Diseases" as recommended by the OIE (OIE, 2012 [Chapter 1.1.4/5]).

By deploying POCTs into diagnostic strategies, the implementation of data sharing and storage procedures are also necessitated, to ensure instant reporting and monitoring of results. Some of the newer POCT platforms, such as the T-COR™ 8 and Genie® III, are equipped with data transmission capacity, enabling real-time reporting to laboratories. Furthermore, with programs such as EpiCollect now freely-available (which utilises mobile phones for data entry), results can easily be collated in internet databases for centralised analysis (Aanensen *et al.*, 2009). For example, EpiCollect has now been utilised in preliminary trials with Kenyan Maasai farmers to monitor diseases of veterinary importance (FAO, 2010). The advent of the "Internet of Things" (IoT), an interconnection of physical devices with network infrastructure to enable collection and exchange of data (Xia *et al.*, 2012), may further revolutionise decentralised testing. By connecting POCT through internet-based software, which is a current initiative of Toshiba for the Genie® III, real-time data exchange and analysis can be automated, negating the requirement for manual report preparation.

6.9 Future research directions

The development of a molecular toolbox, suitable for decentralised detection of FMDV, has inevitably raised a number of new questions and opened up directions for future research. In particular, there are two main areas which require further investigation.

6.9.1 Application of point-of-care tests to answer FMD research questions

Recent data have suggested that FMD outbreaks occur as continuous repeating patterns of alternating serotypes, driven by livestock movements and livestock contact networks (Casey-Bryars, 2016). If true, this suggests that infection cycles may be predictable, in terms of temporal and spatial patterns of serotypic dominance, opening up possibilities and opportunities for disease control. For example, although vaccination is cited as an existing FMD control strategy for East Africa (Hunter, 1998; Rweyemamu and Astudillo, 2002), the ability to predict waves of serotypic dominance would improve the

effectiveness of vaccination strategies (Vosloo *et al.*, 2002; Mumford, 2007). However, further investigation of this hypothesis is difficult considering the current diagnostic capacity in East Africa (Namatovu *et al.*, 2013). The lyophilised FMDV-typing assay and T-COR™ 8 offer a new avenue to investigate this hypothesis, as FMDV serotype can be determined in under two hours (from sample collection to result calling) in either laboratory or field-settings. Furthermore, with the communities of East Africa having previously shown effective engagement with disease control programmes, such as rabies control in northern Tanzania (Cleaveland *et al.*, 2003) and Rinderpest eradiation throughout East Africa (Mariner *et al.*, 2012), such enthusiasm could enable such hypothesis testing and FMD surveillance to be performed.

6.9.2 Extending GoPrime to alternate molecular assays

The current version of GoPrime allows researchers to quantitatively evaluate the performance of rPCR primers and probes *in silico* across multiple sequence data. However, parameterisation of GoPrime should be extended to include other molecular assays, such LAMP. The complex primer requirements for LAMP result in primer-template mismatches often being unavoidable, as four to six primers are necessary to target six to eight sequence regions (Notomi *et al*. 2000; Nagamine *et al*. 2002). Very limited work has been conducted into investigating the effects of these mismatches (Wang, 2016), and further research is necessary. Therefore, the study conducted in Chapter 5 should be expanded, firstly to ascertain the effects of each mismatch type on LAMP performance, and secondly to include and evaluate this as a function of GoPrime. Furthermore, GoPrime has been designed for rPCR in such a way to accommodate any primers, probes and targets, regardless of the pathogen, however validation within this thesis has been limited to primers and probes which target FMDV. Consequently, a further research avenue would be to challenge GoPrime with alternative targets, to ascertain the prediction accuracy in these circumstances.

6.10 Concluding remarks

Overall, this thesis has demonstrated that sophisticated molecular assays can be simplified into formats which can be used easily and quickly by unskilled staff, with the potential to be rolled out immediately into FMDV outbreak scenarios, or within endemic FMD settings where there is often a high turnover of staff.

Predictions indicate that the POCT market will increase in value to US$ 38.2 billion by 2024 (researchandmarkets.com, 2017), demonstrating a real commitment of investors and stakeholders towards implementation of these technologies. Even within the duration of this project, technologies have advanced so rapidly that prototype devices, which combine automated sample collection, are beginning to appear on the horizon (Regen *et al.*, 2008; Figure 6.6). With continuous investment and progression being made within the field of POCTs, it is only a matter of time before decentralised detection of FMDV becomes a practical reality.

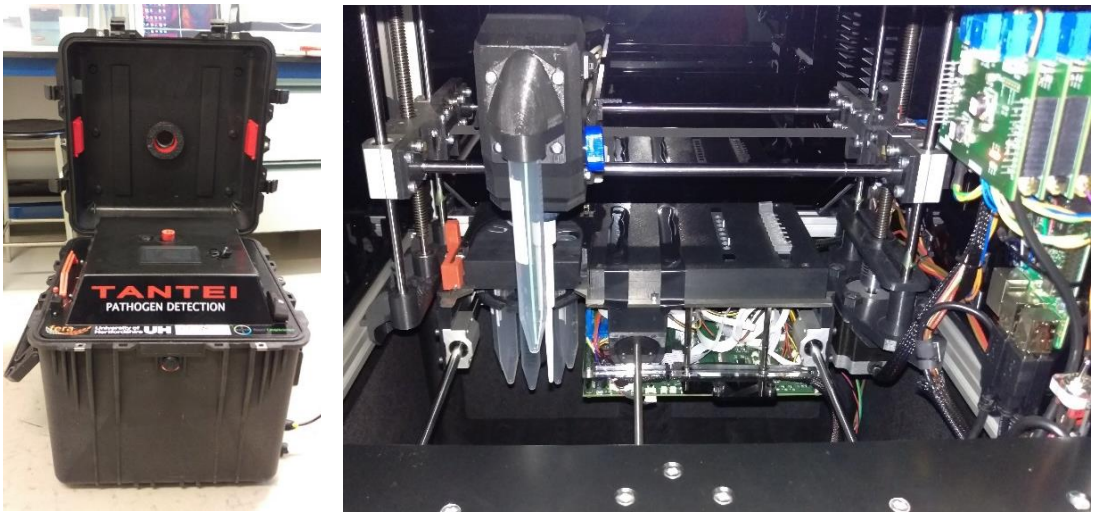


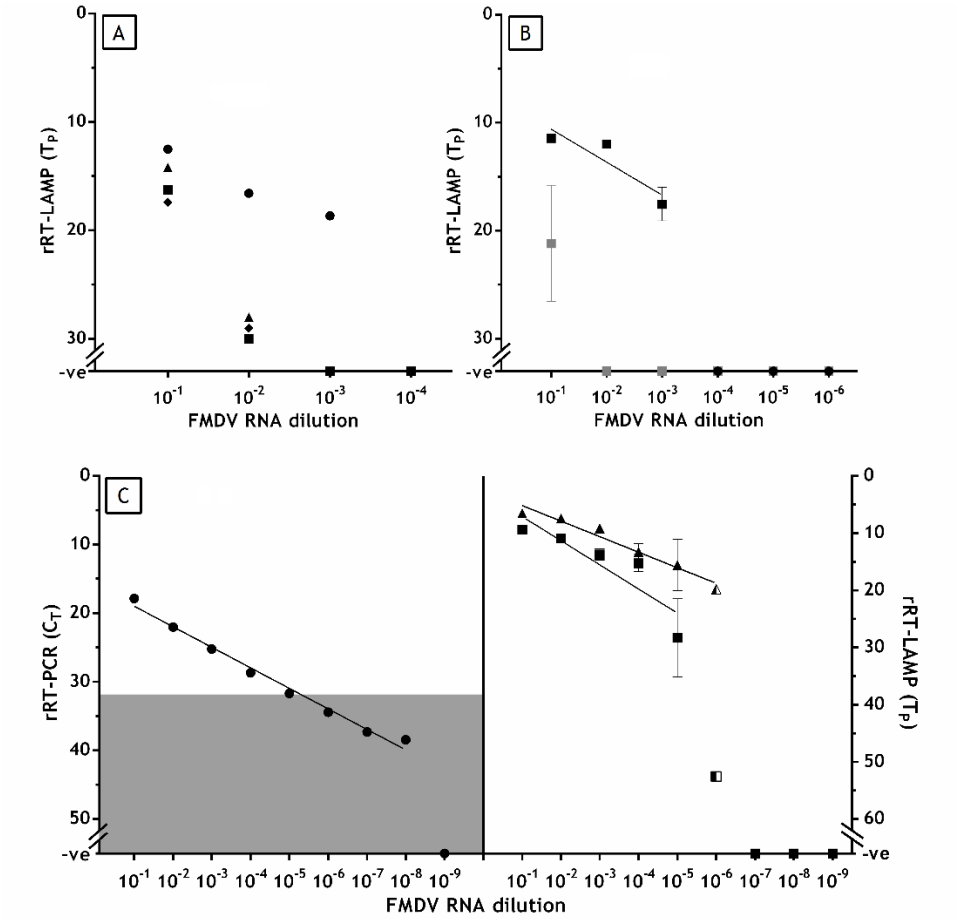
Figure 6.6 The future of point-of-care tests. The Tantei, developed by the Food and Environment Research Agency (FERA), is a fully automated prototype designed for detection of plant pathogens. The platform performs automated air sampling, nucleic acid extraction, loop-mediated isothermal amplification (using lyophilised regents), result interpretation and transmission of results without operator intervention, enabling real-time, remote pathogen surveillance.

Appendices

Appendix 1

Optimisation of isothermal reactions

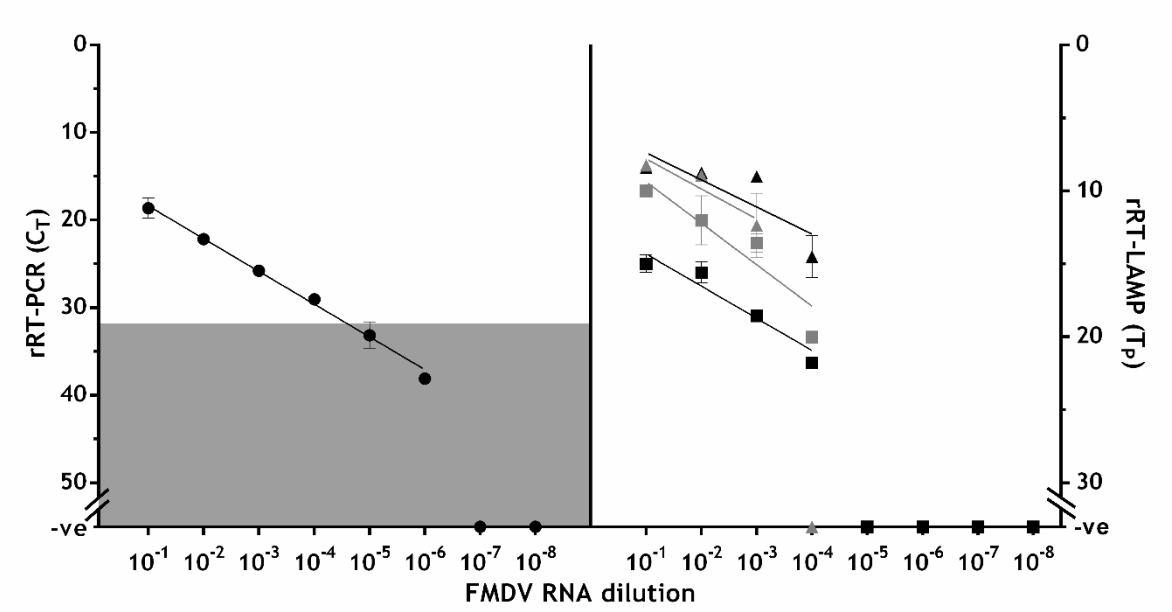
1a) Optimisation of reverse transcription loop-mediated isothermal amplification using wet reagent formats (Chapter 2):



RT-LAMP-LFD									
ISO-001	+	+	+	+	+	+/-	-	-	-
ISO-004	+	+	+	+	+	+/-	-	-	-

Figure 7.1 Optimisation of reverse transcription loop-mediated isothermal amplification (RT-LAMP) conditions for wet reagents. (A) Primer concentration comparison for ISO-001 real-time RT-LAMP (rRT-LAMP) (internal primers [µM]: loop primers [µM]: external primers [µM]): (●) 2 : 1: 0.2; (▲) 1.6 : 0.8 : 0.2; (■) 1.2 : 0.6 : 0.2; (◆) 0.8 : 0.4 : 0.2. (B) Addition of Avian Myeloblastosis Virus (AMV) reverse transcriptase for ISO-001 rRT-LAMP: (■) rRT-LAMP plus 2 U AMV; (■) rRT-LAMP with no AMV. (C) Reaction time for ISO-001 and ISO-004 reagents: (●) reference real-time RT-PCR (rRT-PCR); (■) ISO-001 rRT-LAMP performed for 60 minutes; (▲) ISO-004 rRT-LAMP performed for 60 minutes. The table presents results for RT-LAMP with end-point lateral-flow detection (RT-LAMP-LFD). All points represent the mean of two replicates (bars display the range), half-shaded points indicate where one replicate was positive and the other negative.

1b) Optimisation of reverse transcription loop-mediated isothermal amplification using lyophilised reagent formats (Chapter 3):



RT-LAMP-LFD								
ISO-001 wet	+	+	+	+	-	-	-	-
ISO-001 lyophilised	+	+	+	+	+/-	-	-	-
ISO-004 wet	+	+	+	+	+/-	-	-	-
ISO-004 lyophilised	+	+	+/-	-	-	-	-	-

Figure 7.2 Selection of reverse transcription loop-mediated isothermal amplification (RT-LAMP) lyophilised reagents. A comparison was performed between wet and lyophilised reagents for real-time RT-LAMP (rRT-LAMP) and RT-LAMP combined with lateral-flow detection (RT-LAMP-LFD) using a dilution series of RNA extracted from a FMDV cell culture isolate (Chapter 2.3.6). Grey shaded area represent cycle threshold (C_T) values over the diagnostic cut-off threshold of C_T < 32 (Shaw *et al.*, 2007). All points represent the mean of two replicates (bars display the range): (■) ISO-001 in a wet format; (■) ISO-001 in a lyophilised format; (▲) ISO-004 in a wet format; (▲) ISO-004 in a lyophilised format. For RT-LAMP-LFD (table): (+) both duplicates positive; (+/-) one duplicate positive; (-) both duplicates negative.

Appendix 2

Comparison of sample preparation methodologies

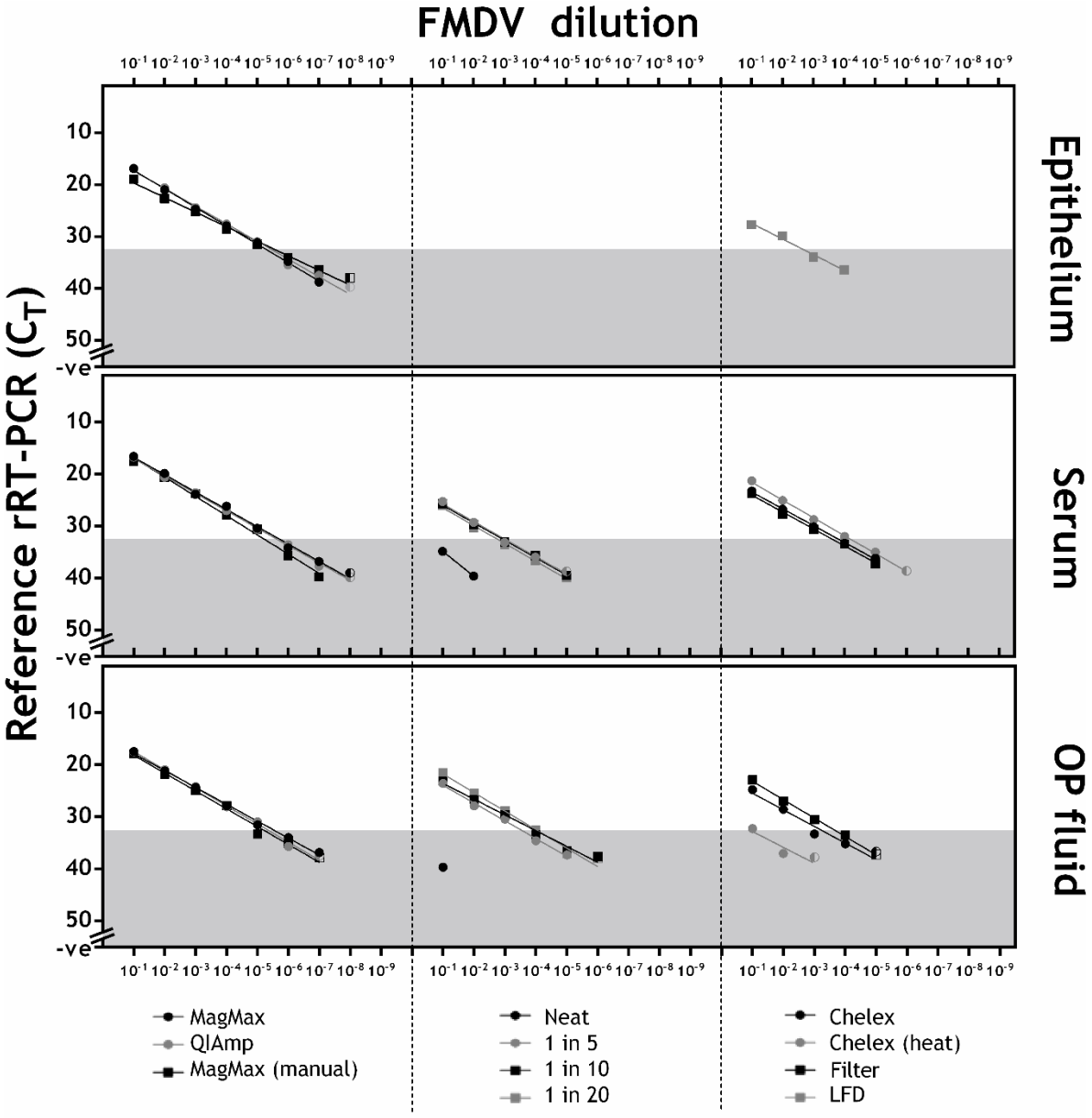


Figure 7.3 Comparison of sample preparation methods for the reference real-time reverse transcription PCR (rRT-PCR). Performed on a bench top real-time PCR machine (Stratagene Mx3005P™). Grey shaded area represent cycle threshold (C_T) values over the diagnostic cut-off threshold of C_T < 32 (Shaw *et al.*, 2007). A total of 11 sample preparation methods were trialled: nine simple preparation techniques, compared to automated robotic extraction and the use of neat samples. Extraction from antigen-detection lateral-flow devices (Ag-LFDs) was trialled for epithelial suspensions only. All points represent the mean of two replicates; half shaded points represent when one replicate was positive and the other negative.

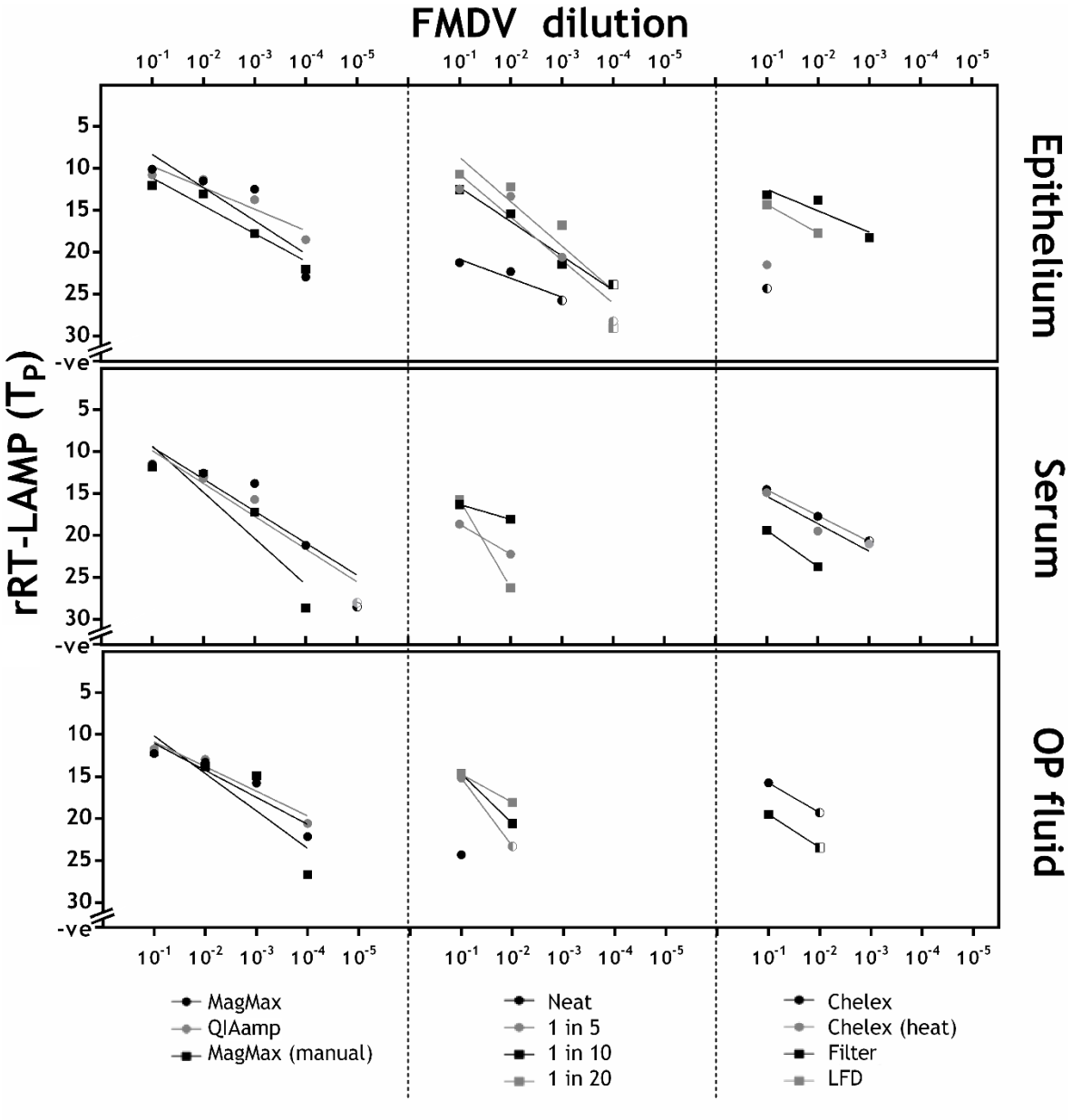


Figure 7.4 Comparison of sample preparation methods for real-time reverse transcription loop-mediated isothermal amplification (rRT-LAMP) (wet reagents). Performed on a Genie® II. A total of 11 sample preparation methods were trialled: nine simple preparation techniques, compared to automated robotic extraction and the use of neat samples. Extraction from antigen-detection lateral-flow devices (Ag-LFDs) was trialled for epithelial suspensions only. All points represent the mean of two replicates; half shaded points represent when one replicate was positive and the other negative.

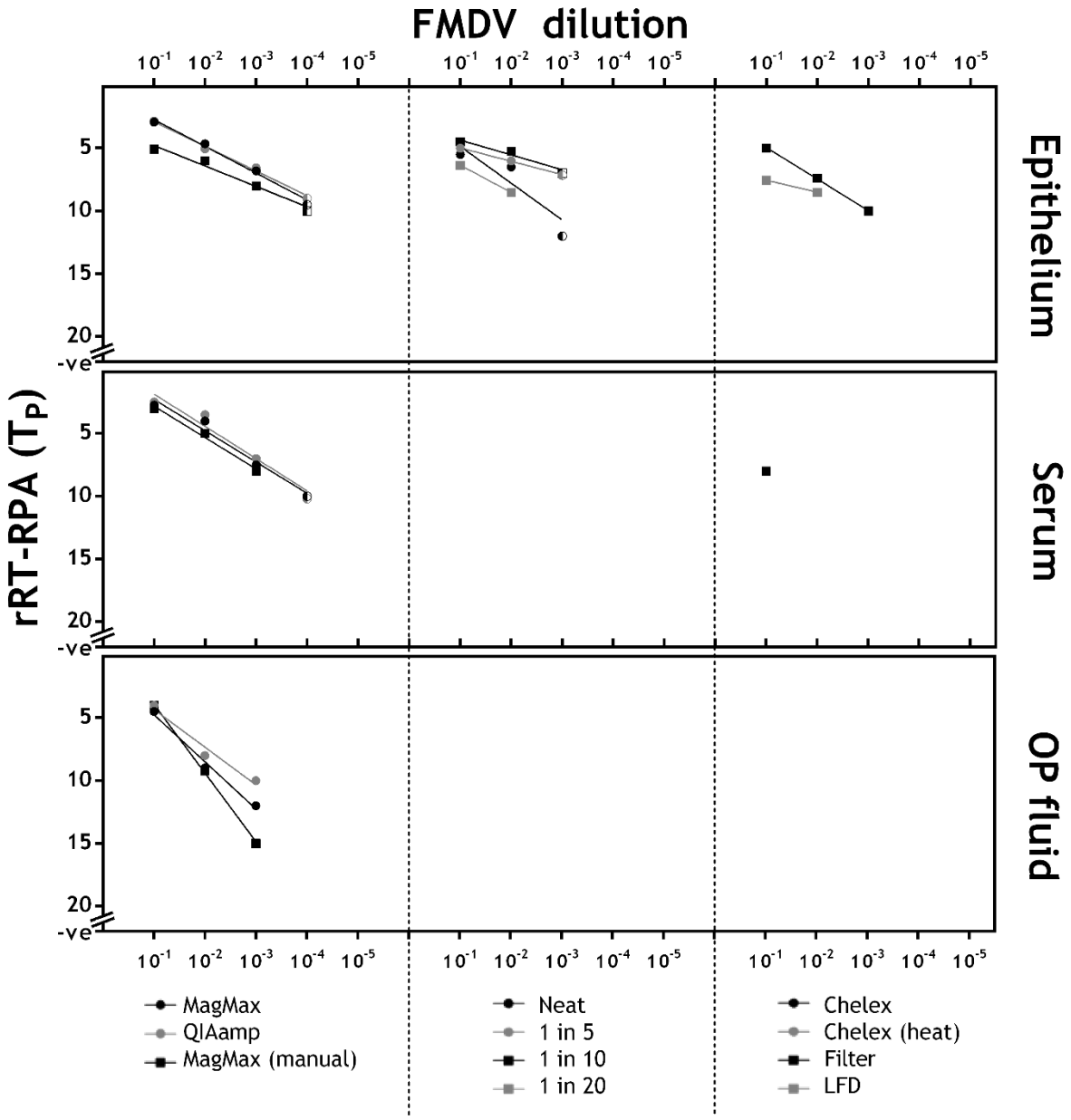


Figure 7.5 Comparison of sample preparation methods for real-time reverse transcription recombinase polymerase amplification (rRT-RPA). Performed using the TwistAmp® exo RT kit, on a Genie® II. A total of 11 sample preparation methods were trialled: nine simple preparation techniques, compared to automated robotic extraction and the use of neat samples. Extraction from antigen-detection lateral-flow devices (Ag-LFDs) was trialled for epithelial suspensions only. All points represent the mean of two replicates; half shaded points represent when one replicate was positive and the other negative.

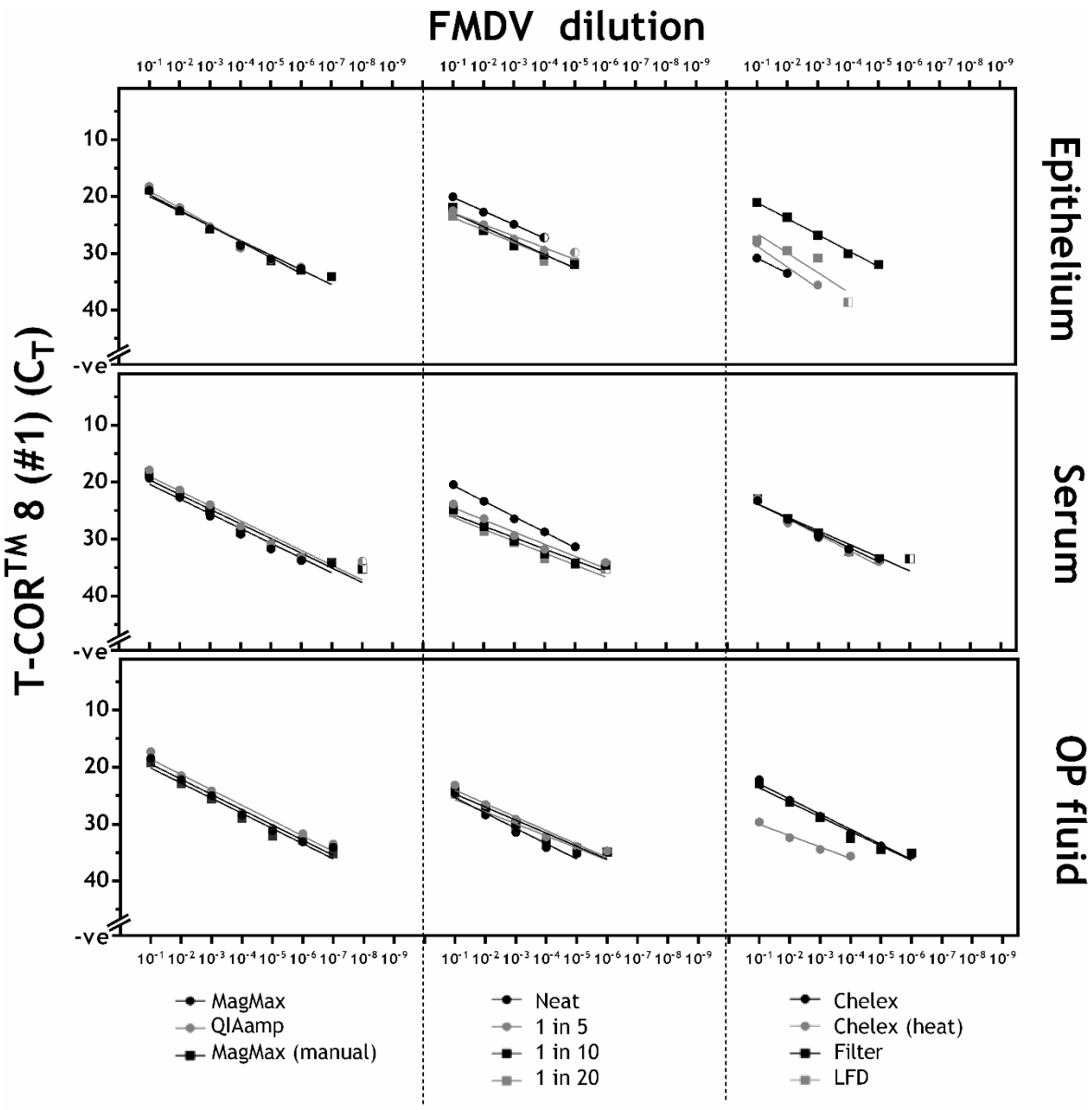


Figure 7.6 Comparison of sample preparation methods for T-COR™ 8 (#1) real-time reverse transcription PCR (rRT-PCR). Performed on a T-COR™ 8. A total of 11 sample preparation methods were trialled: nine simple preparation techniques, compared to automated robotic extraction and the use of neat samples. Extraction from antigen-detection lateral-flow devices (Ag-LFDs) was trialled for epithelial suspensions only. All points represent the mean of two replicates; half shaded points represent when one replicate was positive and the other negative.

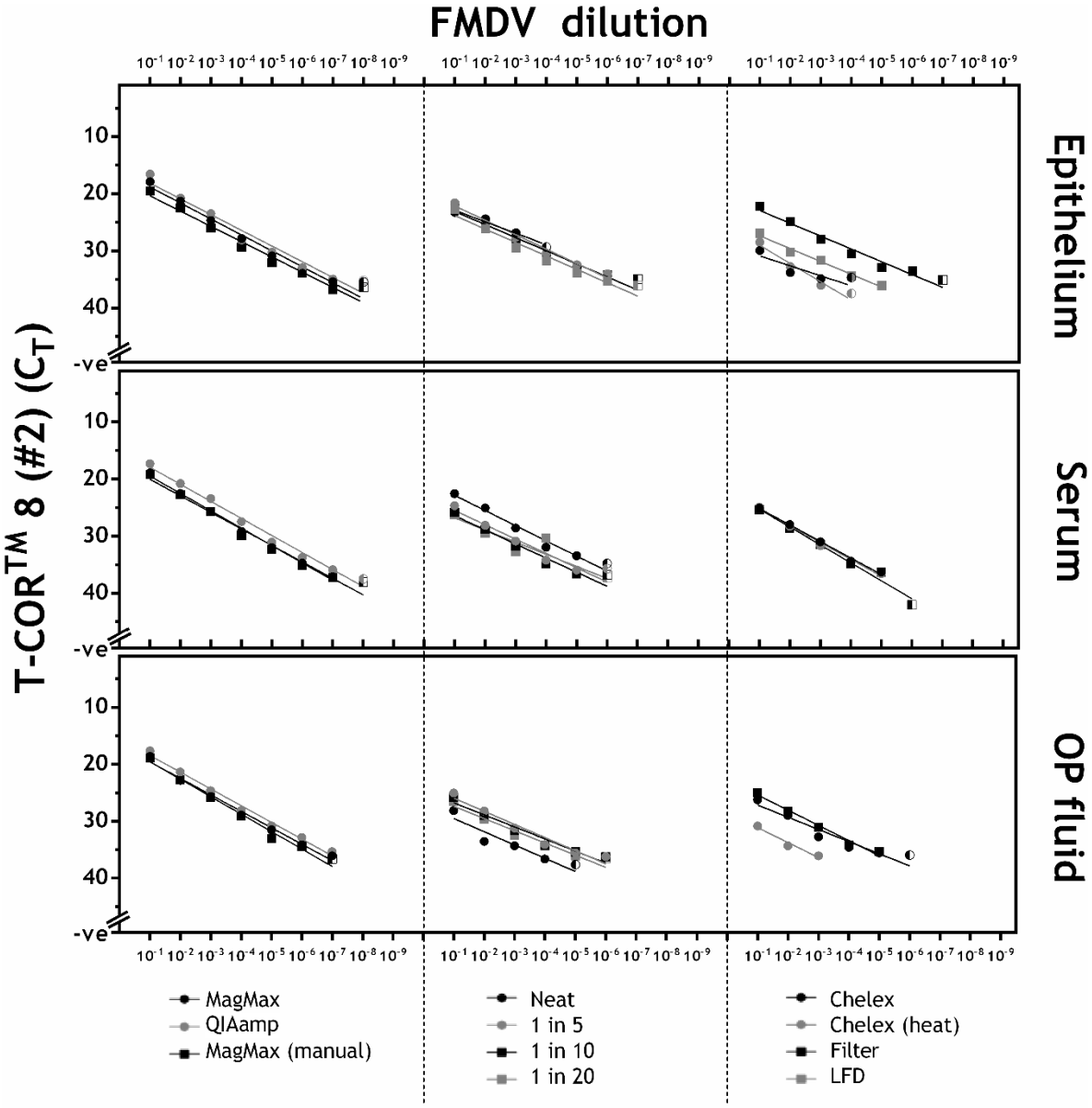


Figure 7.7 Comparison of sample preparation methods for T-COR™ 8 (#2) real-time reverse transcription PCR (rRT-PCR). Performed on a T-COR™ 8. A total of 11 sample preparation methods were trialled: nine simple preparation techniques, compared to automated robotic extraction and the use of neat samples. Extraction from Ag-LFDs (antigen-detection lateral-flow devices) was trialled for epithelial suspensions only. All points represent the mean of two replicates; half shaded points represent when one replicate was positive and the other negative.

Appendix 3

Samples for evaluation of isothermal reactions

Appendix 3a: Samples used to evaluate reverse transcription loop-mediated isothermal amplification (RT-LAMP) performance in laboratory settings

Table 7.1 Samples used to evaluate RT-LAMP

Purpose	Sample type	Sample/s
Comparison of RT-LAMP master mixes	Cell culture isolate (dilution series in 10% [w/v] bovine epithelial tissue suspension)	O/UAE/2/2003
Evaluation of lyophilised reagents on clinical samples	Archival field epithelial tissue suspensions	Asia 1/TUR/2/2013 A/IRN/24/2012 A/TUR/7/2013 A/TUR/4/2013 Sat 1/TAN/50/2012 SAT 2/TAN/14/2012 SAT 2/BOT/15/2012
Evaluation of sample preparation methodologies	Experimental bovine sera (n = 19) and oesophageal-pharyngeal fluid samples (n = 21) from cattle challenged with FMDV	O/UKG/34/2001
Evaluation of rRT-LAMP as a laboratory surveillance tool	Field samples (OP fluid) collected from cattle between September 2011 and November 2013 (n = 158)	Representing serotypes O, A, SAT 1 and SAT 2
	Experimental samples (serum = 38; OP fluid = 50) from buffalo challenged directly with three FMDV isolates	KNP/196/91/1 (SAT 1) KNP/19/89/2 (SAT 2) KNP/1/08/3 (SAT 3)
Evaluation of rRT-LAMP and RT-LAMP-LFD in endemic laboratory settings	Archival field epithelial tissue suspensions	O/Musoma Rural O/Tabora O/Tabora O/Mara O/Njombe O/Kilimanjaro O/Ntwara A/Kagera SAT 1/Dar es Salaam SAT 1/Morogoro SAT 2/Morogoro GD/Unknown NVD/Kilimanjaro NVD/Kilimanjaro
Evaluation of the AMPlite®	Cell culture isolate (dilution series in 10% [w/v] bovine epithelial tissue suspension)	O/UAE/2/2003

(RT-LAMP) reverse transcription loop-mediated isothermal amplification; (rRT-LAMP) real-time RT-LAMP; (RT-LAMP-LFD) RT-LAMP with molecular lateral-flow detection; (FMDV) foot-and-mouth disease virus; (SAT) Southern African Territories; (OP fluid) oesophageal-pharyngeal fluid.

Appendix 3b. **Table 7.2** Clinical samples used to evaluate RT-LAMP in field settings

Animal reference	Location	Time since start of clinical signs	Clinical signs	Heart rate (bpm)	(°C) Rectal temperature	Sample	Antigen LFD	Enigma FL	RT-LAMP (T_P)	RT-LAMP (T_a)	RT-LAMP-LFD	RT-LAMP-LFD (T_a)
Mcow1	Morogoro, Tanzania	ca. 2 - 4 weeks	none	N/A	N/A	serum	N/A	-	-	-	N/A	-
						OP fluid	+	33	15.50	88.81	+	88.16
Mcow2	Morogoro, Tanzania	ca. 2 - 4 weeks	none	N/A	N/A	serum	N/A	-	-	-	N/A	-
						OP fluid	-	-	29.5	88.5	-	88.29
Mcow3	Morogoro, Tanzania	ca. 2 - 4 weeks	none	N/A	N/A	serum	N/A	-	-	-	N/A	-
						OP fluid	N/A	-	-	-	-	-
Mcow4	Morogoro, Tanzania	ca. 2 - 4 weeks	none	N/A	N/A	serum	N/A	-	-	-	N/A	-
						OP fluid	N/A	-	-	-	-	-
Mcow5	Morogoro, Tanzania	ca. 2 months	none	N/A	N/A	serum	N/A	N/A	-	-	N/A	-
						OP fluid	N/A	N/A	-	-	N/A	-
Mcow6	Morogoro, Tanzania	ca. 2 months	bleeding gums	N/A	N/A	serum	N/A	N/A	-	-	N/A	-
						OP fluid	+	38	6.75	88.75	+	88.71
Mcow7	Morogoro, Tanzania	ca. 2 months	none	N/A	N/A	serum	N/A	N/A	-	-	N/A	-
						OP fluid	N/A	N/A	-	-	N/A	-
Mcow8	Morogoro, Tanzania	ca. 2 months	none	N/A	N/A	serum	N/A	N/A	-	-	N/A	-
						OP fluid	N/A	N/A	-	-	N/A	-
7647	Serengeti, Tanzania	ca. 7 days	lesions, lameness and salivation	21	39.9	serum	N/A	N/A	-	-	-	-
						OP fluid	N/A	N/A	-	-	-	-
7801	Serengeti, Tanzania	ca. 4 days	severe foot and mouth lesions salivation and lameness	26	36.8	serum	N/A	N/A	20.75	88.90	+	89.34
						OP fluid	-	N/A	26.25	88.49	+	89.34
						LF epi	+	28	10.25	88.93	+	88.93
						LH epi	+	N/A	9.75	88.98	+	89.38
						RF epi	+	N/A	9.75	89.04	+	89.63
						RH epi	+	N/A	10.50	89.09	+	89.58
						gum epi	-	N/A	11.75	88.94	+	89.53
7802	Serengeti, Tanzania	ca. 1 days	severe foot / mouth lesions salivation and lameness	23	38.1	serum	N/A	N/A	23.00	88.84	+	89.05
						OP fluid	-	N/A	11.25	89.04	+	88.84
						RF epi	-	N/A	13.75	89.43	+	89.59
						gum epi	-	-	17.75	88.96	+	88.94
		ca. 7 days	healing lesions	30	36.9	serum	N/A	N/A	22.50	88.27	+	88.77
						OP fluid	-	37	13.5	88.95	+	89.13
7803	Serengeti, Tanzania	ca. 1 days	severe foot lesions, salivation and lameness	37	38.5	serum	N/A	N/A	17.75	89.40	+	89.41
						OP fluid	-	N/A	13.00	89.44	+	89.30
						LF epi	+	27	11.75	89.44	+	88.99
		ca. 7 days	healing lesions	31	37.5	serum	N/A	N/A	25.00	88.37	+	89.07
						OP fluid	-	35	13.00	89.05	+	89.07
7804	Serengeti, Tanzania	ca. 2 days	severe foot and mouth lesions salivation/lameness	36	38.4	serum	N/A	N/A	13.25	89.39	+	89.39
						OP fluid	+	N/A	11.25	89.35	+	89.10
						RH epi	+	22	9.75	88.98	+	88.89
		ca. 8 days	healing lesions	28	37.1	serum	N/A	N/A	-	-	-	-
						OP fluid	N/A	N/A	-	-	-	-
						gum epi	-	33	15.75	88.67	+	88.67

7805	Serengeti, Tanzania	ca. 1 day	severe foot lesions, salivation and lameness	38	39.9	serum	N/A	N/A	17.50	88.93	+		88.88
						OP fluid	-	N/A	17.50	89.24	+		89.29
						LH epi	+	26	11.50	89.68	+		88.98
		ca. 7 days	healing lesions	37	37.0	serum	N/A	N/A	-	-	-		-
						OP fluid	-	36	12.50	89.16	+		89.10
7806	Serengeti, Tanzania	ca. 1 days	severe foot lesions, salivation and lameness	24	38.3	serum	N/A	N/A	25.00	88.90	+		89.25
						OP fluid	+	N/A	7.50	89.39	+		89.29
						LH epi	+	N/A	12.75	89.25	+		89.54
						gum epi	+	N/A	11.00	88.88	+		89.33
		ca. 7 days	healing lesions	26	37.6	serum	N/A	N/A	25.00	88.35	+		88.95
						OP fluid	N/A	N/A	-	-	-		-
7818	Serengeti, Tanzania	NA	NA	25	35.5	serum	N/A	N/A	-	-	-		-
						OP fluid	N/A	N/A	-	-	-		-
7807	Serengeti, Tanzania	ca. 1 - 2 weeks	healing lesions and lameness	23	36.9	serum	N/A	N/A	-	-	-		-
						OP fluid	-	N/A	13.25	88.95	+		88.91
						gum epi	-	36	14.50	89.15	+		89.10
7808	Serengeti, Tanzania	ca. 1 - 2 weeks	healing lesions and lameness	21	36.8	serum	N/A	N/A	-	-	-		-
						OP fluid	N/A	N/A	-	-	-		-
						RF epi	+	24	11.75	88.89	+		88.69
						gum epi	-	N/A	-	-	-		-
7809	Serengeti, Tanzania	ca. 1 - 2 weeks	healing lesions, lameness and salivation	29	36.4	serum	N/A	N/A	-	-	-		-
						OP fluid	+	N/A	17.75	88.85	+		88.82
						tongue epi	+	25	11.00	89.23	+		88.99
7810	Serengeti, Tanzania	ca. 1 - 2 weeks	healing lesions and lameness	23	37.4	serum	N/A	N/A	-	-	-		-
						OP fluid	-	N/A	8.50	89.30	+		89.02
						gum epi	-	24	12.50	88.95	+		88.74
7811	Serengeti, Tanzania	ca. 1 - 2 weeks	healing lesions	23	37.6	serum	N/A	N/A	25.25	88.78	+		88.84
						OP fluid	-	N/A	8.75	89.29	+		89.29
7812	Serengeti, Tanzania	ca. 1 - 2 weeks	healing lesions and salivation	24	38.3	serum	N/A	N/A	-	-	-		-
						OP fluid	N/A	N/A	-	-	-		-
						gum epi	-	36	25.50	89.44	+		89.25
7813	Serengeti, Tanzania	ca. 1 - 2 weeks	healing lesions and lameness	26	37.6	serum	N/A	N/A	-	-	+		88.87
						OP fluid	-	N/A	13.50	89.25	+		88.90
						gum epi	-	39	14.25	88.98	+		88.84
7814	Serengeti, Tanzania	ca. 1 - 2 weeks	healing lesions and lameness	28	39.4	serum	N/A	N/A	-	-	-		-
						OP fluid	+	N/A	13.50	88.90	+		89.15
						RH epi	+	24	12.50	89.38	+		88.84
7730	Serengeti, Tanzania	ca. 2 months	healed mouth and foot lesions	25	38.0	serum	N/A	N/A	-	-	-		-
						OP fluid	-	29	8.00	88.83	+		88.92
7731	Serengeti, Tanzania	ca. 2 months	healed mouth lesion	22	37.9	serum	N/A	N/A	-	-	-		-
						OP fluid	+	26	6.75	88.93	+		88.92
7743	Serengeti, Tanzania	ca. 2 months	none	22	38.5	serum	N/A	N/A	-	-	-		-
						OP fluid	+	33	8.00	89.02	+		89.04
7744	Serengeti, Tanzania	ca. 2 months	slight loss of condition	23	38.3	serum	N/A	N/A	-	-	-		-
						OP fluid	-	33	9.00	88.89	+		88.89

7746	Serengeti, Tanzania	ca. 2 months	none	21	37.6	serum	N/A	N/A	-	-	-	-	-
						OP fluid	N/A		-	26.75	66.47	-	66.51
7732	Serengeti, Tanzania	ca. 2 - 4 weeks	none	19	38.5	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	-	-	-	-
7733	Serengeti, Tanzania	ca. 2 - 4 weeks	none	20	38.2	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	-		-	25.00	88.59	+	88.83
7734	Serengeti, Tanzania	ca. 2 - 4 weeks	none	20	38.5	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	+		-	27.50	88.76	+	88.89
7735	Serengeti, Tanzania	ca. 2 - 4 weeks	healed gum lesion	19	39.0	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	-	-	-	-
7737	Serengeti, Tanzania	ca. 2 - 4 weeks	none	21	38.4	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	27.50	90.76	-	88.54
7739	Serengeti, Tanzania	ca. 2 - 4 weeks	none	20	38.0	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	27.50	89.48	-	-
7741	Serengeti, Tanzania	ca. 2 - 4 weeks	none	20	38.8	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	-	-	-	-
7742	Serengeti, Tanzania	ca. 2 - 4 weeks	none	24	38.1	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	-	-	-	-
7645	Serengeti, Tanzania	N/A	N/A	20	39.6	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	-	-	-	-
7648	Serengeti, Tanzania	N/A	N/A	20	38.5	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	-	-	-	-
7649	Serengeti, Tanzania	N/A	N/A	22	38.6	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	27.50	-	-	-
7650	Serengeti, Tanzania	N/A	N/A	21	36.6	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	-	-	-	-
7601	Serengeti, Tanzania	ca. 2 - 4 weeks	none	25	36.4	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	-	-	-	-
7602	Serengeti, Tanzania	ca. 2 - 4 weeks	none	26	36.9	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	+		N/A	29.50	88.88	-	-
7603	Serengeti, Tanzania	ca. 2 - 4 weeks	healed gum lesion	22	37.4	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	27.50	90.21	-	-
7607	Serengeti, Tanzania	ca. 2 - 4 weeks	none	20	36.8	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	+		32	10.50	88.67	+	89.20
7609	Serengeti, Tanzania	ca. 2 - 4 weeks	none	23	36.0	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	-		N/A	27.50	89.43	-	-
7610	Serengeti, Tanzania	ca. 2 - 4 weeks	none	24	37.1	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	-		30	14.00	88.93	+	89.19
7615	Serengeti, Tanzania	N/A	N/A	26	36.3	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	-	-	-	-
7625	Serengeti, Tanzania	N/A	N/A	20	37.6	serum	N/A	N/A	N/A	-	-	-	-
						OP fluid	N/A		N/A	-	-	-	-
Kcow1	Nakuru, Kenya	N/A	none	N/A	39.1	serum	N/A	N/A	N/A	-	-	-	-

Kcow2	Nakuru, Kenya	N/A	none	N/A	39.0	serum	N/A	N/A	-	-	-	-
Kcow3	Nakuru, Kenya	N/A	none	N/A	38.7	serum	N/A	N/A	-	-	-	-
Kcow4	Nakuru, Kenya	ca. 2-4 days	lesions, drop in milk production	N/A	37.6	serum gum epi	N/A -	N/A N/A	- 9.45	- 88.93	- +	- 88.93
Kcow5	Nakuru, Kenya	N/A	none	N/A	37.1	serum	N/A	N/A	-	-	-	-
Kcow6	Nakuru, Kenya	ca. 3-5 days	lesions, drop in milk production	N/A	37.9	serum	N/A	N/A	-	-	-	-
Kcow7	Nakuru, Kenya	ca. 7-8 days	Healed lesions	N/A	37.0	serum	N/A	N/A	-	-	-	-
Kcow8	Nakuru, Kenya	ca. 3-5 days	lesions, drop in milk production	N/A	37.8	serum OP fluid	N/A N/A	N/A N/A	22.00 -	88.63 -	- -	- -
Kcow9	Nakuru, Kenya	ca. 2-4 days	lesions	N/A	37.0	serum OP fluid gum epi	N/A N/A -	N/A N/A N/A	- 12.75 12.00	- 88.99 88.68	- + +	- 88.99 88.58
Kcow10	Nakuru, Kenya	ca. 1-2 days	lesions, drop in milk production	N/A	37.4	serum OP fluid	N/A N/A	N/A N/A	19.75 15.75	88.68 88.78	- +	- 88.78
Kcow11	Nakuru, Kenya	N/A	none	N/A	38.4	serum	N/A	N/A	-	-	-	-

(OP fluid) oesophageal-pharyngeal fluid; (epi) epithelium; (Ag-LFD) antigen-detection lateral-flow device; (Enigma FL) Enigma® Field Laboratory; (rRT-PCR) real-time reverse transcription PCR in the field, values represent the cycle threshold (C_T); (rRT-LAMP) real-time reverse transcription loop-mediated isothermal amplification; (RT-LAMP-LFD) RT-LAMP combined with molecular lateral-flow detection; (N/A) not applicable (test not performed); (T_P) time to positivity; (T_a) anneal temperature. T_P and T_a values represent the mean of two replicates.

Appendix 4

Preliminary evaluation of the AMPlite®

Field validation of reverse transcription loop-mediated isothermal amplification using molecular lateral-flow detection (RT-LAMP-LFD) highlighted a number of important factors for consideration in future assay design, including the requirement to minimise cross-contamination. Consequently, in order to reduce the number of pipetting stages required in the field and minimise the opportunities/risk for cross-contamination between assays, preliminary trials were performed on the AMPlite®, which is a prototype closed RT-LAMP-LFD system developed by The Animal and Plant Health Agency (APHA, Addlestone, UK).

RT-LAMP-LFD reactions were performed in a total reaction mix of 50 µl, containing labelled primers, ISO-001 master mix (wet) and reverse transcriptase at equivalent final concentrations as previously stated (Chapter 2.3.4). In addition, reactions contained a proprietary internal control (IC) (10^4 copies) and detecting primers (0.4 µM internal primers: 0.04 µM external primers: 0.2 µM loop primers labelled with digoxigenin [forward] and biotin [reverse]). Reactions were incubated in the AMPlite® heat block for 30 minutes at 65°C. Once the incubation has completed, the AMPlite® automatically dispensed the reaction onto the LFD (Figure 7.8): a positive result was signified by the presence of three lines (test, RT-LAMP IC and LFD read lines), a negative result was indicated by the presence of the IC and LFD read lines only.

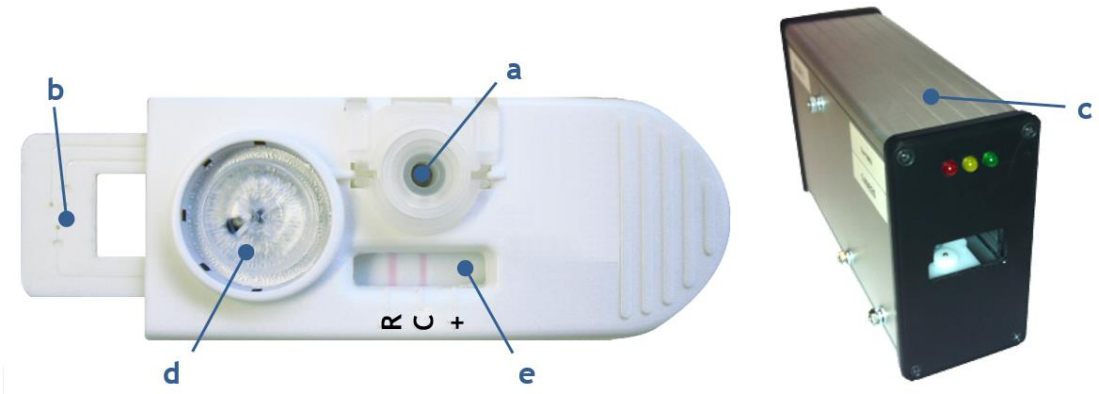
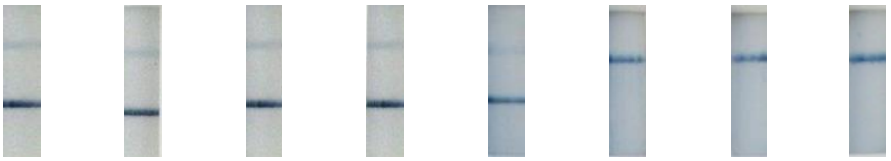


Figure 7.8 AMPlite® device and heat block. Reaction mixture was added to the AMPlite® sample well (a). Upon closing of the lid, the reaction was dispensed into the reaction chamber (b), and device inserted into the AMPlite® heat block (c). Following incubation, the heat block automatically releases lateral-flow device (LFD) running buffer (d) through reaction chamber, dispensing the reaction onto the LFD (e). (R) LFD read line; (C) internal control line; (+) test-line.

The analytical sensitivity of the AMPlite®, established using RNA extracted from a FMDV cell culture isolate (as described in Chapter 2.3.6), was maintained in comparison to RT-LAMP-LFD (Table 7.3).

Table 7.3 Analytical sensitivity of the AMPlite® comparatively to RT-LAMP-LFD

	10-fold dilution series of FMDV (O/UAE/2/2003) RNA							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Reference rRT-PCR	15.61	18.89	22.02	25.62	29.09	32.08	36.66	No C_T
RT-LAMP-LFD								
AMPlite®								

(rRT-PCR) real-time reverse transcription PCR; (RT-LAMP-LFD) RT-LAMP combined with molecular lateral-flow detection. For RT-LAMP-LFD, the upper band represents the LFD control line, and lower band the test line. For the AMPlite®, the upper band represents the LFD read control, middle the internal control and lower band the test line. In samples with high amounts of FMDV, the control lines are often feint due to reagent competition.

During field work in Kenya (Chapter 3.3.8) preliminary trials were performed using two lyophilised RT-LAMP-LFD pellets (Chapter 3.3.3), re-suspended with 30 µl of re-suspension buffer, 10 µl sample and made up to 50 µl total volume with NFW. The heat block was powered through a vehicle auxiliary, using a 200 W inverter. 100% concordance between rRT-LAMP, RT-LAMP-LFD and the AMPlite® was present across the three samples (serum = 1; epithelium = 2) tested (data not shown).

Appendix 5

Laboratory evaluation of lyophilised real-time reverse transcription PCR

Table 7.4 Clinical samples for evaluation of rRT-PCR in laboratory settings

Serotype	WRLFMD sample ID	Topotype	Lineage	Location	Year	Type	Reference rRT-PCR	Lyophilised rRT-PCR	Typing rRT-PCR
O	HKN/12/2015	CATHAY	unnamed	Hong Kong	2015	OS	28.26	28.70	N/A
	IRN/26/2015	ME-SA	PanAsia-2BAL-09	Iran	2015	OS	28.67	27.75	N/A
	KUW/1/2016	ME-SA	PanAsia-2BAL-09	Kuwait	2016	OS	16.86	18.35	N/A
	KUW/4/2016	ME-SA	PanAsia-2BAL-09	Kuwait	2016	OS	14.06	15.75	N/A
	PAK/30/2015	ME-SA	PanAsia-2BAL-09	Pakistan	2015	OS	21.20	19.60	N/A
	PAK/32/2015	ME-SA	PanAsia-2BAL-09	Pakistan	2015	OS	21.30	20.80	N/A
	PAK/34/2015	ME-SA	PanAsia-2BAL-09	Pakistan	2015	OS	20.70	20.55	N/A
	PAT/4/2015	ME-SA	PanAsia	Palestine	2015	OS	27.90	28.30	N/A
	TAN/5/2009	EA-2	unnamed	Tanzania	2012	OS	23.82	24.10	23.75
	TAN/39/2012	EA-2	unnamed	Tanzania	2012	OS	24.03	27.45	25.95
	TAN/3/2014	EA-2	unnamed	Tanzania	2014	OS	26.68	29.15	27.80
	TAN/6/2014	EA-2	unnamed	Tanzania	2014	OS	26.57	28.75	28.95
	KEN/10/2009	EA-2	unnamed	Kenya	2009	OS	27.18	29.85	No C_T
	KEN/146/2010	EA-2	unnamed	Kenya	2010	OS	16.87	19.10	18.95
	KEN/151/2010	EA-2	unnamed	Kenya	2010	OS	20.54	23.70	28.35
	KEN/152/2010	EA-2	unnamed	Kenya	2010	OS	20.15	21.90	25.65
	KEN/154/2010	EA-2	unnamed	Kenya	2010	OS	25.33	24.50	26.20
	KEN/15/2011	EA-2	unnamed	Kenya	2011	OS	19.61	21.90	22.45
A	IRN/21/2015	ASIA	G-VII	Iran	2015	OS	27.39	26.80	N/A
	IRN/24/2015	ASIA	Iran-05SIS-10	Iran	2015	OS	21.68	19.85	N/A
	SAU/8/2015	ASIA	G-VII	Saudi Arabia	2015	OS	26.78	25.30	N/A
	PAK/31/2015	ASIA	Iran-05FAR-11	Pakistan	2015	OS	18.63	19.55	N/A
	PAK/56/2015	ASIA	Iran-05FAR-11	Pakistan	2015	OS	22.63	25.80	N/A
	TAN/42/2009	AFRICA	G-I	Tanzania	2009	OS	21.79	23.15	23.65
	TAN/56/2012	AFRICA	G-I	Tanzania	2012	OS	35.64	35.50	No C_T
	TAN/61/2012	AFRICA	G-I	Tanzania	2012	OS	26.96	31.35	28.55
	TAN/70/2012	AFRICA	G-I	Tanzania	2012	OS	20.36	23.00	23.05
	TAN/71/2012	AFRICA	G-I	Tanzania	2012	OS	24.91	27.60	25.75
	TAN/15/2013	AFRICA	G-I	Tanzania	2013	OS	15.78	18.85	21.05
	KEN/28/2008	AFRICA	G-I	Kenya	2008	OS	30.61	30.45	27.20
	KEN/22/2009	AFRICA	G-I	Kenya	2009	OS	28.09	29.10	28.00
SAT 1	TAN/50/2012	I (NWZ)	unnamed	Tanzania	2012	OS	25.21	27.10	26.45
	TAN/23/2013	I (NWZ)	unnamed	Tanzania	2013	OS	15.87	17.95	20.75
	TAN/29/2013	I (NWZ)	unnamed	Tanzania	2013	OS	15.38	17.50	21.85
	TAN/22/2014	I (NWZ)	unnamed	Tanzania	2014	OS	17.44	21.75	25.40
	KEN/26/2008	I (NWZ)	unnamed	Kenya	2008	OS	16.05	18.35	22.95
	KEN/9/2009	I (NWZ)	unnamed	Kenya	2009	OS	23.37	25.25	25.20
	KEN/12/2009	I (NWZ)	unnamed	Kenya	2009	OS	24.54	23.80	25.20
SAT 2	ZIM/9/2015	II	unnamed	Zimbabwe	2015	OS	25.44	26.05	N/A
	ZIM/21/2015	II	unnamed	Zimbabwe	2015	OS	26.16	26.95	N/A
	TAN/3/2011	IV	IV	Tanzania	2011	OS	25.08	25.95	24.40
	TAN/7/2011	IV	IV	Tanzania	2011	OS	22.63	24.35	24.25
	TAN/5/2012	IV	unnamed	Tanzania	2012	OS	20.37	23.30	21.25
	TAN/10/2012	IV	unnamed	Tanzania	2012	OS	19.77	22.30	23.35
	TAN/14/2012	IV	unnamed	Tanzania	2012	OS	22.44	25.10	22.65
	TAN/19/2012	IV	unnamed	Tanzania	2012	OS	25.78	24.75	22.20
	TAN/64/2012	IV	unnamed	Tanzania	2012	OS	25.56	26.20	24.80
	KEN/2/2007	IV	unnamed	Kenya	2007	OS	30.65	32.15	No C_T
	KEN/12/2011	IV	unnamed	Kenya	2011	OS	16.74	18.85	19.00
	KEN/21/2011	IV	unnamed	Kenya	2011	OS	17.19	19.15	18.45
	KEN/4/2012	IV	unnamed	Kenya	2012	OS	25.48	27.70	24.30
	SUD/7/2014	VII	Alx-12	Sudan	2015	OS	35.03	35.90	N/A
Asia 1	IRN/20/2015	ASIA	Sindh-08	Iran	2015	OS	22.37	20.60	N/A
	PAK/28/2015	ASIA	Sindh-08	Pakistan	2015	OF	20.27	22.30	N/A
	PAK/29/2015	ASIA	Sindh-08	Pakistan	2015	OF	18.13	21.1	N/A
	PAK/33/2015	ASIA	Sindh-08	Pakistan	2015	OS	19.31	19.20	N/A
	PAK/43/2015	ASIA	Sindh-08	Pakistan	2015	OS	23.11	22.80	N/A
SVDV	UKG/24/1972	Undefined	Undefined	UK	1972	CC	No C_T	No C_T	No C_T
	UKG/50/1972	Undefined	Undefined	UK	1972	CC	No C_T	No C_T	No C_T
	UKG/51/1972	Undefined	Undefined	UK	1972	CC	No C_T	No C_T	No C_T
	UKG/68/1972	Undefined	Undefined	UK	1972	CC	No C_T	No C_T	No C_T
VSIV	Colorado/1942	Undefined	Undefined	USA	1942	CC	No C_T	No C_T	No C_T
VSNJV	Columbia/1964	Undefined	Undefined	Colombia	1964	CC	No C_T	No C_T	No C_T

(rRT-PCR) real-time reverse transcription PCR; (OS) original suspension; (OF) original fluid e.g. vesicular fluid; (CC) cell culture; (C_T) cycle threshold value is the average value across two replicates; (WRLFMD) World Reference Laboratory for Foot-and-Mouth Disease; (FMDV) foot-and-mouth disease virus; (SAT) Southern African Territories; (SVDV) swine vesicular disease virus; (VSIV) vesicular stomatitis Indiana virus; (VSNJV) vesicular stomatitis New Jersey virus.

Appendix 6

Field evaluation of real-time reverse transcription PCR

Table 7.5 Clinical samples for evaluation of rRT-PCR in field settings

Animal reference	Location	Approximate lesion age	Sample	T-COR™ 8 pan-FMDV	T-COR™ 8 serotype specific A (FAM)	O (DFO)	SAT 1 (Cy®5)	SAT 2 (TxR)	Reference rRT-PCR
Cow_1	Kericho County, Kenya (Farm 1)	4 days	Serum	36.40	No C_T	No C_T	No C_T	No C_T	No C_T
Cow_2		7 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_3		10 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_4		6 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_5		10 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_6		10 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_7		7 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_8		10 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_9	Kericho County, Kenya (Farm 2)	5 days	Epithelium	20.25	21.00	No C_T	No C_T	No C_T	19.44
			Serum	29.55	27.15	No C_T	No C_T	No C_T	28.88
Cow_10		5 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_11		3 days	Epithelium	20.15	22.20	No C_T	No C_T	No C_T	23.14
			Serum	32.30	30.50	No C_T	No C_T	No C_T	32.02
Cow_12		2 days	Epithelium	17.80	22.25	No C_T	No C_T	No C_T	20.17
Cow_13		4 days	Epithelium	19.55	21.70	No C_T	No C_T	No C_T	17.64
Cow_14	Nakuru County, Kenya (Farm 3)	4 days	Lesion swab	25.40	No C_T	26.70	No C_T	No C_T	30.53
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_15		4 days	Lesion swab	29.80	No C_T	32.80	No C_T	No C_T	33.09
Cow_16		7 days	Lesion swab	32.75	No C_T	34.40	No C_T	No C_T	40.97
Cow_17		10 days	Lesion swab	39.00*	N/A	N/A	N/A	N/A	No C_T
Cow_18	Nakuru County, Kenya (Farm 4)	7 days	Lesion swab	No C_T	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_19		5 days	Epithelium	36.2	No C_T	No C_T	No C_T	No C_T	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_20		8 days	Lesion swab	No C_T	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_21		7 days	Lesion swab	28.85	No C_T	No C_T	No C_T	No C_T	27.17
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
			OP fluid	26.85	No C_T	25.25	No C_T	No C_T	26.45
Cow_22		5 days	Lesion swab	No C_T	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_23		NCS	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_24		10 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_25		6 days	Lesion swab	29.00	No C_T	No C_T	No C_T	No C_T	34.90
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_26		10 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_27		7 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_28		NCS	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_29		2 days	Epithelium	17.35	No C_T	16.90	No C_T	No C_T	21.21
			Lesion swab	30.80	No C_T	No C_T	No C_T	No C_T	39.14
			Serum	36.90	No C_T	No C_T	No C_T	No C_T	No C_T
Cow_30	Morogoro Region, Tanzania (Farm 5)	ca. 1 month	Lesion swab	No C_T	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_31		ca. 1 month	Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_32		ca. 2 weeks	Lesion swab	No C_T	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_33		ca. 1 month	Lesion swab	No C_T	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_34		ca. 2 weeks	Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_35		ca. 2 weeks	Lesion swab	No C_T	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_36		ca. 1 month	Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	No C_T

Cow_37	Morogoro Region, Tanzania (Farm 6)	ca. 1-2 months	Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	35.95	No C_T	No C_T	No C_T	No C_T	32.66
Cow_38			Serum	No C_T	No C_T	No C_T	No C_T	No C_T	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_39			Serum	No C_T	No C_T	No C_T	No C_T	No C_T	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_40			Lesion swab	No C_T	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T	No C_T	No C_T	No C_T	No C_T	N/A
Cow_41			Serum	No C_T	No C_T	No C_T	No C_T	No C_T	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_42			Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	32.65	No C_T	No C_T	No C_T	No C_T	30.44
Cow_43			Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_44	Morogoro Region, Tanzania (Farm 7)	ca. 2 weeks	Lesion swab	No C_T	N/A	N/A	N/A	N/A	38.31
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_45		5 days	Epithelium	28.60	No C_T	28.25	No C_T	No C_T	20.10
			Lesion swab	32.25	No C_T	No C_T	No C_T	No C_T	25.37
			Serum	No C_T	N/A	N/A	N/A	N/A	37.24
Cow_46		4 days	Epithelium	26.25	No C_T	23.45	No C_T	No C_T	17.05
			Lesion swab	24.75	No C_T	22.30	No C_T	No C_T	18.83
			Serum	37.50	No C_T	No C_T	No C_T	No C_T	27.57
Cow_47		4 days	Epithelium	23.75	No C_T	20.20	No C_T	No C_T	18.62
			Lesion swab	23.30	No C_T	21.05	No C_T	No C_T	18.67
			Serum	No C_T	N/A	N/A	N/A	N/A	38.03
Cow_48		2 days	Epithelium	19.85	No C_T	17.95	No C_T	No C_T	17.77
			Serum	35.70	No C_T	No C_T	No C_T	No C_T	35.10
Cow_49		NCS	Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_50		NCS	Mouth swab	No C_T	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_51		NCS	Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_52	Morogoro Region, Tanzania (Farm 8)	ca. 1-2 months	Lesion swab	No C_T	No C_T	No C_T	No C_T	No C_T	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_53		NCS	Serum	No C_T	No C_T	No C_T	No C_T	No C_T	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_54		NCS	Serum	No C_T	No C_T	No C_T	No C_T	No C_T	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_55		NCS	Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_56		NCS	Mouth swab	No C_T	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_57		7 days	Lesion swab	No C_T	N/A	N/A	N/A	N/A	38.11
			Serum	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_58		NCS	Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_59	Morogoro Region, Tanzania (Farm 9)	5 days	Lesion swab	30.55	No C_T	No C_T	No C_T	No C_T	23.08
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_60		9 days	Lesion swab	36.00*	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_61		5 days	Epithelium	24.10	No C_T	27.00	No C_T	No C_T	15.24
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_62		5 days	Lesion swab	33.50	No C_T	No C_T	No C_T	No C_T	30.83
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_63		4 days	Epithelium	36.30	No C_T	No C_T	No C_T	No C_T	34.97
			Lesion swab	34.25	No C_T	No C_T	No C_T	No C_T	26.30
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_64		7 days	Lesion swab	No C_T	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_65		4 days	Epithelium	27.65	No C_T	36.00	No C_T	No C_T	23.10
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
		7 days	Epithelium	29.90	No C_T	28.05	No C_T	No C_T	22.61
Cow_66		7 days	Epithelium	37.50*	N/A	N/A	N/A	N/A	No C_T
			Serum	No C_T					N/A
Cow_67	Morogoro Region, Tanzania (Farm 10)	NCS	Serum	No C_T	N/A	N/A	N/A	N/A	N/A

Cow_68	Morogoro Region, Tanzania (Farm 11)	4 days	Epithelium	33.85	No C_T	No C_T	No C_T	No C_T	31.08
			Lesion swab	29.20	No C_T	No C_T	No C_T	No C_T	21.26
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_69		4 days	Epithelium	34.95	No C_T	No C_T	No C_T	No C_T	31.48
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_70		7 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
			OP fluid	35.20	No C_T	No C_T	No C_T	No C_T	26.11
Cow_71		10 days	Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
			OP fluid	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_72		4 days	Epithelium	24.75	No C_T	26.05	No C_T	No C_T	16.65
			Serum	No C_T	N/A	N/A	N/A	N/A	No C_T
Cow_73	Adama, Ethiopia (Farm 12)	2 days	Epithelium	22.50	No C_T	21.70	No C_T	No C_T	N/A
			Lesion swab	29.15	No C_T	35.00	No C_T	No C_T	N/A
		5 days	Serum	No C_T	N/A	N/A	N/A	N/A	N/A
			OP fluid	29.70	No C_T	35.00	No C_T	No C_T	N/A
Cow_74		NCS	Serum	No C_T	N/A	N/A	N/A	N/A	N/A
Cow_75		10 days	OP fluid	36.40	No C_T	No C_T	No C_T	No C_T	N/A
Cow_76	Adama, Ethiopia (Farm 13)	6 days	Lesion swab	32.05	No C_T	No C_T	No C_T	No C_T	N/A
Cow_77		7 days	Lesion swab	38.05	No C_T	No C_T	No C_T	No C_T	N/A
Cow_78		7 days	OP fluid	No C_T	N/A	N/A	N/A	N/A	N/A

All results represent the average cycle threshold (C_T) across two real-time reverse transcription PCR (rRT-PCR) replicates. *represents replicates where one was positive and the other negative. (NCS) no clinical signs: swabs in these animals were taken from the mouth; (N/A) not applicable, test not performed; (OP fluid) oesophageal-pharyngeal fluid; (FMDV) foot-and-mouth disease virus. Shaded rows represent positive pan-FMDV results, for which the rRT-PCR typing assay was performed (blue: positive typing results for serotype A; red: positive typing results for serotype O; grey: did not type). The reference rRT-PCR was performed in laboratory settings within East Africa, using extracted RNA (this was not possible in Ethiopia due to a lack of facilities).

Appendix 7

Evaluation of GoPrime

Appendix 7a: *The addition of published data into the parameters of GoPrime:*

The effect of primer/probe-template mismatches have previously been quantified and published for a number of different targets and *Taq*-based kits (Süß *et al.*, 2009; Stadhouders *et al.*, 2010). In order to ascertain whether the use of alternative parameters affected the prediction ability of GoPrime, data from these publications were added to the linear model analysis, in addition to the experimental data collected in Chapter 5. Results of the linear model were used to update the underlying mathematical model behind GoPrime (Table 7.6).

Table 7.6 The effect of different mismatches calculated from linear model analysis

Factor	Mismatch type	ΔC_T	SE	t value	*p* value
	% mismatch (forward/reverse combined)	1.00	0.03	-35.39	< 0.001
	nt 1 mismatch (type 1)	2.41	0.24	10.05	< 0.001
	2x nt 1 mismatch (type 1)	6.12	0.82	7.45	< 0.001
	nt 1 mismatch (type 2)	4.87	0.34	14.38	< 0.001
	2x nt 1 mismatch (type 2)	9.94	1.25	7.94	< 0.001
	nt 2 mismatch (type 1)	1.79	0.35	5.17	< 0.001
Primer	2x nt mismatch (type 1)	4.47	0.77	5.85	< 0.001
	nt 2 mismatch (type 2)	3.71	0.37	10.05	< 0.001
	2x nt 2 mismatch (type 2)	7.36	1.06	6.92	< 0.001
	nt 3-4 mismatch (type 1)	0.89	0.37	2.39	0.0172
	2x nt 3-4 mismatch (type 1)	1.68	0.94	0.73	0.468
	nt 3-4 mismatch (type 2)	2.14	0.31	6.88	< 0.001
	2x nt 3-4 mismatch (type 2)	4.16	2.96	1.40	0.161
	% mismatch	0.27	0.02	11.91	< 0.001
Probe	3'-end mismatch	0.68	0.43	1.60	0.110
	5'-end mismatch	-1.08	0.33	-5.62	< 0.001

(nt) nucleotide; (ΔC_T) change in cycle threshold; (SE) standard error. For multiple mismatches, the linear model was able to calculate the effect of having the same mutation in both the primers (2x). Mismatches were grouped as one of two types: (type 1) purine-pyrimidine mismatch (G-T; C-A: minor conformational change in the primer/probe-template duplex); (type 2) purine-purine or pyrimidine-pyrimidine mismatch (G-A; A-A; G-G; C-T; T-T; C-C: major conformational change in the primer/probe-template duplex). *If a type nt 1 mismatch was present, the percentage mismatch ΔC_T would be calculated and additional nt 1 mismatch ΔC_T penalty added. ΔC_T, SE and t value given to 2 decimal places.

Using these data as the underlying parameters, evaluation of GoPrime was repeated using the oligonucleotide templates containing the 90 artificial variations and seven naturally occurring variations within the FMDV $3D^{pol}$-coding region. Using the templates containing artificial variations, the predictions for GoPrime were slightly improved, with the change in cycle threshold (ΔC_T) predictions on average being 2.75 away from the observed result (SD 1.95; RMSD 3.37) (Figure 7.9A; 7.9B). Similar results were evident using the templates containing naturally occurring variations: GoPrime on average predicted the ΔC_T of reactions 1.33 away from the observed result (SD 1.07; RMSD 1.69) (Figure 7.9C; 7.9D).

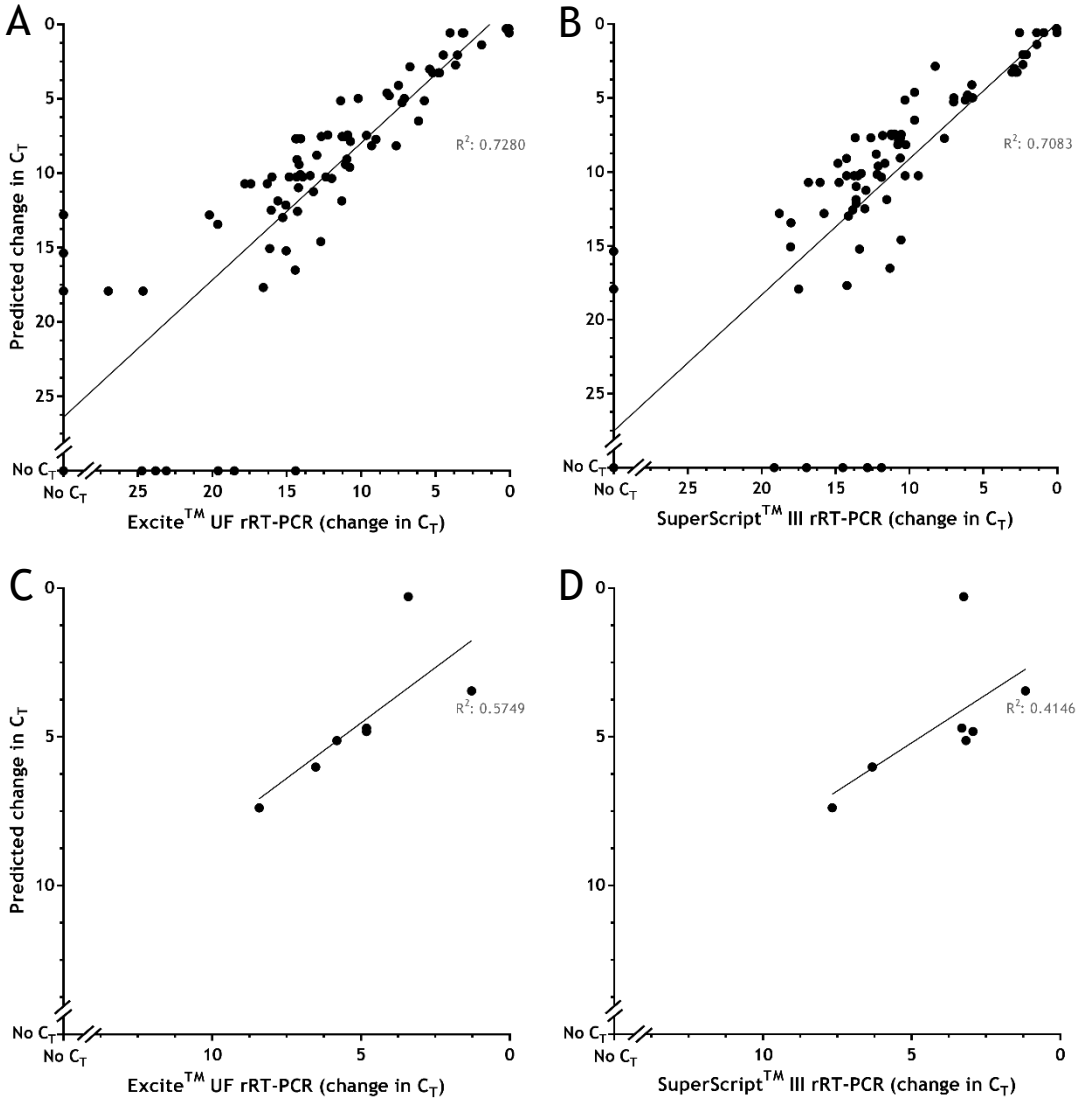


Figure 7.9 The addition of published data into GoPrime. GoPrime was evaluated using both the templates containing artificial (n = 90) and naturally occurring (n = 7) sequence variations. (A) observed change in cycle threshold for Excite™ UF 2x Master Mix for artificial variations; (B) observed change in cycle threshold for SuperScript™ III Platinum™ One-Step qRT-PCR Kit for artificial variations; (C) observed change in cycle threshold for Excite™ UF 2x Master Mix for natural variations; (D) observed change in cycle threshold for SuperScript™ III Platinum™ One-Step qRT-PCR Kit for natural variations. For the observed results, points represent the average change in cycle threshold or limit of detection across all dilutions (10^6-10^0) of starting template.

Appendix 7b: Impact of primer-template mismatches on one-step real-time reverse transcription PCR

GoPrime, in its current form, was designed to evaluate real-time PCR and two-step real-time reverse transcription PCR (rRT-PCR) (where mismatches persist through to the complementary DNA [cDNA]). To ascertain the effect of mismatches in one-step rRT-PCR, in which gene-specific primers are used in both the reverse transcription and rPCR stages, linear dsDNA constructs of 178 bp (gBlocks®: Integrated DNA Technologies Inc., IA, USA) were designed to contain M13 forward and reverse primer targets (commonly used as a cloning site), a T7 promoter and a 109 bp $3D^{pol}$-coding region (containing the Callahan *et al*. [2002] target region [as stated in Chapter 5.3.1]) (Figure 7.10; Table 7.7).

Figure 7.10 Simplified schematic of the double-stranded DNA (dsDNA) constructs. Grey regions represent the M13 bacteriophage primer targets; the black arrow represent the T7 promoter; the blue region represents the 109 bp $3D^{pol}$-coding region (Table 5.5).

Table 7.7 $3D^{pol}$-coding regions contained within the DNA constructs (5'-3') used to prepare RNA oligonucleotides

	Forward primer target	Probe target	Reverse primer target
R	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
1	ACTGGGTTTTACAAACCTGTG**C**	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
2	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	**G**CCGTGGCAGGACTCGC
3	ACTGG**T**TT**C**TAC**G**AAC**A**TGTGA	TCCTTTGCACGCCGTGGGAC	TCCGTGGCAGGACTCGC
4	ACTGGGTTTTACAAACCTGTGA	TCCTTTGCACGCCGTGGGAC	TCCGT**T**GC**G**GGACT**T**GC

The primer/probe binding regions of the four templates. Regions between the primer/probe sites were identical to O/UKG/35/2001 (Accession number KR265074: nucleotides 7862-7970). Black sequences represent the reference template (R); grey sequences represent the varying DNA templates, with red underlined based depicting primer/probe-template mismatch sites.

Synthetic RNA transcripts (137 bp in length) were produced by *in vitro* transcription using a MEGAshortscript™ T7 Transcription Kit (Invitrogen™, Thermo Fisher Scientific, MA, USA), followed by DNase digestion using TURBO™ DNase (Ambion®, Thermo Fisher Scientific). Transcripts were purified using a MEGAclear™ Transcription Clean-Up Kit (Ambion®). Quantification and quality of the RNA transcripts were examined using a NanoDrop ND-1000 spectrophotometer at A_{260} (Thermo Fisher Scientific) and Qubit® Fluorometer with Qubit® RNA HS Assay Kit (Invitrogen™). A dilution series of the RNA standard (10^6 to 10^0 copies) was then prepared in 0.1 µg/ml carrier RNA (Ambion®) and used as template in rRT-PCR reactions which were performed as previously stated

(Chapter 2.3.2), using a ABI ViiA™ 7 Real-Time PCR system and SuperScript™ III Platinum™ One-Step qRT-PCR Kit (as described in Chapter 5.3.1).

Differences were evident between mismatches that occurred in the forward and reverse primers. The presence of forward primer-template mismatches resulted in similar change in cycle threshold (ΔC_T) when used in rPCR and one-step rRT-PCR (Figure 7.11). Conversely, when the reverse primer-template mismatches were present, the ΔC_T was reduced in one-step rRT-PCR comparatively to rPCR (Figure 5.13). This is likely due to the Callahan *et al*. (2002) assay studied utilising the reverse primer for the reverse transcription stage, therefore cDNA generated will contain the reverse primer sequence. However, further analysis of the effects of these mismatches are required in order to add one-step rRT-PCR as a function of GoPrime.

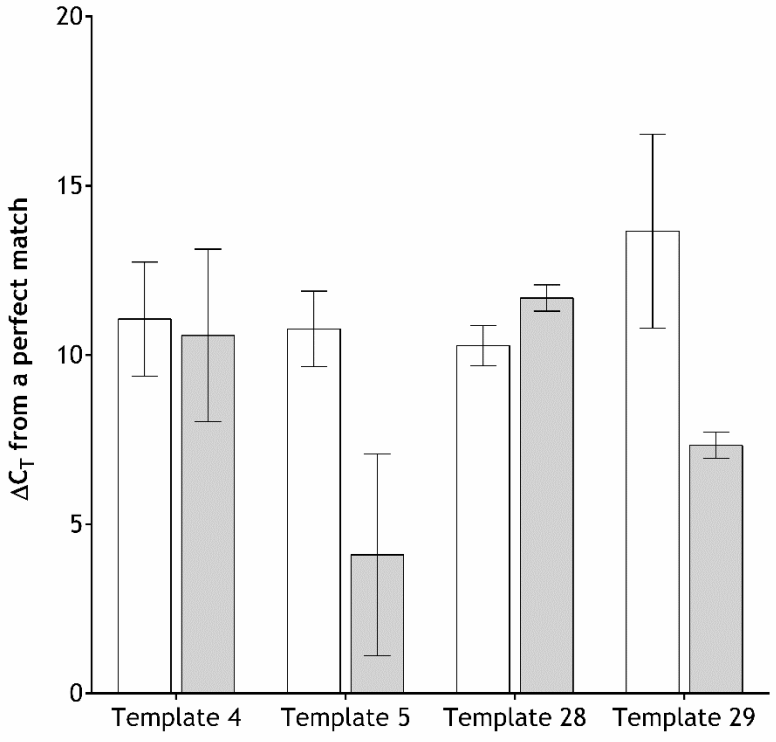


Figure 7.11 The impact of 3'-end mismatches on real-time PCR (rPCR) and one-step real-time reverse transcription PCR (rRT-PCR). Results show the raw experimental data collected, using the SuperScript™ III Platinum™ One-Step qRT-PCR Kit (for rPCR the reverse transcription step was omitted). (ΔC_T) change in cycle threshold from the reference template. White bars represent the ΔC_T for rPCR; grey bars represent the ΔC_T for one-step rRT-PCR. Error bars represent the standard error. Templates 4 and 28 (Chapter 5, Table 5.1) contained forward primer-template mismatches; templates 5 and 29 (Chapter 5, Table 5.1) contained reverse primer-template mismatches.

Appendix 8

Protocols for the use of molecular point-of-care tests

Point-of-care test selection

Molecular-based point-of-care tests should be selected based on the estimated FMD-status of the animal and required results (detection/characterisation), according Figure 1.

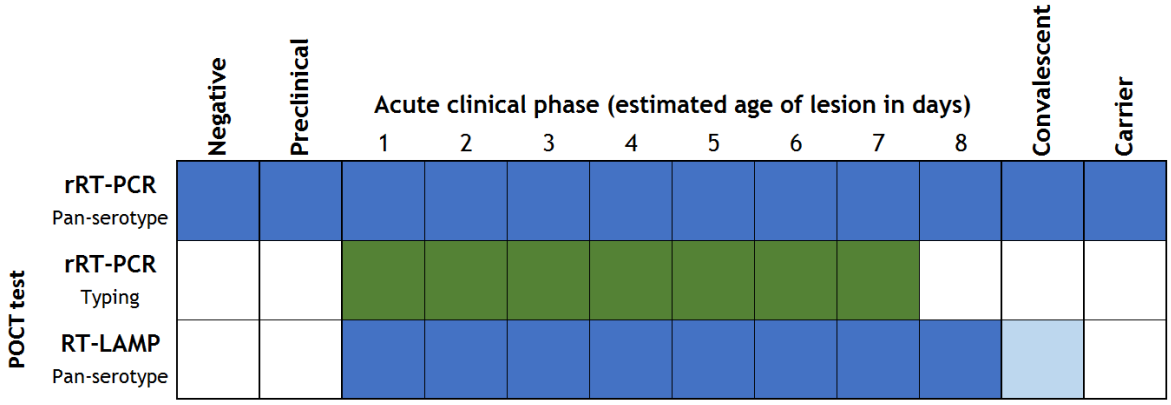


Figure 1. Point-of-care test selection chart. ■ For pan-FMDV diagnosis (■ assay be used, however alternatives may be better); ■ For FMDV-typing.

Sample processing

Samples should be collected according to Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012), and processed as stated in Table 1.

Table 1 Processing of samples for molecular POCTs

Sample	Processing	Dilution in NFW rRT-PCR	Dilution in NFW RT-LAMP
Epithelium	Epithelial tissue should be prepared using a SVANODIP® FMDV-Ag Extraction Kit (Boehringer Ingelheim), by homogenising 0.2 g of tissue in the sample extraction vial with 1 ml of sample buffer from the SVANODIP® FMDV-Ag LFD kit (Boehringer Ingelheim). Note. Epithelial samples from the feet (interdigital space/coronary band) should be briefly washed in sterile water prior to processing to remove soil contaminants.	1 in 10	1 in 5
Serum	Blood (collected using serum tubes) should be centrifuged either in a low-resource laboratory or using a field-based centrifuge (minimum 1400 x g for 3 min) at room temperature. Alternatively, blood can be left to clot at room temperature for 30 minutes, with serum pipetted from the top.	1 in 10	1 in 5
Mouth and foot swabs	Once taken, swabs should be agitated by hand in 1 ml NFW, which can be used directly in analysis. Note. If using foot swabs, the feet should be briefly cleaned in sterile water prior to swabbing to remove soil contaminants.	Neat	Neat
OP fluid	Once collected, OP fluid only requires dilution in NFW for processing. Note. Allow the sample to settle before processing to reduce contaminants.	1 in 10	1 in 10

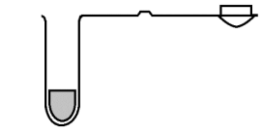
(rRT-PCR) real-time reverse transcription PCR; (RT-LAMP) reverse transcription loop-mediated isothermal amplification; (OP fluid) oesophageal-pharyngeal fluid; (NFW) nuclease-free water; (POCT) point-of-care tests.

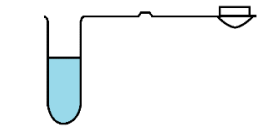
rRT-PCR using the T-COR™ 8

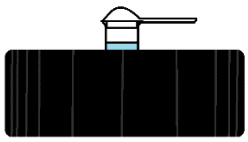
Materials supplied	Additional materials required
• T-COR 8™ device • AC Adapter • Magnetic stirrer • T-COR 8™ reactions (eight reactions per packet) • Resuspension buffer (in 1 ml aliquots) • T-COR 8™ quick start guide	• Nuclease-free water • Pipettes and tips for 5 µl and 20 µl volumes • DNAZap™ DNA Degradation Solutions (Thermo Fisher Scientific)

Note: The T-COR 8™ can be powered by battery (one charge is enough for approximately eight runs) or through mains power.

Protocol for set-up







1) Prepare work surfaces and pipettes using **DNAZap™**
2) Remove reactions from the **sealed packet**
3) Add 20 µl of **resuspension buffer** to each of the pellets
4) Add 5 µl of the **sample** to be tested (processed as above)
5) Close the lid, tap tubes to remove air bubbles and place each reaction within the **magnetic stirrer** for 5 - 10 seconds
6) Switch on the **T-COR™ 8** using the circular side button
7) Select **[Barcode]** under the **Guided** start-up option
8) Hold the **barcode** (on the sealed packet) 3 inches from the scanner. This will automatically enter the correct thermocycling conditions for each reaction
9) Follow the screen's instructions to set-up reactions and enter relevant sample details
   (see T-COR 8™ quick start guide for troubleshooting)

Analysis

T-COR 8™ software will automatically analyse results and report samples as FMDV-positive, FMDV-negative or re-test (for instance if the internal control has failed to amplify). Results can be easily saved and shared (instructions within the T-COR 8™ quick start guide).

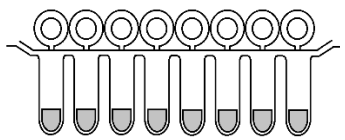
Note. The T-COR™ 8 should be surface disinfected between runs to prevent cross-contamination. Ensure to follow appropriate biosecurity procedures throughout.

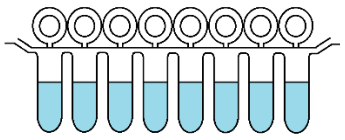
rRT-LAMP using the Genie® II

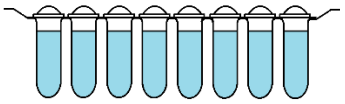
Materials supplied	Additional materials required
• Genie® II device • AC Adapter • Genie® II reactions (eight reactions per strip) • Resuspension buffer (in 1 ml aliquots) • Genie® II user manual	• Nuclease-free water • Pipettes and tips for 5 µl and 15 µl volumes • DNAZap™ DNA Degradation Solutions (Thermo Fisher Scientific)

Note: The Genie® II can be powered by battery (one charge is enough for approximately four hours use) or through mains power.

Protocol for set-up







1) Prepare work surfaces and pipettes using **DNAZap™**
2) Remove reactions from the packet and remove the seal from the top of the tube strip
3) Add 15 µl of **resuspension buffer** to each of the pellets
4) Add 5 µl of **nuclease-free water** to each reaction
5) Add 5 µl of the **sample** to be tested (processed as above)
6) Pipette up and down ten times to mix reactions
7) Close the reaction tube lids and tap tubes to remove air bubbles
8) Turn on the **Genie® II** using the back switch
9) Insert the reaction strip into either heat block A or B
10) Select **FMDV** on the start-up menu and select the heat block chosen
11) Follow the screen's instructions to set-up reactions and enter relevant sample details

Analysis

Genie® II software will automatically analyse results and report samples as FMDV-positive, FMDV-negative or re-test (for instance if the anneal temperature of amplicons is not within the defined range for the reaction). Results can be easily saved and shared (instructions within the Genie® II user manual).

Note. Alternatively, reactions can be heated in a water bath and analysed using PCRD-2™ lateral-flow devices (Abingdon Health, York, UK) as per manufacturer's instructions (see Waters *et al.*, 2014). The Genie® II should be surface disinfected between runs to prevent cross-contamination. Ensure to follow appropriate biosecurity procedures throughout.

Appendix 9

Responding to new emerging disease threats

Preliminary trials were performed on a field-ready real-time reverse transcription PCR (rRT-PCR) assay from Tetracore Inc. (MD, USA) to differentiate between foot-and-mouth disease virus (FMDV) and Seneca Valley virus 1 (SVV) (Tetracore Ltd., 2017). SVV, a vesicular disease of pigs, has recently been linked as one of the possible causes of idiopathic vesicular disease in the USA, Canada, Brazil, China and Thailand (Pasma *et al.*, 2008; Singh *et al.*, 2012; Leme *et al.*, 2015; Vannucci *et al.*, 2015; Wu *et al.*, 2016; Saeng-Cuto *et al.*, 2017).

Reactions were performed in duplicate on the T-COR™ 8 (Tetracore Inc.) as stated in Chapter 2.3.3. Probes for were modified for multiplex detection using the following fluorescence channels: SVV (Dragonfly Orange™ [DFO]), FMD virus (FMDV) (6-fluorescein amidite [FAM]) and exogenous internal control (Cy®5).

To determine the relative diagnostic performance, RNA was extracted from a panel of 36 clinical samples previously submitted to WRLFMD. These included 27 FMDV-positive samples, representing four serotypes (O, A, Southern African Territories [SAT] 2 and Asia 1) from 11 countries (Table 7.8), seven SVV samples originating from the USA and two swine vesicular disease virus (SVDV) samples (specificity controls) (Chapter 2.3.6). Results were compared against a reference rRT-PCR for FMDV (performed as stated in Chapter 2.3.2), SVV (performed as stated in Fowler *et al.* [2017]) and SVDV (performed as stated in Reid *et al.* [2004]).

Table 7.8 Clinical samples to evaluate the performance of a multiplex FMDV/SVV assay

							Singleplex	Multiplex		
Virus / Serotype	WRLFMD sample ID	Topotype	Lineage	Location	Year	Type	Reference rRT-PCR C_T	FMDV C_T	SVV C_T	IC C_T
O	TAI/20/2015	SEA	Mya-98	Thailand	2015	OF	15.61	19.65		33.55
	ISR/5/2015	ME-SA	PanAsia	Israel	2015	OF	26.30	23.70		32.20
	ISR/4/2015	ME-SA	PanAsia	Israel	2015	OF	20.46	21.90		32.45
	TAI/9/2015	SEA	Mya-98	Thailand	2015	OF	14.10	17.30		33.40
	SKR/6/2016	SEA	Mya-98	South Korea	2016	OF	29.75	25.15		31.95
	PAT/2/2015	ME-SA	PanAsia	Palestine	2015	OF	22.73	24.95		31.75
	ISR/9/2015	ME-SA	PanAsia	Israel	2015	OF	17.54	20.30		32.80
	NEP/15/2015	ME-SA	Ind-2001d	Nepal	2015	OF	18.28	21.90		32.85
	NEP/19/2015	ME-SA	Ind-2001d	Nepal	2015	OF	23.15	22.05		31.90
	SUD/4/2013	EA-3	unnamed	Sudan	2013	OS	20.79	21.50		32.20
A	TAI/15/2015	ASIA	Sea-97	Thailand	2015	OF	15.02	17.30		32.95
	TAI/23/2015	ASIA	Sea-97	Thailand	2015	OF	15.02	18.30		32.60
	SAU/8/2015	ASIA	G-VII	Saudi Arabia	2015	OS	22.73	24.80		32.10
	IRN/27/2015	ASIA	G-VII	Iran	2015	OS	25.26	26.00		31.80
	PAK/25/2015	ASIA	Iran-05^{FAR-11}	Pakistan	2015	OF	23.49	27.05		31.75
	PAK/55/2015	ASIA	Iran-05^{FAR-11}	Pakistan	2015	OS	15.93	20.25		33.90
	SUD/12/2013	AFRICA	G-IV	Sudan	2013	OS	27.95	25.40		31.80
	SUD/10/2013	AFRICA	G-IV	Sudan	2013	OS	27.85	25.85		31.80
	SUD/13/2013	AFRICA	G-IV	Sudan	2013	OS	31.07	27.70		31.80
SAT 2	ZIM/5/2015	II	unnamed	Zimbabwe	2015	OS	37.63	35.70		31.70
	SUD/5/2013	VII	Alx-12	Sudan	2013	OS	33.81	33.30		31.90
	ETH/10/2015	VII	Alx-12	Ethiopia	2015	OS	32.99	34.00		32.20
	SUD/7/2014	VII	Alx-12	Sudan	2014	OS	34.86	34.85		31.65
Asia 1	PAK/27/2015	ASIA	Sindh-08	Pakistan	2015	OF	7.82	14.75		33.80
	PAK/33/2015	ASIA	Sindh-08	Pakistan	2015	OS	19.81	21.90		32.45
	PAK/37/2015	ASIA	Sindh-08	Pakistan	2015	OS	21.19	24.35		32.25
	PAK/54/2015	ASIA	Sindh-08	Pakistan	2015	OS	21.94	23.25		31.80
SVV	NJ-90-10324	SVV1		New Jersey	1990		13.07		14.60	33.35
	LA-97-1278	SVV1		Los Angeles	1997	OS	11.19		13.30	33.70
	CA-01-131395	SVV1		California	2001		11.92		12.20	33.45
	IL-92-48963	SVV1		Illinois	1992		13.53		14.70	32.65
	IA-89-47552	SVV1		Iowa	1989		11.89		12.75	32.95
	MN-88-36695	SVV1		Minnesota	1988		12.40		13.50	33.15
	NC-88-23626	SVV1		North Carolina	1988		14.07		15.35	32.95
SVDV	UKG/4/73	SVDV		UK	1973	OS	11.48			31.50
	UKG/155/80	SVDV		UK	1980	OS	13.17			31.75

(OS) original suspension; (OF) original fluid e.g. vesicular fluid; (CC) cell culture. Cycle threshold (C_T) value is the average value across two replicates for real-time reverse transcription PCR (rRT-PCR). (WRLFMD) World Reference Laboratory for Foot-and-Mouth Disease; (FMDV) foot-and-mouth disease virus; (SAT) Southern African Territories; (SVDV) swine vesicular disease virus; (SVV) Seneca Valley virus 1.

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