Development and validation of a field deployable test for the diagnosis of high-priority infectious animal diseases in New Zealand

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Rudolfo Bueno

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Abstract

In the event of infectious disease incursions, rapid and accurate diagnosis is essential for ensuring appropriate and prompt control measures are put in place to minimise further transmission. Foot and mouth disease (FMD) is one example of an exotic disease that could severely affect New Zealand's livestock industries if introduced to this country. Pen-side testing can help by providing a rapid confirmation of a provisional diagnosis without the delays and risks associated with sending samples to a diagnostic laboratory. The aim of the work presented in this thesis was to develop and validate a field deployable diagnostic test system for prompt and accurate detection of FMD virus (FMDV). In addition, the test can be used to simultaneously detect two other viruses that would be expected to be on the differential diagnosis list: bovine viral diarrhoea type 1 (BVDV-1) and type 2 (BVDV-2).

Chapter 1 comprises a brief literature review of FMDV infections in susceptible species, followed by a review of the current and emerging trends in field deployable diagnostics as applicable to animal diseases.

In Chapter 2, a multicriteria scoring and ranking model for identifying the best test platform for development of the deployable field test is presented. The general flow of the method consisted of defining the requirements for the ideal test platform, identifying, and shortlisting potential candidate systems, describing the criteria for evaluation, and scoring the candidate platforms against the criteria by a panel of recruited experts. This participatory and collective opinion provided a basis for selecting T-COR 8™ (Tetracore®) as the best overall fit-for-purpose.

In Chapter 3, several easy techniques for processing clinical samples compatible with the selected test platform were examined. These protocols were applied to test panels comprising serial dilutions of BVDV-1 or equine rhinitis A virus (ERAV) in serum or oral swab samples. The latter was used as a proxy for FMDV. The protocols were compared to a reference extraction method based on the observed detection limit, as judged by quantification cycle (Cq) values generated in virus-specific reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays. The complexity of sample manipulation and time required were also considered. Dilution of the sample with phosphate-buffered saline (PBS), with or without a pre-

heating step, was chosen as the most suitable method for integration in the pen-side PCR testing.

Development of the field assay's controls is described in Chapter 4. These included a synthetic positive control transcript (R3+) that could be safely used with assays aimed at the detection of several pathogens associated with development of vesicular disease in cattle. The universal control transcript also incorporated an exogenous internal control (IC) target, which was designed to be used with a phage based (Qβ) internal control (IC) system. Optimization of a Qβ IC assay for use in the pen-side multiplex RT-qPCR (mRT-qPCR) is also included in this Chapter.

In Chapter 5, development, and optimisation of mRT-qPCR for the differential detection of FMDV, BVDV-1 and BVDV-2, including detection of a Qβ as exogenous IC, is presented. The optimised mRT-qPCR showed linearity over five 10-fold dilutions of R3+ transcript, good efficiency, and low intra-and inter-assay variability. The mRT-qPCR was highly specific for the detection of representative FMDV serotypes and was also able to simultaneously detect BVDV-1 and BVDV-2 isolates. The assay did not react with other viruses that can produce vesicular lesions, nor did it react with unrelated bovine pathogens endemic in New Zealand. Multiplexing the four primer- and probe sets did not affect the performance and analytical sensitivity of the assay for the detection of individual components when compared to the respective singleplex assays.

The diagnostic performance of the optimised mRT-qPCR for detecting FMDV, BVDV-1 and BVDV-2 is presented in Chapters 6 and 7. Diagnostic specificity was evaluated using sera and oral swabs from New Zealand cattle. Diagnostic sensitivity for FMDV detection was assessed using mock oral swabs from outbreak samples in two endemic countries (Lao PDR and Myanmar). The robustness of the field PCR was evaluated at three field locations with varied environmental conditions (New Zealand, Lao PDR, and Myanmar). Overall, the diagnostic specificity (DSp) of the field mRT-qPCR for three target viruses (FMDV, BVDV-1 and BVDV-2) was close to 100%, which was similar to the performance of respective reference PCRs. Although the diagnostic sensitivity (DSe) of the FMDV component was comparable to that obtained with the reference method, care must be taken in interpreting the result since FMD positive samples used for evaluation of the sensitivity of the mRT-qPCR were not sourced from New Zealand cattle. The mRT-qPCR also had high DSe for detecting BVDV-1 infected cattle when the BVDV RNA levels expected to be present in clinical samples from either persistently infected (PI) or

transiently infected animals were considered. Pre-heating of samples increased the sensitivity of the BVDV-1 component of the assay. Further validation using additional FMDV-positive and negative clinical specimens should be attempted in the future.

Overall, the work presented in this thesis resulted in the development of a simple, extraction-free pen-side PCR test that can be deployed around New Zealand for rapid and reliable detection of FMDV in the event of a suspected incursion. Future work to enhance its use would involve exploration of other methods of preparing samples so that the test can be utilised in screening sub-clinical FMDV infections during post-outbreak surveillance.

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List of abbreviations

AHL	Animal Health Laboratory
AHL-MPI	Animal Health Laboratory-Ministry for Primary Industries
ASe	analytical sensitivity
ASF	African swine fever
ASp	analytical specificity
BLCM	Bayesian latent class model
BVDV-1	bovine viral diarrhoea virus type 1
BVDV-2	bovine viral diarrhoea virus type 2
CI	confidence interval
CP	cytopathic
CPE	cytopathic effect
CPMV	cowpea mosaic virus
Cq	quantification cycle
CSF	classical swine fever
CV	coefficient of variation
DIVA	differentiating infected from vaccinated animal
DNA	deoxyribonucleic acid
DSe	diagnostic sensitivity
DSp	diagnostic specificity
ELISA	enzyme-linked immunosorbent assay
ERAV	equine rhinitis A virus
FMD	foot and mouth disease
FMDV	foot and mouth disease virus
IC	internal amplification control
LAMP	loop mediated isothermal amplification
LFD	lateral flow device
LFIA	lateral flow immunoassay
LOD	limit of detection
MD	mucosal disease
mRT-qPCR	multiplex reverse transcription quantitative polymerase chain reaction
NA	nucleic acid
NC	negative control
NCP	non cytopathic
NI	North Island
nM	nanomolar
NPV	negative predictive value
NSP	non-structural protein
nt	nucleotide
NTC	non template control
NZ	New Zealand
ORF	open reading frame
PBS	phosphate buffered solution

PC	positive control
PCR	polymerase chain reaction
PI	persistently infected
POC	point-of-care
PPV	positive predictive value
PRRS	porcine respiratory and reproductive syndrome
qPCR	quantitative polymerase chain reaction
Qβ	Q beta bacteriophage
QβIC	Q beta internal control
RFW	RNAse-free water
RNA	ribonucleic acid
RT	room temperature
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SAT	Southern African Territories
SD	standard deviation
SI	South Island
SP	structural protein
SVDV	swine vesicular disease virus
SVV	Seneca Valley virus
TAD	transboundary animal disease
TCID	tissue infective dose
UK	United Kingdom
µL	microlitre
µM	micromolar
UTR	untranslated region
VESV	vesicular exanthema of swine virus
VSV	vesicular stomatitis virus
VTM	virus transport media
WOAH	World Organisation for Animal Health
SEACFMD	South-East Asia and China Foot and Mouth Disease Campaign

Chapter 1. Review of Literature

Transboundary animal diseases (TADs) can be transmitted rapidly among susceptible animals across national borders (FAO, 1996). High-priority TADs include but are not limited to the following diseases: foot and mouth disease (FMD), highly pathogenic avian influenza (HPAI), Rift Valley fever, Newcastle disease, African swine fever (ASF), classical swine fever (CSF), peste des petits ruminants, and contagious bovine pleuropneumonia (Domenech et al., 2006; Rossiter & Al Hammadi, 2009; Siembieda et al., 2011). A TAD epidemic in a previously free country has tremendous implications on trade, socio-economic welfare, animal welfare and food security (Rossiter & Al Hammadi, 2009). In such situations, it is usually necessary to establish cooperation among affected neighbouring countries to control or eradicate the disease (Domenech et al., 2006). Examples of recent outbreaks with high economic consequences are the FMD outbreak in the United Kingdom (UK) in 2001 (Davies, 2002) or the HPAI H5N1 epidemic in poultry in Asia from 2003 to 2007 (Otte et al., 2008). The estimated cost of the UK FMD epidemic to the public and private sectors was about £3 and £5 billion, respectively (Scott et al., 2004; Thompson et al., 2002). In addition, the loss of livelihood brought social and emotional costs to farming communities (Mort et al., 2008). Due to the massive culling and disposal of millions of animals, animal welfare issues also caused undue stress to affected farmers (Convery et al., 2005). In the HPAI H5N1 disease outbreak, poultry producers in the Southeast Asian region incurred high direct costs due to massive mortalities and the culling of millions of birds. An unwanted effect of this epidemic was the contribution of the H5N1 influenza virus to cases of human infections and deaths (Rushton et al., 2005).

A robust surveillance program to detect an exotic disease incursion rapidly and accurately is of primary importance, particularly in countries where specific TAD agents are absent. Currently, developed nations rely on a state reference laboratory network to perform diagnostic testing to confirm the diagnosis of infection with an exotic pathogen. Laboratory procedures include culture of a pathogen of interest; detection of pathogen-specific antigens (e.g., enzyme immunoassay), nucleic acids (e.g., polymerase chain reaction or PCR), antibodies (e.g., enzyme-linked immunosorbent assay or ELISA), and direct visualisation of the pathogen (e.g., microscopy) (Fenner et al., 2014).

The new Biocontainment Animal Health Laboratory of the Ministry for Primary Industries (AHL-MPI), located at Wallaceville, Upper Hutt, is the national reference laboratory for animal diseases in New Zealand. AHL-MPI plays an essential role in protecting New Zealand's primary industry against TAD incursions, including the ability to perform laboratory testing for specific exotic pathogens. Indeed, rapid, reliable diagnostic tests are crucial to minimising the effect of new disease incursions and subsequent spread. However, one diagnostic challenge for AHL-MPI when confronted with high-impact disease such as FMD is the risk of delayed transit of collected samples from the field to the laboratory, especially from remote locations.

Field-based diagnostic testing is one approach AHL-MPI can employ to corroborate provisional diagnosis based on the clinical presentation of diseased animals on-farm. "Field-based diagnostic test" refers to testing that can be completed at the farm or in the field near sick animals. Other descriptors of such test include "field deployable diagnostic test" and "field-based test", however in this thesis, the term "pen-side test" will be commonly used. In such cases, a rapid presumptive diagnosis can be made, allowing immediate implementation of interventions and controls while awaiting a confirmatory diagnosis from AHL. Field-based tests have been introduced as first response tests to detect bio-threat organisms (Ivnitski et al., 2003; McAvin et al., 2003); to provide rapid and reliable diagnostic tools in low resource areas (Cordray & Richards-Kortum, 2012; Escadafal et al., 2014); and to support animal, plant, and aquatic disease investigations (King et al., 2010; King et al., 2012; Liu, Benyeda, et al., 2016). Several field-based diagnostic assays for specific TADs such as FMD, ASF, CSF and avian influenza have been developed (http://rapidia.eu/workshop-2015/). These deployable tests offer complementary advantages over current laboratory capability in terms of speed of diagnosis in certain situations.

Therefore, the main purpose of the project presented in this thesis was to explore the utility of pen-side tests in enhancing AHL's efficiency of investigation, diagnosis, and early response to potential exotic disease incursions in New Zealand. FMD, a highly consequential disease, including an endemic differential agent, were prioritised in developing a "proof-of-concept" pen-side test. The use of FMD as a model disease for field-based test development was timely due to the recent FMD outbreak in Indonesia that could threaten New Zealand. An important aspect of diagnostic capability is multiplexing. A multiplex test can be used to analyse multiple pathogens simultaneously in one reaction. The bovine viral diarrhoea (BVD) virus, the causative agent of an important cattle disease in New Zealand, was selected for

multiplex pen-side test development because the mucosal disease form of BVD can be confused with the clinical diagnosis of FMD. Also, BVD virus (BVDV), a ribonucleic acid (RNA) virus like FMDV, was preferred over other deoxyribonucleic acid (DNA) viruses that may produce vesicular-like lesions in New Zealand cattle.

In the first part of Chapter 1, a brief literature review of FMD virus (FMDV) infection in susceptible species and BVDV infection in cattle is presented. In the second part the current and emerging trends in field deployable diagnostics applicable to animal health are reviewed. The basis for selecting a deployable test platform is described in Chapter 2.

1.1. Foot and mouth disease

Foot and mouth disease is highly contagious and one of the earliest animal diseases recorded in history. Italian cattle with vesicular-like lesions in the mouth and on the feet described by Fracastorius in 1541 were believed to be due to FMDV infection (Bachrach, 1968). Almost four centuries later, Loeffler and Frosch (1897) described the identification of a filterable infectious agent from similarly affected animals, later referred to as the virus that causes FMD (Grubman & Baxt, 2004). In modern times, FMD has been reported by almost all primary livestock-producing nations except New Zealand. The virus is circulating in two-thirds of the World Organisation for Animal Health (WOAH) member countries, in Asia, Africa, the Middle East, and a small area in South America (Anonymous, 2022). FMD is a WOAH-listed disease and requires member countries to report it if detected.

The most recent incursion of FMD to a previously disease-free country was in Indonesia in April of 2022, with subsequent spread among the country's livestock population. Indonesia has been FMD-free without vaccination since 1983. Due to geographic proximity and an elevated risk of FMD introduction, Australia, and New Zealand, intensified their disease preparedness through surveillance, border prevention measures, public awareness, and updated existing response plans. FMD is considered one of the costliest animal diseases with negative socio-economic consequences, as demonstrated during the UK epidemic in 2001 (Mort et al., 2008; Park et al., 2020; Thompson et al., 2002).

1.1.1. The causative virus

Foot and mouth disease virus is a member of the genus *Aphthovirus* of the *Picornaviridae* family (Zell et al., 2017). The virion has a diameter of 25-30 nm with an icosahedral capsid composed of four proteins, 1D (VP1), 1B (VP2), 1C (VP3), and 1A (VP4), with 60 copies each forming 12 pentamers (Acharya et al., 1989; Belsham & Martinez-Salas, 2019). The capsid, specifically the VP1, plays an essential role in virus affinity and entry to the host cells, immunity, and serotype identification. Within the non-enveloped capsid is a positive sense, single-stranded genomic RNA (approximately 8400 nt) consisting of a 5' untranslated region (5' UTR), a large open reading frame (ORF), and the 3' UTR (Jamal & Belsham, 2013, 2018). The large, 7000 nucleotide ORF encodes one long polyprotein (Figure 1-1) that includes two proteases (Lpro and 3Cpro) (Mason et al., 2003). Primary autocatalytic polyprotein cleavage occurs between L and 1A, followed by cleavage at the 2A oligopeptide and 2B junction. 3Cpro mediates the cleavage between 2C and 3A proteins. Protein precursor products after the three primary proteolytic events consisted of Lpro, P1-2A, P2 and P3. The 3Cpro protease then processes the protein precursor products into four structural proteins from the P1 region and ten non-structural proteins from P2 and P3 regions (Mason et al., 2003). The structural proteins form the capsid, where most antigenic sites are located. The non-structural proteins are involved in the replication of the virus genome. The gene coding of the VP1 protein has been commonly used to characterise the phylogenetic and epidemiological relationships of various FMDV variants and lineages (Knowles & Samuel, 2003).

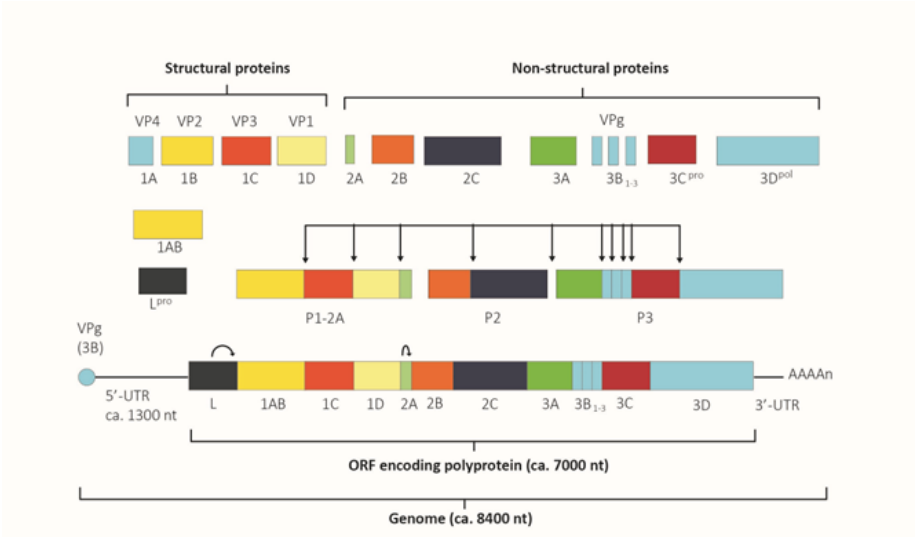

Figure 1-1. The FMDV genome composition. Auto-catalytic cleavage occurs between L and 1AB and 2A and 2B proteins. 3Cpro mediates the cleavage between 2C and 3A protein, and the processing of the protein precursors (P1-2A, P2 and P3) to produce four structural proteins and ten non-structural proteins. UTR – untranslated region, ORF – open reading frame, VP- viral protein. Image was based on Jamal & Belsham, 2018.

Seven antigenically distinct FMDV serotypes exist: A, O, C, Asia 1, Southern African Territories 1 (SAT 1), SAT 2 and SAT 3 (Jamal & Belsham, 2013; Klein, 2009). Within each serotype are subtypes or topotypes with many genetically and antigenically diverse variants and lineages due to the error-prone replication typical for RNA viruses (Knowles & Samuel, 2003). Serotypes O and A are the most common worldwide (Jamal & Belsham, 2018). Serotype O, however, has caused most of the reported FMD outbreaks (Maradei et al., 2014; Park et al., 2014; Valdazo-González et al., 2013). Serotype Asia 1, except for occasional incursion to another geographic region, is primarily confined to Asia (Ali et al., 2020; Bo et al., 2019). The same pattern was also seen with the three SAT viruses, which are restricted to the African continent (Ahmed et al., 2012; Blignaut et al., 2020; Vosloo et al., 2002). Serotype C is considered extinct after the last reported outbreak in South America in 2004 (Paton et al., 2021; Sanchez-Vazquez et al., 2019).

1.1.2. FMDV geographic pools

Genetic analyses of the VP1 coding region of FMDV worldwide revealed the circulation of related or similar FMDV subtypes or topotypes within a particular region (Rweyemamu et al., 2008) despite the ability of the virus to spread irrespective of national boundaries (Jamal & Belsham, 2013). The characteristics of virus distribution were believed to reflect concurrent animal movement patterns, trading practices, and wildlife maintenance reservoirs of a specific region (Brito et al., 2017; Freimanis et al., 2016). Overall, seven geographical FMDV pools, consisting of at least three serotypes, were identified across the continents of Asia, South America, and Africa (Brito et al., 2017; Paton et al., 2009) (Figure 1-2). Trans-pool migration of FMDV, however, does occur. For example, serotype O/ME-SA/Ind-2001d lineage escaped the South Asian subcontinent multiple times in different directions. The virus was re-introduced in the Middle East as early as 2009, spreading to North Africa in 2014 (Jamal & Belsham, 2018). After a year, this O lineage was also detected for the first time in Southeast Asian countries, with subsequent spread to the Republic of Korea and the Russian Federation. The recent outbreak in Indonesia in 2022 (Figure 1-2) was also due to the sub-lineage O/ME-SA/Ind-2001e (Susila et al., 2022).

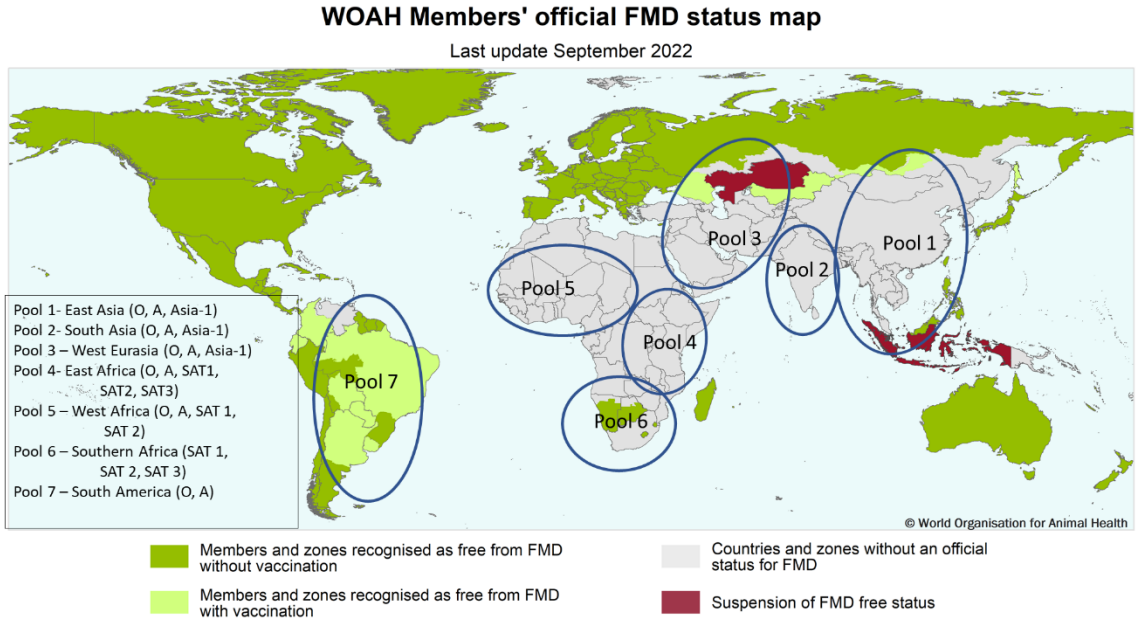

Figure 1-2. The conjectured global distribution of foot and mouth disease (FMD) viruses are divided into seven regional pools (Paton et al., 2009). Pool 1- Southeast Asia, East & Central Asia - (O, A, Asia-1), Pool 2- South Asia (O, A, Asia-1), Pool 3 – West Eurasia (& Middle East (O, A, Asia-1), Pool 4- East Africa (O, A, SAT1, SAT2, SAT3), Pool 5 – Central and West Africa (O, A, SAT 1, SAT 2), Pool 6 – Southern Africa (SAT 1, SAT 2, SAT 3), Pool 7 – South America (O, A). Serotype O followed by A is the most common serotype. Overlapping between pools may also occur. The FMD status map (updated May 2023) was taken from the World Organisation of Animal Health website (https://www.woah.org/en/disease/foot-and-mouth-disease/#ui-id-2).

1.1.3. Host range and clinical disease

Domestic cloven-footed animals, including cattle, sheep, pigs, goats, and deer, are the most important animal species affected by FMD under natural conditions (Alexandersen & Mowat, 2005). FMD also affects at least 70 species of wild ungulate animals (e.g., kudu, impala, gazelle, African buffaloes etc.) (Grubman & Baxt, 2004), with relatively mild clinical signs (Berkowitza et al., 2010; Rout et al., 2017; Vosloo et al., 2003; Ward et al., 2009), although severe disease is not uncommon (Berkowitza et al., 2010). However, African buffaloes in endemic locations (Di Nardo et al., 2015; Wekesa et al., 2015) or wild boar and wild deer in areas of existing outbreaks (Wekesa et al., 2015) have been found to seroconvert without any overt disease. As Alexandersen and Mowat (2005) pointed out, it is important to understand each animal species' role in the transmission and maintenance of FMD in a given region. For example, the African buffalo is the only known maintenance host of FMDV and plays an essential role in the disease epidemiology in southern Africa (Vosloo et al., 1996; Vosloo et al., 2007).

Clinical severity of FMD and disease transmission varies (Alexandersen & Mowat, 2005) depending on the individual or a combination of the following factors: virulence of the virus (García-Nuñez et al., 2010), infective dose (Moreno-Torres et al., 2018), animal species (Donaldson, 2019), and differences between animals of the same species (Yoon et al., 2012) which can also be influenced by previous infection or vaccination. In highly susceptible cattle, acute signs most often start with depression and fever (≥40°C) for 1-2 days (Arzt et al., 2010). This is followed by the appearance of vesicles on the epithelial surface of the tongue, hard palate, dental pad, lips, muzzle, coronary band, and interdigital spaces (Onozato et al., 2014; Yoon et al., 2012). The teats are also a primary site for vesicle formation in dairy cattle (Yoon et al., 2012). Young calves may die without clinical signs due to myocardial necrosis (Kitching, 2002). As the disease progresses, erosive ulcerations, hypersalivation, lip smacking, and pain associated with the formation of vesicles in the interdigital space leading to lameness can be observed in affected cattle. Cattle usually assume a tucked-up posture and are reluctant to move due to the painful condition in the affected feet. Serous to mucopurulent nasal discharge is also common during the acute stage of FMD. Despite the high morbidity in adult cattle, mortality is generally low (<5%) (Donaldson, 2019).

Clinical disease in pigs is similar to cattle (Alexandersen, Zhang, et al., 2003). Vesicles appear to manifest initially as blanched spots between the interdigital cleft of the hooves, coronary band, heel bulb and accessory digits in the feet (Yamada et al., 2018). Vesiculation and ulcerations are more common in the feet and snout than in the oral cavity's tongue and epithelium (Alexandersen, Kitching, et al., 2003; Yoon et al., 2012). A severe form of the disease in pigs is characterised by lameness, reluctance to move, recumbency and claw shedding and can be complicated by secondary infections (Yoon et al., 2012). In the South Korean outbreak (2010/2011), Yoon et. al. (2012) also reported high piglet mortality.

In contrast to cattle and pigs, FMD in small ruminants (sheep, goats, and deer) is generally milder with less visible signs (reviewed by Arzt et al., 2011). Experimental infection of sheep or goats by a combination of both intra-dermo-lingual and coronary band routes only produced mild signs such as poor appetite, panting, and fever (≥40 ◦C) (Muthukrishnan et al., 2020). In that study, vesicles were only observed between day 2 to day 5 post-inoculation in animals inoculated with FMDV O/IND/R2/75. Kittelberger et al. (2017) also reported similar clinical observations in red deer when only 1/10 animals showed mild clinical signs after intranasal inoculation with FMDV O UKG 11/2001. No pyrexia was noted in the affected deer, with mild lameness and two small erosions (2 mm wide) underneath the tongue

observed on day six post-inoculation. Whilst these experiments only comprised very small numbers, the clinical findings generally agreed with the milder disease manifestation in small ruminants during the Korean outbreak (2010/2011) caused by FMDV serotype O from Southeast Asian lineage (Yoon et al., 2012). It is important to note that all studies described above (two experimental and an outbreak report) involved only serotype O and do not represent the disease spectrum caused by other genotypes and serotypes. Some FMDV strains were believed to have species-specific traits, which produce severe disease in one specific host but not in others (Arzt et al., 2011). One example was the existence of a porcinophilic variant (serotype O) with low virulence in cattle and small ruminants (reviewed by Arzt et al., 2011). Nevertheless, subclinical infection is still generally regarded as more common among small ruminants than in cattle and pigs (Arzt et al., 2011), with the severity of disease resulting from the interplay between the virus, animals, and environment (Donaldson, 2019).

Except for the reported outbreak in impala (South Africa) and gazelle (Israel) during the late 19th century (reviewed in Thomson et al., 2003), the clinical disease seen in wildlife (deer, kudu, wild pigs, and African buffalo) under natural condition was often considered to be mild (Donaldson, 2019; Thomson et al., 2003; Vosloo et al., 2007; Vosloo et al., 2009).

The appearance of the FMD lesions can be used as a guideline to estimate the age of the lesions and hence the time of infection in an outbreak response (Anonymous, 1995). During the 2001 FMD outbreak in the UK, animal health personnel were able to approximate the timing of FMD introduction into a property by deducting the incubation period (1-14 days) from the likely date of emergence of the old lesions (Ryan et al., 2008). This was very useful for tracing contact animals or herds during the outbreak.

1.1.4. Pathogenesis

In general, the pathogenesis of FMD can be influenced by the differences in animal species and strain-specific viral properties. The pathogenesis described for cattle is similar to other animals. However, some notable differences with other animal species were specified. The infection pathway of the FMD virus was recently examined for the following primary susceptible animals: cattle (Arzt et al., 2011; Arzt et al., 2010), pigs (Stenfeldt, Segundo, et al., 2016; Yamada et al., 2018), sheep and goat (Arzt et al., 2011; Muthukrishnan et al., 2020). Pathogenesis has also been elucidated in young lambs (Ryan, Horsington, et al., 2008) and

neonatal animals (pigs, calves, and lambs), with a particular focus on the non-epithelial tropism of FMDV (Zhang et al., 2021).

1.1.4.1. Route of infection

Inhalation of expelled respiratory droplets with FMDV is commonly accepted as the primary route of infection in ruminants, particularly cattle, due to their large lung capacity (Alexandersen, Zhang, et al., 2003). This may be why cattle succumb to FMD to lower doses than pigs (Sellers & Gloster, 2008). In pigs, the disease is more likely through ingestion of virus-contaminated feed during communal feeding and by infected bodily secretions (saliva, nasal fluid, tears, expired breath, urine, or faeces) rather than the respiratory route (Stenfeldt, Segundo, et al., 2016). Experimental inoculation has also demonstrated infection through wounds or breakage in the epithelial layer of the skin (reviewed by Alexandersen, Zhang, et al. (2003).

1.1.4.2. Incubation period

The incubation period between infection and the appearance of the clinical lesions in cattle during farm-to-farm (direct, indirect, or airborne) spread appears to range between 2 and 14 days (Alexandersen & Mowat, 2005). The length of this period can be impacted by several variables, such as infective dose, the variant of the virus, proximity to infected animals, animal species present at the farm, population density, and environmental conditions (Alexandersen, Zhang, et al., 2003). Animal-to-animal spread within the farm has a shorter incubation period (1-6 days), especially if there is high infection pressure on the farm through close contact with diseased animals. Recent mathematical modelling involving three serotypes (O, A, Asia 1) supported this shorter incubation period in cattle (3.6 days, 95% CI = 2.7-4.8) (Yadav et al., 2019) and pigs (2.9 days, 95% CI = 2.67-3.17) (Moreno-Torres et al., 2022) using experimental infection studies (direct inoculation or direct contact with experimentally inoculated animals).

1.1.4.3. Pre and viraemic phase

Arzt et al. (2011) described three phases of FMD in cattle: pre-viraemic (initial incubation and pre-clinical), viraemic (virus dissemination and clinical), and post viraemic phase (clinical resolution and viral persistence). The pre-viraemic phase was defined as the

time from the virus entry (infection) until virus detection in the bloodstream. In earlier reviews, the pharynx was implicated as the site of primary viral replication after exposure (Alexandersen & Mowat, 2005; Alexandersen, Zhang, et al., 2003). This was based on the consistent detection of FMDV in probang samples and previous *in situ* hybridisation studies. FMDV RNA was also observed to increase temporally in the nasopharyngeal and laryngeal sites from 3 to 12 hours post-aerosol exposure (hpa) (Arzt et al., 2010). More specifically, localised FMDV antigen was observed by immunohistochemistry in the rostro dorsal nasopharynx as early as six hpa (Arzt et al., 2011; Arzt et al., 2010). Within 24-48 hours after virus exposure in cattle, a pan-respiratory viral distribution was assumed after abundant viral RNA was also detected in the pulmonary tissues and antigens in pneumocytes (Arzt et al., 2011). The increase in RNA detection in the lungs coincided with viremia. Whilst not so well studied, the primary site of replication in small ruminants is presumed to be the same as for cattle (Arzt et al., 2011). In the viraemic phase, FMDV is disseminated to almost all tissues and organs, followed immediately by secondary replication in the epithelium of the feet, mouth, and teats producing the typical vesicular lesions (Arzt et al., 2010). It is speculated that sustained viraemia in cattle is due to the high viral concentration in the lungs (Arzt et al., 2011; Arzt et al., 2010). However, the exact link between lymph nodes draining the lungs and oral cavity during the early phase of infection as a pathway towards the onset of the viraemic phase has yet to be clearly established (Arzt et al., 2010). Virus titre in serum starts to decay as soon as neutralising antibodies circulate 4-7 days post-infection (PI), and the presence of viral RNA is variably detected between 4-14 days.

In contrast to cattle, the primary site of viral replication in pigs upon entry via the gastrointestinal tract is believed to be the epithelium of the oropharyngeal tonsils (reviewed by Stenfeldt, Segundo, et al., 2016). The dorsal nasopharynx in cattle and oropharyngeal tonsils in pigs have distinct mucosa-associated lymphoid tissue underneath the epithelium (Stenfeldt, Segundo, et al., 2016). Regardless of the animal species, FMDV was detected (PCR, virus isolation) in the bloodstream (viraemia) 1-2 days before the appearance of any clinical signs of disease, including fever (Alexandersen, Quan, et al., 2003; Alexandersen et al., 2002; Arzt et al., 2010; Pacheco et al., 2016; Yamada et al., 2018). In pigs, viraemia was believed to be due to the virus entering the bloodstream from the high virus-concentrated vesicular lesions (Arzt et al., 2011; Yamada et al., 2018).

1.1.4.4. Post-viraemia

The post viraemic phase is characterised by viral persistence. FMDV persists in about 50% of infected cattle beyond 28 days post-infection, with no associated symptoms and circulating and excreted virus (Arzt et al., 2011). Although the cellular and molecular basis of virus persistence needs further investigation (Zhu et al., 2020), FMDV localised in the same pharyngeal site for primary viral replication (Stenfeldt et al., 2018) for an extended period (Arzt et al., 2011; Zhang & Alexandersen, 2004). The mean duration of probable viral persistence in cattle was predicted to be 18 to 24 months after infection using two statistical analyses (survival analysis model and generalised linear model) (Bertram et al., 2020).

The infectious clinical phase is estimated to be longer in pigs (11 days, 95% CI = 10.75-11.33) (Moreno-Torres et al., 2022; Stenfeldt, Pacheco, et al., 2016) than in cattle (8.5 days, 95% CI = 6.2-11.6) (Yadav et al., 2019). Except for one report of possible persistence, FMDV clearance in pigs was considered efficient (Stenfeldt, Segundo, et al., 2016).

1.1.4.5. Virus clearance and immune responses

Although incompletely elucidated, McCullough et al. (2017) reviewed the various mechanisms of innate and adaptive host responses to FMDV infection. It is important to note that variation in the immune response against the virus exists amongst different host species (e.g., cattle and pigs) (Toka & Golde, 2013). Notwithstanding, the rapid induction of humoral-mediated immune response plays a key role in virus clearance (Arzt et al., 2011). Virus-specific antibodies can be detected in cattle and pigs as early as 3-4 days post-infection (dpi) (Alexandersen & Mowat, 2005; Pacheco et al., 2010). The surge of an anti-FMDV IgM peak at 5 to 7 dpi, followed by a high IgG response (Pacheco et al., 2010). Comparative to antibodies produced from infection, vaccine-induced antibodies were found protective in cattle 6-7 days post-vaccination (Golde et al., 2005).

Anti-FMDV antibodies can be induced both by the structural capsid protein, which is serotype-specific, and the pan-serotypic non-structural proteins (Paton et al., 2006). Antibodies against capsid protein appeared to develop earlier than antibodies for the non-structural protein. Neutralising antibodies could last beyond 100 days after infection in pigs (Stenfeldt, Segundo, et al., 2016) and cattle (Alexandersen, Zhang, et al., 2003).

Comparatively, protective antibodies were also observed in cattle six months after vaccination (Cox et al., 2010).

Coinciding with the appearance of neutralising antibodies in cattle and pigs, FMDV is cleared in the blood between 7 and 14 dpi (Stenfeldt, Pacheco, et al., 2016; Zhang & Alexandersen, 2004). However, Zhang and Alexandersen (2004) pointed out that virus clearance in other tissue sites (lymph nodes, nasopharynx, soft palate) has a slower pace in cattle.

1.1.5. Disease transmission

In endemic countries, dissemination of FMDV commonly occurs through direct or indirect contact between infected and naive animals (Pacheco et al., 2012). The virus can be transmitted via infectious droplets, respiratory aerosols, milk, semen or indirectly through farm personnel (Amass et al., 2003), contaminated environment, and fomites (Bravo De Rueda et al., 2015; Colenutt et al., 2021). Pigs are often designated as virus amplifiers because of their ability to produce and expel high quantities of infectious respiratory droplets, which can be aerosolised under certain environmental conditions (Stenfeldt et al., 2020). High virus titres can be detected in oral and nasal fluids, serum, exhaled breaths, and milk 1-2 days before the appearance of clinical disease (Alexandersen, Quan, et al., 2003; Alexandersen et al., 2002). The total duration of potential viral shedding (pre-clinical and clinical) post-infection is about 10 days (95% CI = 8.2-14.2 days) in cattle (Yadav et al., 2019) and 11 days (95% CI = 10.74-11.33 days) in pigs (Moreno-Torres et al., 2022).

Airborne FMDV transmission has also been reported and could be an essential aspect of the virus spread (Brown et al., 2022). Aerosolised virus particles (<5 μm diameter), described as partially evaporated respiratory droplets, stay infectious and can be dispersed by wind currents over long distances (Gloster et al., 1982; Mekibib & Ariën, 2016). The aerosolised virus can remain infective over a prolonged period, especially when relative humidity (>55%) is conducive to virus survival (Gloster et al., 1982). With no evidence of animal movement or personnel from infected farms, airborne transmission from Brittany, France, was suspected as the likely path of the FMD outbreak in Jersey (1974) and Isle of Wight (1981) (Donaldson et al., 1982). The airborne transmission was also reported to occur during the UK 2001 epidemic between proximity farms with a distance between 1 and 16 km (Gloster et al., 2005).

Infected pig farms, exhaling high viral load in the air, can be the primary source of FMDV airborne transmissions, and susceptible cattle are likely to be the recipient of the new outbreak because of their low infective dose threshold (Brown et al., 2022; Gloster et al., 1982).

Survivability of FMDV in the environment may contribute to the indirect spread of infection (Colenutt et al., 2020), as shown by the UK outbreak in 2007, where the virus was believed to escape from the faulty effluent pipe from a vaccine plant at The Pirbright Institute (Ellis - Iversen et al., 2011). Although FMDV is sensitive to mildly acidic pH (7–7.5) (Caridi et al., 2015) and high temperatures (Scott et al., 2019), the virus can survive for weeks outside the host, especially when the humidity is favourably high, e.g., rainy wet condition (Mielke & Garabed, 2020), and in the presence of organic material. The FMD virus can be detected in pig carcasses' muscles at a cold temperature (4°C) for seven days. However, vesicular epithelium with lesions remained infective for at least 11 weeks at this temperature (Stenfeldt et al., 2020).

1.1.6. Carrier state

The exact role of carrier animals in FMD epidemiology needs to be better elucidated. Virus persistence in healthy cattle and buffalo (Buckle et al., 2021; Jamal et al., 2012), although considered low risk (Bertram et al., 2018), is believed to have contributed to the maintenance of FMD in endemic regions such as Southeast Asia (de Carvalho Ferreira et al., 2017). FMDV could also persist in African buffalo (approximately five years) (Vosloo et al., 2007), Asian swamp buffalo (1.5 years) (Maroudam et al., 2008; Verin, 2011), and small ruminants (<1 year) (Arzt et al., 2011). The presence of protective antibodies after vaccinating the animals does not prevent the development of persistent infection (Cox et al., 2006; Hayer et al., 2018). This has implications for countries with recent outbreaks wanting to regain disease-free status immediately with the aid of vaccination (Stenfeldt, Eschbaumer, et al., 2016).

1.1.7. Diagnosis and diagnostic methods

Vesicular disease syndrome is a common feature of several diseases (Table 1-1) that are endemic to New Zealand (Holliman, 2005). Any suspicion of FMDV incursion based on clinical lesions associated with epidemiological features must be investigated using

laboratory methods to rule FMDV in or out. The WOAH (2018) recommends several virological and serological assays to detect FMDV in suspected samples or demonstrate exposure of the animal to the virus through the presence of virus-specific antibodies. Shown in Figure 1-3 is an example of an FMD diagnostic algorithm that can be employed in outbreaks in both endemic and non-endemic countries (Ding et al., 2013; Wong et al., 2020).

1.1.7.1. Sample type

Samples of choice for FMDV detection using RT-qPCR or by virus isolation include vesicular epithelium and fluid; oral or nasal swab; and probang fluids (Poonsuk et al., 2018) because these samples contain high FMDV load during clinical disease (Alexandersen, Quan, et al., 2003; Alexandersen et al., 2002). In addition, a serum is a valuable specimen for qPCR or virus isolation, aside from being used for serological testing. Alexandersen, Quan, et al. (2003) reported high viral RNA concentration in serum during the peak of viraemia in cattle (10^8 FMDV RNA copies/ml) and pigs (10^9 FMDV RNA copies/ml). Alternative sample types include oral fluid collected in ropes (Vosloo et al., 2015) and milk (Armson et al., 2019).

1.1.7.2. Detection of virus and viral genomic material

Culture of the virus in permissive cells is considered the "gold standard" diagnostic test for FMD, especially in developed countries equipped with pathogen containment laboratories (Ding et al., 2013). Isolation of the virus provides the ability to conduct vaccine matching to aid the selection of the appropriate vaccine antigen for the outbreak-affected region. Primary calf thyroid cells, also referred to as bovine thyroid cells (BTY), are the most sensitive cell to isolate FMDV. Still, isolation can also be done in foetal goat tongue cell line (ZZ-R 127), porcine kidney cells (IB-RS-2) (Ludi et al., 2017) or in a porcine kidney cell line, LFBK-$\alpha_v\beta_6$ (LaRocco et al., 2013). The advantage of virus isolation is that this procedure facilitates the detection of other differential agents, such as swine vesicular disease virus. Cytopathic effect in cell culture can be expected for up to 4 days if blind passages are required. Thus, the turn-around time for virus isolation is slower than RT-qPCR and antigen ELISA, results of which are usually available within a day after receipt of the samples.

Table 1-1. Differential diseases and conditions with clinical syndromes similar to foot and mouth disease (Holliman, 2005).

Diseases/conditions	Aetiology	Species affected	Presence in New Zealand
vesicular stomatitis	vesicular stomatitis virus (genus *Vesiculovirus, Rhabdoviridae* family*)*	cattle, pigs, sheep, goats, horses	No
Bluetongue	bluetongue virus (genus *Orbivirus, Reoviridae* family)	cattle, sheep, goats	No
bovine papular stomatitis	bovine papular stomatitis virus (genus *Parapoxvirus, Poxviridae* family)	Cattle	Yes
wooden tongue	*Actinobacillus lignieresii*	Cattle	Yes
malignant catarrhal fever	ovine herpesvirus-2 and alcelaphine herpesvirus 1 (genus *Macavirus, Herpesviridae* family)	cattle, deer, swamp buffalo	Yes
infectious bovine rhinotracheitis	bovine alphaherpesvirus 1 (genus *Varicellovirus*, *Herpesviridae* family)	Cattle	Yes
Rinderpest	rinderpest virus (genus *Morbillivirus, Paramyxoviridae* family)	cattle, buffalo, even-toed ungulates	No
peste des petits ruminants	peste des petits ruminants (genus *Morbillivirus, Paramyxoviridae* family)	sheep, goats, cattle, buffalo	No
mucosal disease	bovine viral diarrhoea virus type 1 and 2 (genus *Pestivirus, Flaviviridae* family)	Cattle	Yes
vesicular exanthema of swine	vesicular exanthema of slime (genus *Vesivirus, Caliciviridae* family)	Pig	No
swine vesicular diseases	swine vesicular diseases virus (genus *Enterovirus, Picornaviridae* family)	Pig	No
Seneca Valley virus disease	Senecavirus A (genus *Senecavirus, Picornaviridae family*)	Pig	No
Orf	orf virus (genus *Parapox, Poxvirus* family)	sheep, goat	Yes
phototoxic or contact dermatitis	exposure to light and irritants	cattle, sheep, goats, pigs	Yes
Footrot	*Fusobacterium necrophorum, other bacteria*	cattle, sheep, goats, pigs	Yes
traumatic injury	trauma	cattle, sheep, goats, pigs	Yes
idiopathic erosive stomatitis	unknown	cattle, sheep, goats, pigs	Yes

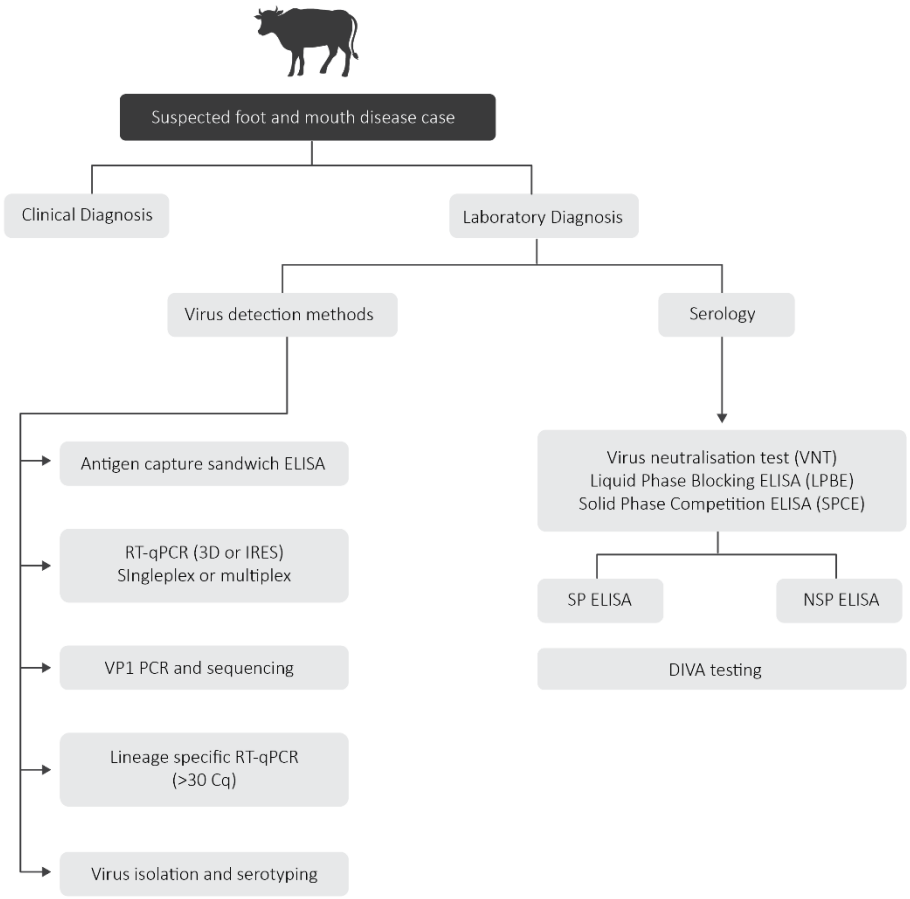

Figure 1-3. Test algorithm for the laboratory diagnosis of FMDV infection. Virological methods consisted of antigen detection by enzyme-linked immunosorbent assay (ELISA) and nucleic acid detection using reverse transcription quantitative polymerase chain reaction (RT-qPCR) tests. Serological methods comprised of ELISA and virus neutralisation test to analyse virus-specific antibodies against structural protein (SP) and non-structural protein (NSP). The NSP ELISA is used to differentiate infected from vaccinated animals. Adopted from Wong et al. (2020) with modifications.

An antigen ELISA may also be performed to detect the capsid protein of the virus. Using characterised polyclonal antisera, it is possible to classify the virus into serotypes (Ludi et al., 2017). Suitable samples for antigen detection ELISA are infected cell culture lysates, vesicular fluid, or epithelial flaps. The sensitivity of the antigen ELISA is generally low (approximately 80%) because the test relies on the amount of virus antigen present in the sample (Oliver et al., 1988). Nonetheless, a positive result in antigen ELISA further increases the probability of classifying the clinical samples as FMD-positive.

The primary virological screening test for suspected FMD cases in New Zealand is a Taqman-based RT-qPCR for amplifying viral RNA fragments due to the assay's high

sensitivity and specificity (100%) (Reid et al., 2009). Two PCRs target the 3D (Callahan et al., 2002) or IRES (Reid et al., 2002) conserved region in singleplex or multiplex format.

Serotype and lineage characterisation of the virus can be performed through sequence analysis of the VP1 PCR product from an appropriate pair of primers (Knowles et al., 2016). However, this conventional PCR approach to producing the VP1 amplicon needs better sensitivity and specificity due to the high diversity of the FMDV genome across the different serotypes (Ludi et al., 2017). In endemic countries, lineage-specific qPCRs have been developed to identify the serotype of an FMDV (Bachanek-Bankowska et al., 2016; Saduakassova et al., 2018). Such lineage-specific qPCRs can be helpful even in PCR-positive samples with high Cq values or low viral load.

1.1.7.3. Detection of antibodies to FMDV

Antibodies to FMDV can be detected by ELISA or virus neutralisation tests (VNT). VNT requires the use of live virus and cell culture and, therefore, can only be used in laboratories equipped to do so. ELISAs use inactivated reagents and are more regularly applied in diagnostic laboratories. Two ELISA methods to check the presence of specific antibodies against non-structural (NSP) protein or structural protein (SP) after exposure to FMDV are usually employed in post-outbreak surveillance (Ludi et al., 2017). The presence of NSP antibodies indicates that active viral replication may have happened recently (Brocchi et al., 2006). On the other hand, the detection of serotype-specific antibodies against SP due to previous exposure to virus infection or vaccination (Chénard et al., 2003). NSP and SP ELISA are used in tandem to differentiate infected from vaccinated animals (DIVA) (Bruderer et al., 2004; Ma et al., 2011).

1.2. Bovine viral diarrhoea virus infection

Bovine viral diarrhoea is present in most cattle populations globally. The disease is targeted for eradication by several countries (Hanon et al., 2018; Wernike et al., 2017), including New Zealand (Han et al., 2018), due to its economic impact on the cattle industry (Reichel et al., 2018). In New Zealand, BVD was detected as early as the 1960s and is now prevalent in beef and dairy herds. Since the focus of this thesis is on FMD with BVD as differentials, therefore only a very short overview of BVDV infection is presented.

1.2.1. Clinical disease

Depending on age, immunocompetence of the host, and virus variant, several disease outcomes may result from BVDV infection in cattle (Goens, 2002). Aside from subclinical infection, the most common signs in non-pregnant cattle are mild to severe bovine viral diarrhoea and respiratory disease. Morbidity may be high with low mortality. Reproductive losses due to poor conception rate, infertility, embryonic death, abortions, and congenital anomaly also result from BVDV infection in immunologically naïve pregnant cows (McGowan et al., 1993). Depending on the gestation status, BVDV infection has different effects on the developing foetus: death or resorption at <40 days; development of congenital defects at 120-150 days; and the birth of normal, antibody positive and virus-free calves when infection occurs after 150 days of gestation (Lanyon et al., 2014). Foetal infection between 30 and 100 days of pregnancy may result in the birth of persistently infected (PI) calves. Apparently healthy PI calves are herds' main reservoir of BVDV. They also develop the mucosal disease (MD) form where the cytopathic (CP) biotype is associated. (Brock, 2003). Mucosal disease is often fatal, characterised by erosions and ulcerations in the mucosal surfaces of the lips, tongue, dental pads, gingival margins, and hard palate (Tautz et al., 1998). In the acute form of MD, the disease is accompanied by fever, weakness, depression, and watery diarrhoea. A severe form of BVDV-2 primary infection is accompanied by bloody diarrhoea, nose bleeding, and ecchymotic haemorrhages (Malacari et al., 2018). Although epidemiologically different to FMD, MD is a significant endemic differential of any cases of suspected vesicular lesions in New Zealand cattle.

1.2.2. The causative agent

Bovine viral diarrhoea virus type 1 and type 2 (BVDV type 1 and BVDV type 2) are classified as two of four *Pestivirus* species (*Pestivirus A* and *Pestivirus B*) of the family *Flaviviridae* (Simmonds et al., 2017). Originally classified as a single species, they were re-classified into two species based on sequence variation of the viral RNA (Peterhans et al., 2010; Ridpath & Bolin, 1998) and differences in antigenic properties (Ridpath et al., 1994). Although no standard basis for segregation has been established, BVDV-1 and BVDV-2 are commonly further classified into several variants based on analyses of three regions of the viral genome, namely the 5' untranslated region (UTR), N^{pro} and E2 (Yeşilbağ et al., 2017).

The bovine viral diarrhoea virus is a spherical-shaped particle consisting of an outer lipid-rich and glycoproteins embedded envelop surrounding the core protein capsid (Neill, 2013). Within the capsid is a single-stranded, positive sense RNA approximately 12.3 kilobases long that comprises an ORF flanked by 5' and 3' UTR (Yeşilbağ et al., 2017). The ORF encodes a large polyprotein that is processed into four structural (C, E^{rns}, E1 and E2) and eight non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (Neill, 2013). Non-cytopathic (NCP) and CP biotypes exist within BVDV-1 and BVDV-2 according to *in vitro* effects on cultured cells (Gamlen et al., 2010). Although most circulating endemic BVDV in cattle are described as NCP, a CP variant can emerge from the NCP one, especially in persistently infected animals (Peterhans et al., 2010). The development of CP from NCP was attributed to the changes in the coding sequences of protein NS2/3, which cleaved into NS2 and NS3 after translation (Gamlen et al., 2010; Neill, 2013).

1.2.3. Pathogenesis

Bovine viral diarrhoea virus enters the animal via the oropharyngeal route through infected nasal secretions, saliva, urine, milk, and faeces (Meyling et al., 1990). Initial virus replication happens in the tonsils and retropharyngeal lymph nodes, followed by transient viremia (10-14 days) in naïve animals with associated leukopenia or lymphopenia (Howard, 1990). The BVDV then localises in enterocytes, Peyer's patches, and other lymphoid organs where secondary replication occurs (Wilhelmsen et al., 1990). Virus infection of the intestinal lining, including Peyer's patches, may cause diarrhoea. In acute infection, animals typically recover within 21 days (Müller-Doblies et al., 2004). An essential feature of BVDV infection is its immune suppressive effect (Marshall et al., 1996; Pedrera et al., 2012). Cattle are highly susceptible to BVDV infection after the disappearance of maternal immunity, but once infected, they mount long-lasting, possibly lifelong immunity (Howard, 1990).

1.2.4. Transmission

Direct animal contact, especially with PI animals (Brownlie, 1990), is the most common means of virus transmission. However, contact with contaminated fomites and flies are also possible (Niskanen & Lindberg, 2003). The birth of PI animals is the most critical aspect of the epidemiology of BVDV (Brock et al., 1998). Persistently infected animals appear

healthy but shed a large amount of virus into the environment via all body secretions and excretions.

1.2.5. Laboratory diagnosis

The RT-qPCR has recently supplanted virus isolation as the laboratory's standard tool to diagnose BVDV infection using samples such as blood, milk, follicular fluid, saliva, or tissues (Bhudevi & Weinstock, 2001; Willoughby et al., 2006; Young et al., 2006). For rapid screening to identify the presence of PI animals, bulk tank milk (Hill et al., 2010) or pooled serum (Smith et al., 2008) has also been used. Another simple method to detect BVDV, although lower in specificity and sensitivity compared to PCR, is an antigen (Ag) ELISA. The Ag ELISA method is more rapid than virus isolation (Mars & Van Maanen, 2005). Virus-specific antibody ELISA detection is also common to examine the immune status or to detect previous exposure to the virus of an individual animal (Beaudeau, Belloc, et al., 2001). Antibody ELISA is also useful for determining herd immunity by testing bulk tank milk (Beaudeau, Assie, et al., 2001).

1.3. Pen-side testing

"Field deployable test platform" discussed here pertains to portable testing devices that can be taken to farms or fields near sick animals where diagnostic assays can be performed without the comfort of dedicated laboratory facilities.

The concept and practice of "pen-side" testing in an animal health setting parallels the practice of point-of-care (POC) testing in human healthcare. POC testing is usually a miniaturised testing method or device that allows an assay to be performed near patients in clinics, hospitals, other healthcare facilities, and low-resource environments (Holland & Kiechle, 2005; Tayo et al., 2012). In contrast to a conventional laboratory with specialised testing machines and highly skilled personnel, a POC test is simple to perform, requires minimal set-up, and is rapid and easy to interpret (St John & Price, 2014). POC test results can effectively guide doctors and clinicians to make immediate, informed decisions on the patient's clinical care. This is especially helpful in remote areas where access to a laboratory is difficult (Niemz et al., 2011). Examples of the earliest and simplest handheld POC test devices include urine dipstick tests, lateral flow pregnancy tests, blood glucose meters or an

INR (International Normalised Ratio) test to monitor warfarin treatment (St John & Price, 2014). Benchtop POC devices that measure blood chemistry, haematological values, or levels of specific antibodies (St John & Price, 2014) are also widely used in small human medical clinics. More recently, portable nucleic acid detection platforms were introduced in human healthcare facilities (Niemz et al., 2011). Most of these portable PCR devices can be "sample to result" systems where the user supplies the sample collected directly from the patient without additional processing or manual interventions.

In veterinary medicine, POC testing refers to testing at veterinary clinics and animal hospitals. Commonly performed in-house tests in small and large animal practices include faecal examination for parasites, heartworm detection (Knott's technique, immuno-chromatographic device, or antigen ELISA), dipstick urinalysis, or vaginal cytology. Portable devices such as i-STAT; (Kim et al., 2015; Yildirim et al., 2015), haemoglobinometers (Magona et al., 2004), and glucometers (Clemmons, 2015) are also available for complete blood count and blood chemistry analysis. The use of digital radiography and portable ultrasound machines is also common.

1.3.1. Deployable field tests to support the detection of infectious pathogens

Current field deployable tests to aid diagnosis of animal infectious diseases can be categorised into two main formats 1.) Lateral flow immunoassay (LFIA) for antigen or antibody detection, and 2.) Nucleic acid detection-based assays based on PCR or loop-mediated isothermal amplification (LAMP) methods. Other promising technologies include microarray and lab-on-a-chip nucleic acid detection using a microfluidic system. All are briefly described below. Test format based on antibody detection is not the interest of this thesis, thus not covered in this review.

1.3.1.1. Lateral flow devices

Lateral flow immunoassays, also known as lateral flow devices (LFD), are quite simple and easy to use as rapid POC or field tests (Yetisen et al., 2013). Among the current POC tests available, lateral flow devices have the most comprehensive range and are the most widely used, as described in detail by O'Farrell (2015) and Yetisen et al. (2013). Patterned after the assembled plastic Clearview™ pregnancy solid chromatographic strip test in the late 80s

(Brüning et al., 1999), the basic principle includes the dispensing of a pre-diluted sample on a sample pad whereby the liquid having the target analyte moves through overlapping membranes by capillary action (Figure 1-4). Upon reaching a conjugate reagent zone, the analyte reacts with a specific antibody conjugated to coloured colloidal gold nanoparticles or latex microspheres. Afterwards, the antigen-antibody complexes migrate through the nitrocellulose or cellulose acetate membrane to an immobilised band of trapping antibodies specific to the analyte. The attached antibody band on the nitrocellulose captures these complexes, and the concentrated dyed particles then give a coloured line in the sample window, showing a positive result. Excess capture antibody in the liquid then moves to the strip of immobilised antibody specific for the conjugate to form a second coloured line that serves as an internal control. To eliminate the visual subjectivity of the test, recent advancement in LFD technology incorporates optical readers to increase detection sensitivity (O'Farrell, 2015).

Several LFDs have been developed to help diagnose various animal diseases such as rinderpest (Brüning et al., 1999), peste des petits ruminants (Baron et al., 2014), swine vesicular disease (Ferris et al., 2010), vesicular stomatitis (Ferris et al., 2012), equine infectious anaemia (Alvarez et al., 2010) and FMD (Ferris et al., 2009; Reid et al., 2001). In general, the clinical performance of LFD is variable depending on the target agent and the design of the test. In one study (Ferris et al., 2009), the diagnostic sensitivity and specificity of LFD to detect FMDV were 84% and 99%, respectively, using 304 positive and 1003 negative samples. This performance was relatively similar to the low sensitivity (85%) of an FMD antigen capture ELISA (Ferris et al., 2009) and other assays for vesicular stomatitis (Ferris et al., 2012) and swine vesicular disease (Ferris et al., 2010). However, the high specificity of LFD is helpful in ruling-in clinical infection.

A newer version of a lateral flow device, the SNAP test (IDEXX), was patterned on an ELISA principle. The SNAP test has a first step of incubating the sample with the conjugate before adding the solution to the sample well. After migration of the sample to the immobilised trapping antibody, an integrated wash step is done by "snapping" the device. This test method is reported to have reasonable diagnostic specificity, but with variable diagnostic sensitivity. In one study, the diagnostic sensitivity of the SNAP test for detecting *Giardia duodenalis* in faecal samples of dogs was only 67-70 %, although the specificity was higher at 92-94% (Geurden et al., 2008; Rishniw et al., 2010). The SNAP tests to detect antibodies against *Leishmania infantum* in dogs (91% sensitivity and 100 % specificity)

(Ferroglio et al., 2007) and FeLV in cats (92 % sensitivity and 97 % specificity) (Hartmann et al., 2007) had better performance.

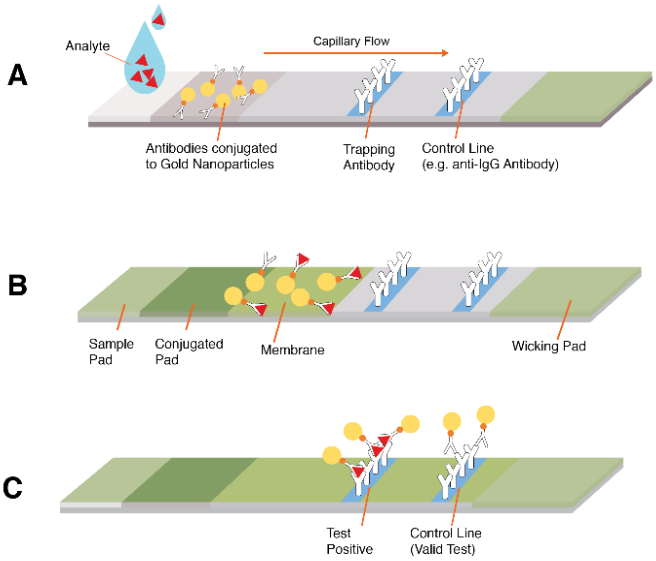

Figure 1-4. Schematic diagram of a lateral flow device (LFD) assay. A. Overall architecture of an LFD device showing the addition of sample containing the target analyte at one end, B. Liquid containing the analyte moves through the membrane by capillary action where analyte-antibody conjugate complexes are formed in the conjugate pad, C. Analyte or antigen-antibody conjugate complexes are sequestered by the trapping antibodies and precipitated the appearance of a coloured line, indicating a positive test. Excess conjugates migrate further in the reaction membrane, attaching to anti-antibody specific immunoglobulins and serves as the control line. Image was based on http://www.cytodiagnostics.com/store/pc/Lateral-Flow-Immunoassays-d6.htm website and reproduced by Cay Bueno (2018).

1.3.1.2. Nucleic acid detection

1.3.1.2.1. PCR

Nucleic acid detection by PCR is commonly performed in diagnostic laboratories despite the need for expensive amplification machines, good laboratory practice, and technically competent staff. This is attributed to PCR's high diagnostic sensitivity and specificity compared to more traditional assays (Hoffmann et al., 2009). PCR nucleic acid amplification involves 30-45 cycles of three temperature changes to enable denaturation of nucleic acids, annealing of primers to target sequences, and extension of the DNA region located between the primer-binding sites, resulting in exponential multiplication of a single copy of the target nucleic acid (Mackay et al., 2002). Accumulation of amplicons, the PCR

product, can be detected through various means, such as endpoint detection by size separation during gel electrophoresis or real-time detection using fluorescent binding dyes or labelled probes (Mackay et al., 2002). The development of a PCR thermocycler instrument has simplified and sped up the entire process.

Several portable PCR thermocyclers adaptable to field conditions have been developed recently to facilitate field-based diagnosis. Callahan et al. (2002) described the detection of the FMDV using a 10-kg portable PCR machine called the SmartCycler (Cepheid). The SmartCycler machine was subsequently used to compare two FMD PCR assays targeting the 3D and IRES genes (King et al., 2006). Its use for detecting FMD RNA was also validated using field samples in Brazil (Paixao et al., 2008). RAPID (Idaho Technology, now called BioFire Diagnostics Inc.), or "ruggedised advance pathogen identification device", is an early PCR instrument developed to detect bioterrorism agents. The RAPID platform was utilised for developing a *Yersinia pestis* PCR assay (McAvin et al., 2003) and dengue virus PCRs for screening and serotyping in the field (McAvin et al., 2005; Pal et al., 2015). A recent version of RAPID, the RAZOR EX BioDetection System (BioFire Diagnostics), a fast, field PCR system, was created as a first response test to detect biothreat agents such as *Brucella* sp., anthrax, *Tularemia*, and smallpox. The RAZOR EX machine utilises a patented freeze-dried, pre-formatted reagent pouch system that allows for testing a single sample in up to 12 independent PCRs. A similar portable PCR platform, the T-COR™ 4 (Tetracore), was also evaluated for field-based detection of classical swine fever and African swine fever viruses in pigs (Liu, Luo, et al., 2016) and Newcastle's disease virus in poultry (Liu, Benyeda, et al., 2016).

The development of portable PCRs for field use was hampered initially by the need for rapid and field-ready nucleic acid extraction methods and thermostable reagents (McAvin et al., 2003). This shortcoming was partially addressed by the "sample-to-result" format, where a single cartridge containing lyophilised sample extraction and temperature-stable PCR reagents is used. The GeneXpert (Cepheid), Enigma ML/FL (Enigma Diagnostics) and BioSeeq (Smith Detection) PCR devices can perform nucleic acid extraction, amplification, detection, and interpretation using a single sample cartridge with limited human input. The closed environment, sample-to-result format drops the need for separate sample processing steps, prevents cross-contamination, maximises the stability of reagents using dried reagents, and simplifies the testing process. The first commercially available assay in this format was the Xpert Flu A panel using the GeneXpert Diagnostic system for human healthcare (Al Johani & Akhter, 2012; Jenny et al., 2010). In veterinary settings, the Enigma FL and BioSeeq

(Smith Detection) machines were trialled to detect animal disease agents such as BVDV (Wakeley et al., 2010) and FMDV (Madi et al., 2012; Reid et al., 2010). Disadvantages of the sample-to-result PCR format include limited throughput (only one sample can be tested in a run) and the inability to develop customised assays for different pathogens. More recently, lyophilised reagents used in an open-format PCR system have been tested for field use (Pal et al., 2015) and are becoming available commercially.

Handheld and smaller PCR devices are increasingly being offered commercially. Examples include the Freedom 4 (Ubiquitome), miniPCR (Amplyus), T-COR 8™ (Tetracore), two3™ (Biomeme), and palm PC (Ahram Biosystems). These devices are usually light (1.5 -4 kg) and have a limited throughput of 2 to 8 samples/run. Most handheld devices have 1 or 2 channels and are thus only suited for running an assay to detect one to two targets. These devices can be run either as standalone units or through mobile phones. Except for the miniPCR (Amplyus) and palmPC (Ahram Biosystems), which use only end-point detection, all the other handheld devices can detect the target in real-time using qPCR. Other portable PCR thermocyclers, including the T-COR 8™, have WIFI capability and are "cloud" ready.

1.3.1.2.2. Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a fast-emerging nucleic acid detection technology (Mannier & Yoon, 2022). The LAMP utilised an auto-cycling mechanism and *Bst* polymerase-mediated DNA strand displacement in one temperature condition (e.g., 60°C) (Panno et al., 2020; Tomita et al., 2008). Tomita et al. (2008) described in detail the principle of LAMP assay. The method uses four primers (forward inner primer, reverse inner primer, and two outer primers), recognising six distinct sequences in the target DNA. The LAMP reaction produces a huge amount of DNA strands (10^9 copies) of various lengths and cauliflower-like structures in a < 1-hour run (Panno et al., 2020). A modified LAMP includes a reverse transcription step for analysing RNA sequences (Fowler et al., 2016).

The LAMP assay can be run with inexpensive equipment (e.g., water bath) using a single low temperature, thus consuming minimal energy. Both crude samples and purified nucleic acids can be used for LAMP as the assay appears unaffected by inhibitors that affect PCR (David de Paz et al., 2014; Tanner & Evans, 2014; Yan et al., 2014). The number of evaluated and validated assays using the LAMP platform continues to grow because of its inherent flexibility and simplicity (Notomi et al., 2015). It could rival PCR (Mannier & Yoon,

2022). Testing trials conducted in Tanzania demonstrated the ability of the RT-LAMP/RT-LAMP-LFD (Genie® II) platform to detect FMDV reliably under field conditions (Howson et al., 2015). Howson et al. (2015) also demonstrated the suitability of LAMP for detecting FMDV in serum and oral fluids without prior nucleic acid extraction. In that study, the analytical sensitivity of an FMD LAMP assay was found to be the same as the qPCR FMD assay. Bath et al. (2020) demonstrated further the robustness of the original FMDV LAMP assay (Howson et al., 2015) using unextracted samples on the Genie® III (OptiGene Ltd) platform with a diagnostic sensitivity of 79 (95% Bayesian credible interval: 65-90%) and diagnostic specificity of 99% (95% Bayesian credible interval: 95-100%).

The LAMP is considered highly specific because six regions of the target segment must be correctly identified first by the set of primers before amplification occurs (Mori & Notomi, 2009). However, the main challenge in developing any LAMP assay is designing the primer set without primer-dimer characteristics (Mori & Notomi, 2009; Panno et al., 2020). For example, Aebischer et al. (2014) suggested the presence of non-specific amplification products from different primer combinations targeting the S and M segment of the Schmallenberg virus (SBV) during the development stage of the LAMP assay.

Difficulty in primer design also limits the ability to develop a LAMP multiplex assay format (Jung et al., 2015). Also, end-point detection methods, which require opening the reaction tubes (e.g., the addition of fluorescent dye and gel electrophoresis), are prone to cross-contamination (Panno et al., 2020). An approach to overcome this problem is the use of intercalated dye in a “closed tube” reaction combined with a Genie (Optigene) fluorescent detection platform (Kurosaki et al., 2016).

Yan et al. (2014) reviewed other isothermal amplification strategies recently. However, the different isothermal amplification approaches, although available, are not widely used for laboratory or field diagnostic testing, therefore, will not be included in the discussion.

1.3.1.2.3. Microarray

Microarray has been used for gene expression research, genotyping, and wide-range pathogen detection applications (Blohm & Guiseppi-Elie, 2001; Uttamchandani et al., 2009). Microarray technology identifies multiple molecular targets in one array assay using a miniaturised device with immobilised recognition (e.g., oligonucleotide) ligands on a solid

matrix (McLoughlin, 2011). Upon labelling the target molecule (e.g., DNA or cDNA), it will hybridise into recognition ligands, generating a signal (Rasooly & Herold, 2008). The FilmArray (BioFire Diagnostics), a commercial microarray, is a simple, fully automated closed system that detects selected lethal viruses and bacteria, including emerging infectious pathogens, in one reagent pouch (Babady, 2013). The closed system, with the advantages of a "sample to result" PCR format, integrates various processes after adding a sample into the pouch. Processes included in the closed system are:

- nucleic acid purification, reverse transcriptions, and primary multiplex PCR reactions
- a second stage multiple, nested singleplex PCR for specific pathogens in each well of the array
- automatic detection by melt curve analysis.

An example of a commercial field deployable platform using the microarray technology is the FilmArray BioThreat Panel (BioFire Diagnostics), which can perform assays to detect 16 important bio-threat agents in a single sample in one run.

1.3.1.2.4. Microfluidic PCR and LAMP (lab on a chip)

Microfluidic miniature PCR and LAMP testing devices have tremendous potential in deployable field diagnostics; however, this method is still in the initial stages of commercialisation. A lab-on-chip nucleic acid amplification using the microfluidic system is an integrated miniaturised analytical device with µL to nL size chambers for performing single or multiplex PCR or LAMP assay (Foudeh et al., 2012). In this novel setup, sample processing, sample transport, manipulation and analysis steps on a micro-scale are performed through high-precision micro-electro-mechanical systems (MEMS) (Chang et al., 2013). Different microfluidic PCR and LAMP systems have been reviewed (Chang et al., 2013). Examples of POC tests in this format are the TruelabTM Real-Time micro-PCR system (Molbio Diagnostics Pvt. Ltd) for detecting malaria parasites, dengue viruses, chikungunya virus and Salmonella; and the Sentinel Nucleic Acid Analysis (SRI international) to analyse samples for the presence of H1N1 influenza virus, dengue virus and human immunodeficiency virus.

1.3.1.3. Comparison of various pen-side test formats

PCR and LAMP (Mori et al., 2013) are more sensitive than LFD (Oem et al., 2009) and microarray ((McLoughlin, 2011) due to their ability to amplify the target nucleic acids from samples with low concentrations of the target virus. Both LFD (Posthuma-Trumpie et al., 2009) and microarray do not include any amplification. Also, it is possible for PCR and LAMP assays to detect all variants of a specific virus by targeting a conserved region. In contrast, LFD and microarray have limited ability to detect viruses that show high genomic variability (Brüning et al., 1999; McLoughlin, 2011), as is the case with FMDV (Ferris et al., 2009).

Portable PCR devices for field deployment are more expensive than LAMP equipment because of the required thermal cycling function for running the PCR assay (Almassian et al., 2013). LAMP reaction uses only one temperature, which makes readily available equipment (e.g., heat block or water bath) suitable for this purpose (Notomi et al., 2000). Also, a specialised device for scanning and acquiring data is needed for the microarray analysis (McLoughlin, 2011). However, LFD has an advantage over all other pen-side tests for its simple format and ease of use, with no equipment needed (Posthuma-Trumpie et al., 2009), as exemplified by the popularity of the rapid antigen test for COVID-19 (Hayer et al., 2021).

PCR assays can be reasonably transferred easily from one device to another. Such transferability means that laboratory-established PCR assays can be adaptable to portable devices for field use with minimal validation effort. Interestingly, LAMP assays are not standard tests at AHL-MPI, thus not available when needed for in-field testing. A primary reason for the non-adoption of the LAMP test platform is the readily accessible PCR assays prescribed by WOAH (2022) for animal disease diagnosis. Another reason could be the complexity of designing and validating the LAMP assay, which requires 4-6 pairs of primers (Notomi et al., 2000). For example, LAMP primers can be affected by changes in the viral RNA genome, especially for viruses susceptible to mutations such as FMDV (Fowler et al., 2016). Microarrays, on the other hand, although it can potentially screen many pathogens in one analysis run, is more common as a research tool than for diagnosing infectious diseases (Uttamchandani et al., 2009).

Developing a PCR test with multiplex capability is also easier than introducing multiplexing to LAMP and LFD testing methods (Haines et al., 2013). Validating a multiplex LAMP is particularly challenging due to the number of primers sets involved,

making it prone to non-specific reactions between primers (Fowler et al., 2016; Wenhui et al., 2015). Similarly, while multiplexing is feasible for the LFD format (Cavalera et al., 2022), it is limited spatially by the small footprint of the device to accommodate more reactive zones for different targets. Microarray can analyse the highest number of targets in a single test run (Uttamchandani et al., 2009). However, because of the considerable number of sequences involved in gene expression or pathogen analyses (Uttamchandani et al., 2009), optimisation for all microarray targets is daunting (Call, 2005), which makes it not ideal for routine diagnostic testing applications.

1.3.2. Challenges of field-based testing

1.3.2.1. Sample preparation method

In a PCR method, initial sample processing or nucleic acid extraction increases the sensitivity of detection (Thatcher, 2015). Depending on the extraction kit used, this process usually takes an average of 30-45 minutes in addition to the PCR assay run time. Ideally, minimal or no sample processing steps should be required for user and eliminate opportunities for contamination, and reduce the testing turnaround time in the field, (Dineva et al., 2007). For example, Fowler (2016) and Howson et al. (2015) employed a minimal sample preparation method for testing FMDV using PCR and LAMP assays, respectively. In these studies (Fowler, 2016; Howson et al., 2015), the users only had to perform simple dilution of serum, oral swabs, and epithelial tissue homogenates to allow amplification of target sequences.

Some field-deployable test devices have integrated sample preparation, amplification, and detection capability. In this format, the testing protocol is easy to follow, and minimal sample handling reduces contamination (Almassian et al., 2013). However, because pre-made assay cartridges are specific to the machine, customised assays for other pathogens must be developed in collaboration with the platform and assay manufacturer.

1.3.2.2. Reagent availability and stability

The thermo-stability and shelf-life of molecular reagents are also important, mainly when conducting field diagnostic work in high ambient temperatures. In several studies, lyophilisation increased the shelf life of reagents and allowed them to be stored or

transported at room temperature (Howson et al., 2015; Pal et al., 2015). Ready-to-use dry format reagents are also available for single-plex assays for FMD, CSF, PRRS and other agents. Such dry format reagents showed no difference in the PCR or LAMP assay performance after rehydration compared to a normal wet reagent combination. However, the performance of lyophilised or other thermostable reagents should be empirically evaluated and validated before being used.

1.3.2.3. Deployability and portability of the test platform

"Deployability" and ruggedness of a field device cover portability, power supply source, and operating environment (Almassian et al., 2013). Portability refers to the device's relative size, weight, and general construction, which makes transportation easy without damaging the unit (Hansen & Abd El Wahed, 2020). The power supply source, whether via a direct power source or through a rechargeable battery, may be acceptable in New Zealand because electricity is available at most farms, including standby generators. However, a battery-operated machine could be an advantage, especially when testing in isolated locations on the farm or when the assay and the device are being validated in an FMD-endemic country with limited resources. The machine's operating environment pertains to its ability to run an assay in a wide range of temperature and humidity field conditions reflecting the range of different seasonal extremes in the country. Although testing in covered barns and animal sheds mitigates the effect of environmental factors, the optimum operating temperature and humidity must be determined.

1.3.2.4. Decontamination of test platform

Developing the field equipment's disinfection process without damaging it is essential for biosecurity reasons. In general, standard disinfection procedures recommended by manufacturers for field test platforms consist of surface disinfection with 10 % sodium hypochlorite or 70% ethanol. Using citric acid as a surface disinfectant is also common, especially when dealing with FMD cases in the field (Howson et al., 2018). The device's tolerance to withstand disinfection procedures is essential to avoid serving as a fomite for transmitting pathogens after being used on an infected farm.

1.3.2.5. Test validation

The WOAH Terrestrial Manual guides for validating assays for infectious diseases (WOAH, 2022). Assay validation assay involves optimising several variables (reagent concentrations, thermal profiles, and reaction parameters), including elucidation of the analytical specificity and limit of detection of the assay (Bustin et al., 2009). In addition, the repeatability and reproducibility of deployable diagnostic tests should be evaluated on factors present in field conditions (Hobbs et al., 2021). Environmental factors that might affect the robustness of a field-based test include testing location, temperature gradient (<0°C in winter to >28°C in summer) during seasonal changes with the testing location, humidity, and various operator/analyst in the field.

1.4. Research aims

Field deployable tests can improve rapid diagnostic capability, especially when we need to detect or exclude the presence of exotic disease agents. Several field deployable tests can be explored to enhance disease diagnostic preparedness. Therefore, the main aim of the current project was to develop a multiplex pen-side test to detect FMDV and two RNA virus differentials, BVDV-1 and BVDV-2.

Specific aims of the work included the selection of a "fit for purpose" field deployable diagnostic platform for New Zealand; the assessment of a most suitable sample processing method; the development of multi-virus positive standard and internal control, the development of rapid multiplex assays for detecting the selected disease agents (FMDV, BVDV-1 and BVDV-2); and lastly, field assessment of the test in New Zealand and in a country where FMD is endemic.

Chapter 2. The use of multi-criteria decision tools in selecting a fit-for-purpose field-deployable test platform

2.1. Introduction

In the event of an FMD outbreak, getting a rapid and accurate diagnosis is essential for ensuring that the appropriate control measures are put in place as quickly as possible to minimise further transmission (King et al., 2012). Pen-side testing can help by giving rapid answers on a farm without the delays and risks associated with sending samples to a diagnostic laboratory (Sammin et al., 2010). In human health, the term pen-side test is synonymous with point-of-care (POC) or point-of-need (PON) testing (Luppa et al., 2011).

Key features of a good pen-side test should only use none or light portable equipment, that can be carried by one person, transported to the required site easily, and be battery operated. (Almassian et al., 2013). In addition, the assay should have high accuracy, short turn-around time (<30 minutes), use thermostable reagents, and require minimal manual handling (Hansen & Abd El Wahed, 2020). Although most pen-side tests are designed to detect single pathogens, it would be beneficial from the point of view of outbreak investigation to have the capacity for multi-pathogen testing. For example, vesicular disease can be caused by infection with FMDV or similar agents such as BVDV. A multiplex assay to differentiate between FMDV and BVDV would be advantageous during index case investigation.

Many different diagnostic test methodologies can potentially be used for pen-side tests, including lateral flow immunoassay system (LFIA) or nucleic acid detection by PCR or isothermal amplification (Hansen & Abd El Wahed, 2020; Kozel & Burnham-Marusich, 2017; Luppa et al., 2011; Moehling et al., 2021; Posthuma-Trumpie et al., 2009). The most common is LFIA which has the advantage of simplicity without needing equipment (O'Farrell, 2015). The principle of LFIA is based on antigen and antibody reaction as the sample with the target analyte migrates along a strip of polymeric material (Figure 1-4). However, it is also limited by low sensitivity (Ferris et al., 2009; Oem et al., 2009; Wiriyachaiporn et al., 2013), which makes it less suitable for certain situations.

In recent years, *in vitro* diagnostics companies have been developing small deployable PCR equipment to take advantage of using laboratory validated assays for field testing. Quantitative PCR (qPCR) technique relies on the amplification of a conserved fragment of the nucleic acid of a pathogen (Hoffmann et al., 2009), making it more sensitive and specific than LFIA, as the latter does not include amplification of the target (Espy et al., 2006). However, the biggest challenge of using qPCR in a pen-side format is the availability of a simple and field-adaptable sample preparation method to replace the standard and contamination-prone nucleic acid extraction procedure (Deregt et al., 2002). Furthermore, this method requires access to stable PCR reagents at ambient temperature (Seise et al., 2013). To mitigate these two limitations, Enigma® ML (Enigma Diagnostics) and GeneXpert Dx System (Cepheid) recently developed novel portable sample-to-result PCR platforms for pathogen identification that do not require human manipulation beyond the addition of the sample to the cartridge that is then inserted into the portable equipment (Goldenberg & Edgeworth, 2015; Jenny et al., 2010; Wakeley et al., 2010). The cartridge already contains freeze-dried nucleic acid extraction and PCR reagents that remain stable under various environmental conditions.

Isothermal amplification is another established nucleic acid amplification technique that works exceptionally well in low-resource settings to address the challenge of working with samples with potentially low pathogen loads. Loop-mediated isothermal amplification (LAMP) is the most common technique, although several other variations have been reported (Moehling et al., 2021). The LAMP setup is very simple because it requires only one temperature to amplify the target nucleic acid (Notomi et al., 2000). Nucleic acid purification of the sample is not necessary before testing (Modak et al., 2016), and the results become available within 30 minutes. The method of detection varies from visual inspection of colour change (Chen et al., 2014), turbidity (Yamazaki et al., 2013), or detection of the product by lateral flow device (James et al., 2010). LAMP performed relatively similar to qPCR with regard to sensitivity and specificity (reviewed by Mannier and Yoon (2022). LAMP, however, is prone to false positivity due to primer-dimer formations and non-specific reactions (Meagher et al., 2018; Schneider et al., 2019).

In New Zealand, there has been significant recent interest in developing the capability to perform rapid diagnostic tests on-farm to assist with investigating disease outbreaks. With so many different platforms and techniques currently available, there was a recognised need to implement a formal selection process to identify the most fit-for-purpose

solution (Shehabuddeen et al., 2006) that considers the strengths and weaknesses of each approach alongside the end-user requirements (Houseman et al., 2004). This work aimed to develop and apply a semi-quantitative multicriteria scoring model in prioritising a suitable deployable diagnostic platform for testing in the field. In this paper, the terms "test platform", "test device", "equipment", or "testing technology" are used interchangeably to describe the physical equipment required to perform the diagnostic test.

2.2. Methodology

The approach for selecting the field test platform was a semi-quantitative, modified Delphi approach based on the subjective-intuitive opinions of an expert panel. The platform selection was adopted from DISCONTOOLS (2012) framework with modifications. The method consisted of three stages (Figure 2-1). This involved 1. identifying the currently available POC platforms, 2. using experts' opinions to rank candidate test platforms, and 3. feedbacking and consensus agreement. Details of the work done for each stage of the method were described in the later sections.

2.2.1. Identification of available field deployable diagnostic platforms

The procedure for identifying available platforms with the potential for performing diagnostic testing in the field was conducted through a literature search using several multidisciplinary databases (Web of Science, MEDLINE, and Scopus) with combinations of the following search queries: PCR, LAMP, POC, "point-of-care test", "field-deployable test", "molecular assay", pen-side, "animal disease", "infectious disease", "veterinary health", "animal health", and "field diagnostics". Further searches for peer-reviewed journal articles were also performed using Google Scholar, Pubmed, and general internet searches. More search terms such as "portable molecular PCR" and "portable isothermal amplification" were also used in Google Scholar.

In addition, review articles on the current and emerging POC tests in human healthcare and innovative technologies from online sites of *in vitro* diagnostics market were examined to identify diagnostic platforms that had the potential for field application.

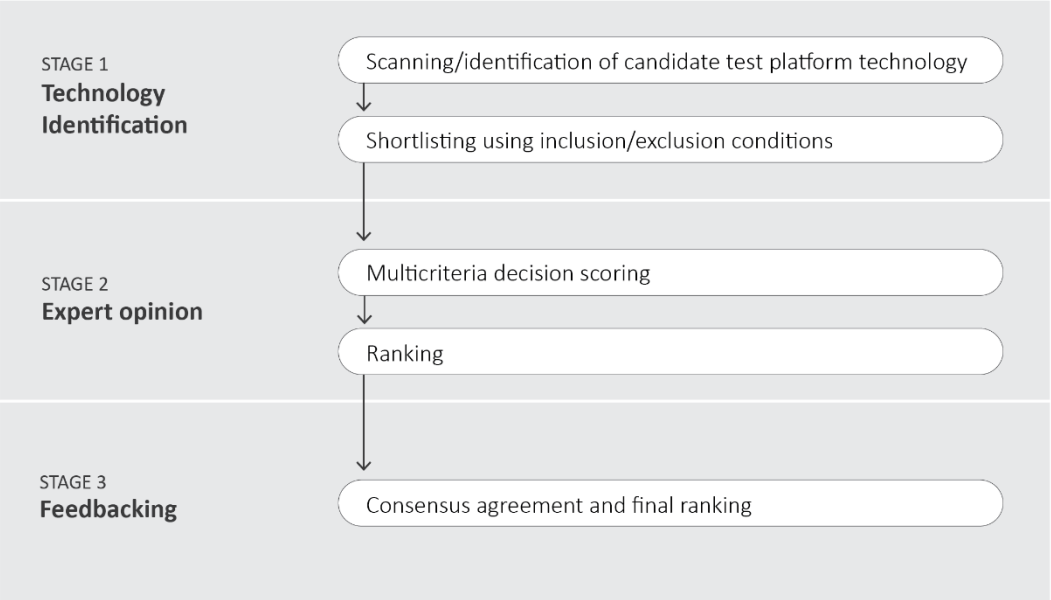

Figure 2-1. Schematic diagram of the general methodology applied in selecting a fit for purpose platform intended for the development of field deployable diagnostic capability.

2.2.2. Short-listing using inclusion and exclusion conditions

The initial list of candidate platforms was filtered with the guidance of the scope and minimum requirements stipulated in the proposed operation research project to eliminate non-feasible options.

Conditions for inclusion were:

a) Nucleic acid detection method – molecular assays tend to have higher sensitivity and specificity when compared to other test methods.

b) Multiplex testing capable – the test platform can detect at least three targets in a single reaction. Diagnostic platforms that can perform simultaneous singleplex assays for at least three agents were also considered.

c) Open type – the field test technology is mature and shares common attributes with several deployable diagnostic platforms, including using standard test reagents and reaction tubes and analysing samples using standard preparation methods.

d) Field ready – the technology was purposely designed for carrying out pen-side or POC settings.

Exclusion conditions included were:

1) High initial capital cost (> NZD 50,000).

2) The available model is a prototype, and the platform's technology requires further development.

3) Test strategy should not be prone to false positives due to cross-contamination.

2.2.3. Selection criteria

The test platforms in the shortlist were assessed against five relevant criteria to ascertain suitability to the overall aim of the study (Chapter 1). The developed criteria (Table 2-1) were (1) fit for project purpose and scope; (2) operational simplicity and flexibility; (3) portability and field deployability; (4) performance; and (5) price and technical support of the device.

To satisfy each criterion, specific parameters and requirements were listed. *Fit for project purpose and scope* contained parameters describing the operational capability of the machine, such as multiplex testing, and holistic traits, such as the potential for a broader range of applications not only in animal health but also for aquatic and plant disease testing, and bio-threat detection. *Operational simplicity and flexibility* consisted of attributes that make the machine easy to use, including the ability to accommodate a range of standard molecular assays and the transferability of assays developed utilising the machine to a similar testing device. *Portability and field deployability* of a test platform refers to ease of transfer from one physical place to another, battery operated, tolerance to disinfection procedure and connectivity to the internet for reporting. *Performance* pertains to available literature describing its suitability for field diagnostic use. Lastly, reasonable *price and technical support* were included to reflect the resources available for developing and maintaining the platform.

Table 2-1. Developed criteria to select the most feasible field diagnostic test platform for New Zealand.

Main selection criteria	Acceptance parameters and or requirements
Fit for the project purpose and scope	- multiplex assay with at least 3 targets - alternatively, it has enough sample reaction sites for simultaneous singleplex reactions - mature platform technology that does not need further development - established laboratory-based assays can be used with minimal validation effort - not prone to cross-contamination
Operational simplicity and flexibility	- the test platform is easy to use - can be used with various reagents, kits, and sample preparation methods - connectivity to the internet for data transmission - ease of storing and reporting data results - easy to maintain
Field deployability and suitability	- portable and easy to transport without damaging the device - it can be operated in various temperature conditions, dust, and humidity - option to be powered by either a rechargeable battery or through a direct power source - recommended disinfection procedure of the equipment is robust and acceptable
Performance	- if possible, the performance of the assays using the platform have been published in journal articles - supported by company data and anecdotal evidence or testimonies from laboratory users
Price and technical support	- supplier's price is affordable, realistic, reasonable, and within the available budget - good track record of supplier/distributor - service engineer is readily available - backup or replacement instrument is readily on hand

2.2.4. Multi-criteria decision support tool

A rating scale shown in Table 2-2 was integrated into the selection matrix scoresheet (Table 2-3) to weigh the contribution of each criterion (Table 2-1) according to perceived importance and score the different test platforms against the individual criterion.

A multi-criteria decision support tool (MCDT) was developed to guide recruited experts in evaluating the candidate test platform in the selection matrix scoresheet (Table 2-4). Relevant data and technical details of the shortlisted platform were compiled and summarised in a Microsoft Excel spreadsheet according to the requirements of the main criteria. Attributes within each criterion were assigned with a score of 1 or 2, corresponding to the relevant descriptor described in (Table 2-4). Information was sourced from company websites, vendor's technical brochures, published literatures and anecdotal testimonies.

Table 2-2. Rating scale to score each field test platform against each criterion listed in Table 2-1.

9 - 10	Excellent - Significantly exceeds the requirements.
7 - 8	Good - Exceeds the requirements with minor additional benefits.
5 - 6	Acceptable - Meets the requirements in full.
3 - 4	Minor reservations - Satisfies the criteria with minor reservations.
1 - 2	Major reservations - Significant issues that need to be addressed
0	Unacceptable - Significant issues unlikely to be resolved

Table 2-3. Selection matrix score sheet. Experts' opinion was elicited by asking them to assign weights (%) to each criterion according to perceived importance for a total of 100 %. Experts then rate each platform against the five criteria listed (Table 2.3) using a scale of 0 to 10 (Table 2 2). A box for any comments to support their scores was also provided in this score sheet.

Panel Member:	[insert name]	FIELD DEPLOYABLE PLATFORMS									
		Platform 1		Platform 2		Platform 3		Platform 4		Platform 5	
Criteria/Requirements	Weighting	Raw Score	Comments	Raw Score	Comments	Raw Score	Comments	Raw Score	Comments	Raw Score	Comments
FIT FOR THE PROJECT PURPOSE AND SCOPE - the deployable platform can perform multiplex assay to detect at least 4 target pathogens in a sample, or it has enough sample reaction site (THROUGHPUT) to perform simultaneous 4 singleplex assays; the technology principle is mature and does not need further development; developed assays from the laboratory can be adopted in the test device or vice versa meaning assays from the deployable device are transferrable to the similar platform with minimal verification and validation effort; cross-contamination throughout the testing process among samples is nil.											
OPERATIONAL SIMPLICITY AND FLEXIBILITY - the test platform is easy to use; adaptable to current laboratory standard assay protocols, reagents, kits, and various sample prep methods for field diagnostic work; connectivity to internet for transmission of data; ease of storing and reporting data results; easy to maintain.											
FIELD DEPLOYABILITY AND SUITABILITY - portable and easy to transport without damaging the device; it can be operated in various temperature conditions, dust, and humidity; preferably powered both by a rechargeable battery or through a direct power source; recommended disinfection procedure of the equipment is robust and acceptable.											
PERFORMANCE - performance of the assays using the platform has been published in journal articles, supported by company data and anecdotal evidence or testimonies from laboratory users.											
PRICE and TECHNICAL SUPPORT - supplier's price is affordable, realistic, reasonable and represents the total cost of delivering the goods & services and are within the available budget; track record of supplier/distributor; service engineer is readily available; backup or replacement instrumentation is readily on hand.											
TOTAL	**0%**	**0.00**		**0.00**		**0.00**		**0.00**		**0.00**	

Table 2-4. Multicriteria decision support tool that was developed in this study to assist experts in scoring each criterion between 0 and 10. Each attribute was assigned with a score of 1 or 2 by the author, depending to the present relevant descriptor.

Company		Platform Name			
Website					
	Attributes/Parameters	Descriptor		Score	Remarks
1. Technical profile	a. Nucleic acid detection method and analysis capability	1	LAMP		
		2	Quantitative PCR		
	b. Multiplex capable (# of channel)	1	<=3 channels		
		2	>=4 channels		
	c. sample throughput	1	< 12 reactions		
		2	> 12 reaction-well		
2. Operational simplicity and flexibility	a. assay protocols - transferability to other similar platforms and vice versa	1	dependent on the machine's specific protocol and recommended assay set-up		
		2	it can run newly developed assays, existing AHL protocols or assays from published literature		
	b. ease of operating the machine	1	it requires a laptop and its software to operate the machine		
		2	it can run assays as a stand-alone device without a laptop		
	c. sample processing/nucleic acid purification requirements	1	conventional processing/NA purification method is needed		
		2	requires no or minimal sample processing before the amplification step		
	d. kits, reagents, primers, probes, and consumables	1	reliant on the vendor for primers, probes, reagents, and consumables		
		2	adaptable to available standard or lyophilised reagents (primers/probes, enzymes etc.), including reaction tubes		
	e. reporting of results and data storage	1	results are downloaded via USB or saved in the machine for reporting later		
		2	connectivity to WIFI/ethernet for transmission of data via the internet		
3. Field testing deployability and suitability	a. ease of transport, relative weight, portability	1	> 15 kg, including associated equipment		
		2	≤ 15 kg, including associated equipment		
	b. performance in various environments (dust, temperature, humidity)	1	no information or the device is not routinely marketed for field use		
		2	the device is marketed as field ready and demonstrated to be suitable in previous studies or testimonies that it can operate in a variety of environments (temperature, dust, humidity)		
	c. power supply	1	direct power source only		
		2	powered either by a rechargeable battery or directly through a power point		
	d. disinfection procedure	1	70% ethanol or 10% bleach decontamination		
		2	can be decontaminated with 0.2-2 % citric acid or similar disinfectant		
4. Maturity of technology, technical support, and performance	a. stage of commercial maturity	1	commercially available with defined research applications only		
		2	commercially available, supported by published reports and literature; accepted and used by other laboratories		
	b. performance of test platform	1	no published reports or testimonies available - needs further proof of testing capability		
		2	performance characteristics of tested assays are provided by vendors or in published studies; testimonies from users are also available		
	c. customer and technical support	1	technical support for troubleshooting is available only online or by telephone		
		2	readily available through the vendors or a distributor in Australia or NZ; backup instrumentation can be obtained.		
5. Price	a. Supplier's cost	1	not within budget, unrealistic		
		2	affordable and reasonable representation of the total cost of the equipment, including services (<40k NZD)		

2.2.5. Evaluation of diagnostic device against criteria

Experts were recruited to participate in evaluating the five shortlisted devices. The aim was to recruit 15-20 experts, which is within the acceptable number of participants (n = 5-50) in various Delphi studies (Dedewanou et al., 2023; Fong et al., 2020; Plüddemann et al., 2010). The target panel members were individuals with expertise in laboratory diagnostics, knowledge or experience performing pen-side testing, veterinary epidemiologists, veterinarians with expertise in infectious diseases, and disease incursion investigators. Laboratory scientists were chosen because they were familiar with the technical aspects and intricacies of developing diagnostic assays. At the same time, veterinary epidemiologists and animal disease investigators were involved for their practical expertise in disease diagnosis. Familiarity with field setting conditions was considered essential for understanding the appropriateness of a specific technology.

A short meeting was held with most expert panel members in attendance to introduce the selection methodology. Those who were unable to attend were subsequently briefed by email. A copy of the selection matrix scoresheet (Table 2-3) and the completed MCDT (Table 2-4) was emailed to all panel members. They were asked to score each shortlisted device against the criteria in their own time during the ensuing two weeks.

In the selection matrix scoresheet, panel members were first asked to assign weights (%) according to the perceived importance of each criterion for a total of 100 %. Second, experts were asked to rate each platform against the five criteria listed (Table 2-1) using a scale of 0 to 10 (Table 2-2), where 0 meant that the device was unacceptable, with significant issues affecting its suitability for purpose against the defined criterium, and 10 meant that the device exceeded the expected requirements. In addition, participants were asked to provide any general comments to support their scores. This process was used to identify the top three devices (section 2.2.8) for further, more in-depth evaluation.

2.2.6. Trial inspection of devices

To verify and to gather missing pieces of information compiled in the MCDT, vendors of the top three devices identified from the ranking methods were invited for a demonstration of the equipment at the Animal Health Laboratory, Wallaceville. Further feedback from local and overseas users was also sought if the device was unavailable for trial (i.e., no NZ-based distributor or vendor of the product).

2.2.7. Feedback to the panel

The results of the evaluation and ranking process were presented to panel members. Scores and corresponding ranks were discussed until a level of agreement between members was achieved. The highest rank at the end of the discussion was recommended as the preferred option for the field deployable device. The second rank device with a similar technology principle was regarded as the alternative option in case the chosen equipment became unreliable due to unforeseen circumstances.

2.2.8. Data analysis

Data analysis was performed using Microsoft® Excel®. The ranking of the field test platforms was determined using four methods.

1. The mean of the raw scores from each evaluator (n=15) for each criterion (n=5) was calculated and used to compare the shortlisted platforms (Table 2-3) without considering the weighting of each criterion.
2. Each criterion's assigned weight was multiplied by the expert's raw score (section 2.2.5) to derive the weighted score of the platform against a criterion. The sum of the platform weighted score against all criteria (n=5) was then calculated for each platform. Test platforms were ranked from highest to lowest for each expert according to the weighted score, with the highest weighted score as number one. The frequency of getting rated 1-3 from the 15 experts was then counted to evaluate the final ranking of the platforms.

3. The third method was similar to the second method, except the assigned mean weight (n=15) for each criterion was used to calculate the weighted score of the platform against a criterion.
4. The mean of the assigned criteria weight (n=15) was multiplied by the corresponding mean of a platform's raw scores (n=15) to obtain the weighted score against a criterion. The total weighted score of each platform against the five criteria was then used to rank the shortlisted platforms. The platform with the highest weighted score was ranked first. The weighted score represented the experts' opinions.

2.3. Results

Overall, the initial online searchers identified 25 diagnostic platforms (Table 2-5) as candidate platforms for this study. Applying the inclusion and exclusion conditions described in section 2.2.2, the platform list (Table 2-5) was reduced to five devices (Table 2-6). Four of the five candidate platform were based on quantitative PCR (SmartCycler II, Mic qPCR, the Genesig q16 and T-COR 8™) and one used LAMP (Genie III).

Three platforms (SmartCycler II, Mic qPCR, and T-COR 8™) had the capability of running multiplex assays (3-4 targets), compatible with common PCR reagents, and nucleic acids prepared using different nucleic acid extraction kits could be used as templates. Although not capable of multiplex testing, two platforms, the Genesig q16 and Genie III, allowed simultaneous runs of multiple singleplex assays with 16 and 48 reactions, respectively. An advantage of the Genesig q16 and T-COR 8™ machine was the availability of customised off-the-shelf commercial assays for testing for several animal disease pathogens and biothreat agents.

One device (SmartCycler II), although compatible with universal PCR tubes, was comparatively heavier (33 kg) than the other devices considered (4-5 kg), which was considered a limitation in its portability and deployability. Except for two platforms (T-COR 8™ and Genie III), the other three devices required a direct power supply, which could limit deployability in certain conditions.

Table 2-5. Test platforms identified as candidates for developing field diagnostic capabilities for detection of foot-and-mouth disease virus incursion in New Zealand through a literature search (section 2.2.1).

Portable PCRs	Manufacturer/Vendor	Description	Links
1. R.A.P.I.D. (Ruggedized Advanced Pathogen Identification Device) PCR	Biofire Defense	The Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.®) is a portable real-time PCR system designed to identify biological agents. This unit is a forced-air thermocycler with an integrated fluorometer for real-time monitoring of PCR reactions.	http://biofiredefense.com/rapid/
2. Enigma ML	Enigma Diagnostics	Fully automated portable real-time PCR. The system is portable and completely self-contained. Reagents in single disposable cartridges are stored at ambient temperature.	http://www.enigmadiagnostics.com/
3. Enigma FL	Enigma Diagnostics	Fully automated portable real time PCR for field use. The system is completely self-contained, using reagents in a single disposable cartridge.	http://www.enigmadiagnostics.com/
4. BioSeeq	Smith Detection	Linear after the exponential (LATE) PCR	http://www.smithsdetection.com/index.php?option=com_k2&view=itemlist&layout=category&task=category&id=114&Itemid=1417
5. BioSeeq Plus	Smith Detection	Hand-held Linear after the exponential (LATE) PCR	http://www.smarttechnology.co.id/mainsite/product/bio-seeq-plus/#2
6. T-COR8	Tetracore, Inc.	A portable real-time PCR machine that runs multiple protocols simultaneously. It can also perform an isothermal amplification assay.	http://www.tetracore.com/t-cor8/index.html
7. SmartCycler II Cepheid	Cepheid	Automated real-time PCR system	http://www.cepheid.com/us/cepheid-solutions/systems/smartcycler
8. GeneXpert Omni	Cepheid	Sample to answer real time PCR	http://www.cepheid.com/us/cepheid-solutions/systems/genexpert-systems/genexpert-i
9. Pikoreal	Thermo Fisher Scientific	Real-time qPCR system	https://www.thermofisher.com/order/catalog/product/TCR0024

Continuation of Table 2-5

10. The Hunter Real-Time PCR System	InstantLAbs	Portable real time PCR position for the food industry	http://instantlabs.com/food-safety-test/food-safety-test-kits-2/hunter/
11. POCKIT Nucleic acid Analyzer (Pockit iiPCR)	GeneReach Biotechnology	Insulated convection isothermal portable PCR	http://www.iipcr.com/
12.The genesig q16	Primerdesign Ltd, UK	qPCR	http://www.primerdesign.co.uk/products/9696
13. RAZOR EX	Biofire Defense	PCR-based system for pathogen detection in the field	http://biofiredefense.com/razorex/
14. Mic qPCR	Bio Molecular Systems	Magnetic induction qPCR	http://mic-qpcr.com/
15. Freedom 4 Realtime PCR system	Ubiquitome	Peltier-based handheld qPCR	http://www.ubiquitomebio.com/
16. miniPCR	Amplyus	End-point detection PCR using gel electrophoresis	http://www.minipcr.com/products/dna-discovery-system/
17. Palm PCR	Ahram Biosystems Inc.	Palm-size portable conventional PCR machine - endpoint detection	http://www.ahrambio.com/
18. Bento Lab beta	Bento Lab	Portable conventional PCR - all in one	https://www.bento.bio/bento-lab/
19. Genie III	OptiGene	LAMP – loop-mediated isothermal amplification of DNA / RNA field-deployable device.	http://www.optigene.co.uk/instruments/instrument-genie-iii/
20. ClariLight™ Platform	Lucigen Dx	LAMP – loop-mediated isothermal amplification of DNA / RNA in 3 basic steps using a disposable cartridge system	https://wedc.org/wp-content/uploads/2015/06/WEDC_QNBV-2014-Program_WEB-1.pdf
21. The Sentinel Nucleic Acid Analysis System	SRI International	Fully contained system with microfluidic card actuation, rapid PCR cycling, compact microarray detection optics, and automated hybridisation analysis.	https://www.sri.com/work/projects/sentinel-portable-bioanalysis-system

Continuation of Table 2-5

22. FilmArray BioSurveillance System	Biofire Defense	The film array extract and purifies all NA from the sample, followed by a nested multiplex PCR; last is an individual singleplex second-stage PCR reaction. Use melt curves data to generate a result for each target	http://biofiredefense.com/filmarray/
23. The TrueLab Real-time micro-PCR system	Molbio Diagnostics	Rapid, microchip-based real-time qPCR;	http://www.molbiodiagnostics.com/molbio_realtime%20micro%20pcr%20system.html
24.. Field Portable Palladium System - beta capability	Integrated Nanotechnologies	The palladium system automates and integrates equipment-intensive laboratory sample prep and testing into a single disposable cartridge. It combines fluidics, sample prep and electronic detection of target nucleic acid.	http://www.integratednano.com/products/palladium-test-cartridges.aspx
25. Biomeme two3	Biomeme	A handheld portable PCR-based platform device can perform isothermal molecular analysis. An iPhone can operate thermocycler, and data are disseminated through the cloud in real-time.	http://www.biomeme.com/

Table 2-6. Features of five candidate field test platforms that were shortlisted using inclusion and exclusion conditions described in section 2.2.2.

	Shortlisted Portable Platform		
Technology Description	**SmartCycler II (Cepheid (Cepheid Holdings Pty Ltd, Au, NZ)**	**Mic qPCR (Bio Molecular Systems)**	**The genesig q16 (Genesig, UK)**
Principle	qPCR	magnetic induction qPCR	qPCR
Footprint	12 x 10 x 12 inches	15x15x13 cm	16 cm height and 12 cm diameter
Weight	33 kg (including processing block, computer, and accessories)	2 kg	2 kg
Reagents	compatible with a range of PCR reagents	compatible with a range of PCR reagents	Freeze-dried reagents are available for specific agents and require their own reaction plastic tubes.
Sample processing	requires nucleic acid extraction of samples	requires nucleic acid extraction of samples	requires nucleic acid extraction of samples
Channels	4 channels	2 or 4-channel mode	2 channels
Disinfection of machine	standard surface disinfection with 70% ethanol	no information	no information
Robustness	Optical system has no moving parts. No danger of misalignment during transport.	operating temperature - 18-35C, 20-80% relative humidity -unsure if field ready	Field ready, it can be used anywhere
Sample throughput	smallest module - 16 independently programmable reactions	48 reactions	16 reactions
Run time	20-40 mins/assay	depend on the assay design	depend on the assay design
Power source	direct power requirement	requires an external power supply	90-watt power consumption, direct power required
Software	pre-installed SmartCycler system software in a laptop computer	operated via laptop thru Bluetooth or ethernet	q16 software requires the laptop to operate and analyse results
Reporting/communication	via the internet thru the attached laptop	via the internet thru the attached laptop	Operate from PC, Mac, via a network, or stand-alone with a USB drive
Support	technical support available	technical support available	replacement unit available during repair
Peer-reviewed references	several published literatures available	no available during the study	no available during the study

Continuation of Table 2-6

	Shortlisted Portable Platform	
Technology Description	**TCOR- 8™ (Tetracore, Inc., USA)**	**Genie III (OptiGene)**
Principle	qPCR	LAMP – loop-mediated isothermal amplification of DNA / RNA
Footprint	approximately laptop size with a 26.4cm touchscreen	25x16.5x85 cm
Weight	< 4.5 kg	1.75 kg
Reagents	compatible to a range of PCR reagents	recommends OmniAmp polymerase for LAMP
Sample processing	require nucleic acid extraction but designed to be a fully automated sample to result platform	no or minimal
Channels	4 channels	dual channel
Disinfection of machine	surface wipe with 10% bleach; use of Virkon S is currently on trial	no information
Robustness	Field deployable	Field deployable instrument -developed for demanding environment
Sample throughput	8 independently programmable amplification wells	8 reactions
Run time	4 hours battery life equivalent to about 4 PCR run	approximately 30 mins
Power source	rechargeable battery or external power supply	Long-lasting rechargeable lithium battery or external power supply
Software	Built-in Smart CT software, no laptop required to analyse results	Can be operated stand-alone or connected to a PC
Reporting/communication	Data stored via SD card or download report via network or USB drive; cloud-ready for remote access.	Wireless communication via Bluetooth or Wi-Fi, Positional information via GPS
Support	USA based support	technical support available
Peer-reviewed references	several published validation and verification data are available	several published literatures available

2.3.1. Ranking of the candidate platform for field diagnostics

In total, 15 animal disease experts participated in scoring and evaluating the five shortlisted devices. The background of the individual experts is shown in Table 2-7. The panel members were animal health laboratory scientists, veterinary scientists and veterinary epidemiologists from the Ministry for Primary Industries, New Zealand (MPI, NZ) (n= 13), and the School of Veterinary Medicine, Massey University (n=2). Except for one participant with incomplete scores, all 14 members completed the assigning of weights to each criterion and scored the device against the weighted criteria. Only 10 participants, however, provided comments in support of their expressed scores.

Table 2-7. Background and affiliation of the fifteen recruited experts.

Professional role and expertise	Number
Laboratory diagnostic scientist	5
Animal disease incursion investigator	2
Veterinary scientist - Poultry Diseases	1
Veterinary epidemiologist	1
Diagnostic scientist - aquatic diseases	2
Diagnostic scientist - Bee diseases	1
Principal Scientist – vesicular diseases	2
Academe - Virology and infectious diseases	1
total number of participants	15
Affiliation	
Government	12
Academe	2
Research	1
total number of participants	15

The main comments and concerns raised were what would be the disinfection procedure in the field for all machines, cost (SmartCycler, T-COR 8™), source of power, operating temperature conditions (SmartCycler, Mic qPCR), flexibility in developing new assays, multiplexing capability (Genie III), and general performance in the field (the genesig q16, Mic qPCR).

2.3.1.1. Method 1

Ranking using mean unweighted scores are shown in Figure 2-2. Three devices (SmartCycler, Mic qPCR and T-COR 8™) scored high for two criteria: fit for the project purpose and scope; and operational simplicity and flexibility. The Genie III and T-COR 8™ platforms were ranked first and second regarding field deployability and suitability. SmartCycler and Genie III were ranked high in terms of performance. Mic qPCR, the genesig q16, and Genie III were identified as the least expensive platforms with easy access to technical support in New Zealand.

2.3.1.2. Methods 2 and 3

In these ranking scenarios (Table 2-8), the T-COR 8™ device was the preferred option among 9/15 (60%) respondents when an individual weighted score was used. General preference for T-COR 8™ did not change remarkably (12/15, 80.00%) when the mean of the assigned criteria weights was used on the individual raw scores. Mic qPCR appeared to be the alternative technology to T-COR 8™ as it was consistently ranked as the second-best choice when either the individual or mean of the assigned criteria weights were used on the individual raw scores.

2.3.1.3. Method 4

In this method, the weighted score derived from the mean of the assigned criteria weight (n=15) and the mean of the device raw score (n=15) against a criterion represented the experts' opinion. Overall, the order of experts ranking using method 4 (section 2.2.8) were T-COR 8™ (1st), Mic qPCR (2nd), and Genie III (3rd) (Table 2-9). The weighted scores of the three devices were very close at 6.41, 5.88, and 5.67, respectively.

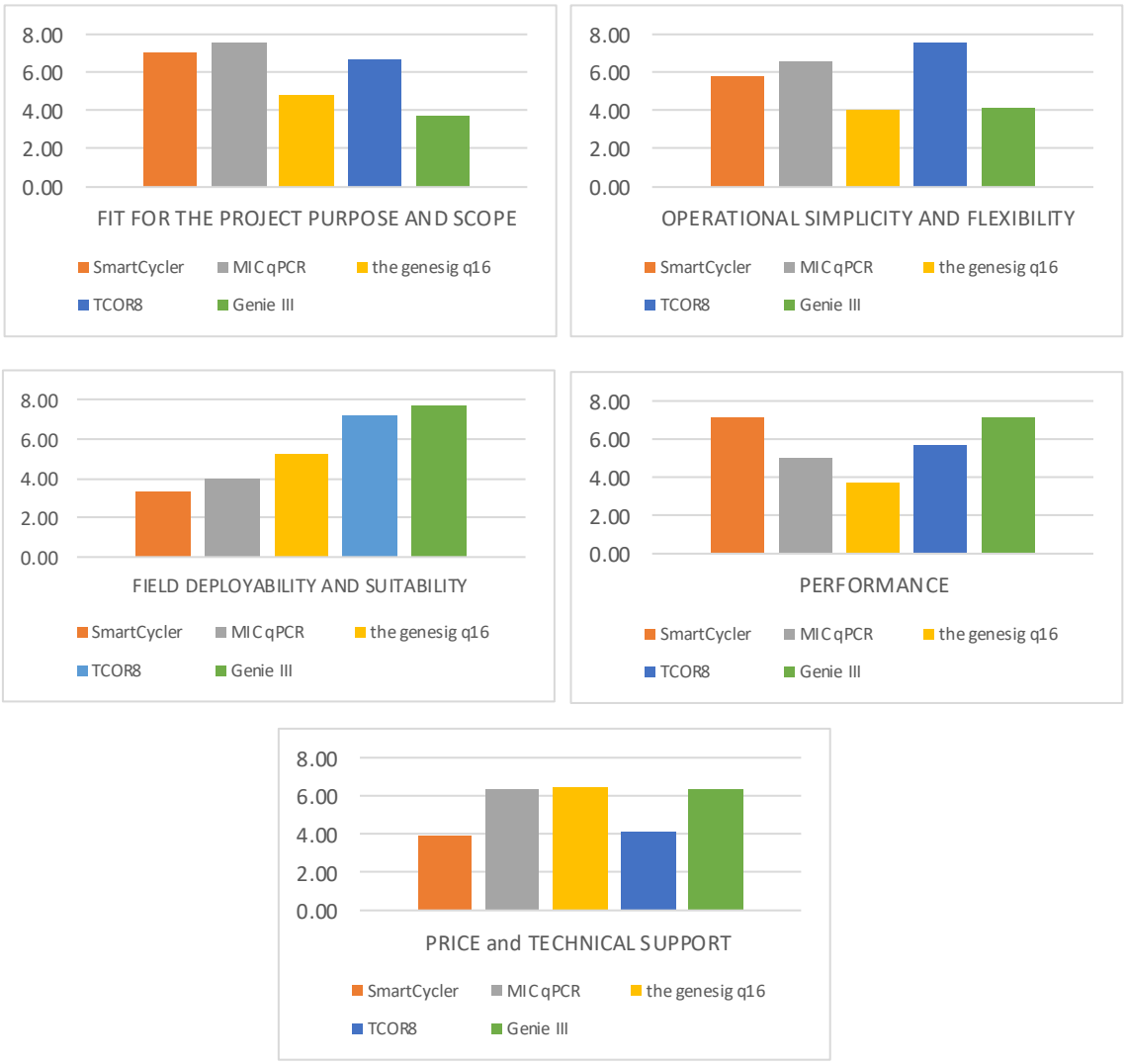

Figure 2-2. Mean of the raw scores based on assessment by experts (n=15) for the criteria listed in Table 2-1 for each of the shortlisted test platforms. Each device was scored against a specific criterion using a scale between 0 and 10 (Table 2-2).

Table 2-8. The platform ranking considered the frequency of getting a rank of 1-3 from each evaluator using the total weighted score of the platform against all criteria. In Method 2 (section 2.2.8), the weighted score was derived by multiplying the criterion's assigned weight and the expert's device raw score against a criterion. Method 3 was similar to Method 2 except that the mean (n=15) of the assigned criteria weight was used to calculate the device weighted score of each expert. In both methods, T-COR 8™ consistently ranked as number one among the experts.

Platforms	Method 2			Method 3		
	Rank 1	Rank 2	Rank 3	Rank 1	Rank 2	Rank 3
SmartCycler	0	4	4	0	3	6
Mic qPCR	4	7	2	3	9	1
the genesig q16	0	0	1	0	0	0
T-COR 8™	9	2	1	12	1	0
Genie III	2	4	5	2	2	7

Table 2-9. Ranking the shortlisted platforms by comparing each cumulative weighted score against all five criteria (Method 4). A device weighted score against each criterion was derived by multiplying the mean of the raw scores (n=15) by the mean of the assigned criterion weighted (n=15) (section 2.2.8). The platform with the highest total weighted score against all criteria was ranked first.

		Expert's MEAN Weighted score (n=15)				
Criteria	Mean weight, %	SmartCycler	MIC qPCR	genesig q16	T-COR 8™	Genie III
FIT FOR THE PROJECT PURPOSE AND SCOPE	25.00	1.75	1.88	1.2	1.67	0.93
OPERATIONAL SIMPLICITY AND FLEXIBILITY	21.33	1.24	1.41	0.85	1.61	0.88
FIELD DEPLOYABILITY AND SUITABILITY	24.00	0.8	0.94	1.25	1.71	1.84
PERFORMANCE	17.83	1.26	0.89	0.66	1.01	1.26
PRICE and TECHNICAL SUPPORT	11.83	0.46	0.75	0.72	0.42	0.75
TOTAL	100%	5.51	5.88	4.68	6.41	5.67
Rank		4	2	5	1	3

2.3.2. Demonstration trial of the three devices

The vendor of the Mic qPCR platform carried out a demonstration for detecting BVDV-1 RNA using an established assay protocol (Appendix 2) on a Mic qPCR instrument to showcase its operational flexibility in accommodating various PCR tests. A vendor-supplied assay was used for demonstration of the capabilities of the Genie III device.

No demonstration trial was conducted for the T-COR 8™ device. However, additional supporting data were obtained from an overseas laboratory (Pirbright Institute, United Kingdom). In unpublished data during the timeframe of this work, the T-COR 8™ was reported to have comparable performance and high sensitivity comparable with a standard laboratory PCR used at The Pirbright Institute during a field trial in Africa (Veronica Fowler, personal communication, 2016). Howson et al. (2018) later published these data.

2.3.3. Final ranking of field test platform

The ranking order was supported by 14/15 of the panel members after presenting the rank analyses, observations from the demonstration trial and anecdotal reference. Overall, the T-COR 8™ platform consistently ranked first using all four scoring methods. The expert panel agreed it would be the best option for developing a field diagnostic test for animal

diseases. The Mic qPCR came second as the alternative device, although one expert strongly preferred this as a top choice due to its higher sample throughput than T-COR 8™.

2.4. Discussion

This study describes the development and application of a semi-quantitative ranking method using a multi-criteria approach, decision support tools, and expert opinions to enable the systematic ranking and selection of the preferred field diagnostic platform. Five platforms satisfied the first pass of short-listing conditions from an initial list of 25 portable test devices. A panel of animal disease experts then ranked the five devices. Overall, the expert panel ranked the T-COR 8™ first, followed by Mic qPCR for developing a POC test to detect FMDV incursion in New Zealand.

The weighted score between T-COR 8™ and Mic qPCR was very close. Both devices scored highly for essential features, including multiplex testing capability and the ability to accommodate previously established PCR assays, allowing these test platforms to be used for applications beyond FMDV/BVDV testing. Multiplex testing is valuable for the differential detection of disease agents (Hindson et al., 2008; Thonur et al., 2012; Wernike et al., 2015). This can be applied, for example, to investigate pig diseases with similar clinical signs, such as African swine fever and classical swine fever (Haines et al., 2013) or avian respiratory disease to differentiate between infection with avian influenza virus, infectious bronchitis virus or Newcastle disease virus (Nguyen et al., 2013). Also, the ability to migrate developed assays for various pathogens into the platform can reduce the time needed for extensive test validations. For instance, PCR assays currently used at AHL could be transferred to the deployable device for field testing if needed.

The T-COR 8™ device differed from the Mic qPCR device in portability and deployability. Whilst both platforms have a relatively small footprint weighing between 2 and 4 kg, its built-in lithium-ion battery can operate the T-COR 8™ for at least four PCR runs in addition to an external power source. The general portability of equipment can be defined by factors such as an instrument's low weight, which makes it easy to be carried by one person, and independence from the availability of the external power source (Almassian et al., 2013; Hansen & Abd El Wahed, 2020). Mic qPCR relies solely on the external power source to operate. Although this is not a problem on most farms in New Zealand where power supply

is readily available, deployment of the device in remote areas may be problematic. The second difference between the machines was the operating temperature. While the operating range was relatively narrow (18-35°C) for Mic qPCR, it was wider (0-50°C) for the T-COR 8™. The narrow temperature range may prevent the Mic machine from performing optimally under field conditions, especially during wintertime in New Zealand. This issue could be mitigated by placing the Mic qPCR platform in a space that offers some temperature control (e.g., a car or a farm shed) during testing. The air conditioner can regulate the temperature inside the car, while an enclosed farm shed could reduce the effect of the low outside temperature. A recent study demonstrated the portability and the deployability of the T-COR 8™ device in detecting FMDV in villages in East Africa (Howson et al., 2018), with outside temperatures reaching up to 28 °C.

One advantage of the Mic qPCR device was the number of samples (n=48) that can be analysed in one run compared to T-COR 8™ (n=8). This was the main reason given by the expert who favoured this option. While high throughput testing capacity is an essential consideration in centralised laboratories for disease surveillance (Fasina et al., 2010; May et al., 2016) or in an outbreak scenario (Kasem et al., 2018; Souley Kouato et al., 2018), it is expected that only a few samples will need to be tested to support the clinical diagnosis of suspected infectious disease incursion. For example, one PCR-positive vesicular fluid taken from an animal with suspected FMD lesions would be sufficient to support a presumptive diagnosis and initiate the response plan (Poonsuk et al., 2018). Similarly, a single positive blood sample taken from a pig with comparable clinical signs would be sufficient to confirm the diagnosis of African swine fever during the investigation of an exotic disease incursion (Oura et al., 2013).

The Delphi-like model of eliciting expert opinion is a systematic technique using multicriteria decision tools commonly applied in forecasting modern technologies in multiple disciplines (Powell, 2003). A group of animal disease experts of diverse specialisations provided a consensus judgement in selecting a fit-for-purpose field test platform. In this case, diversity was necessary for considering aspects of test development and practical application in various field settings. However, the recruitment of like-minded people here could have introduced a framing bias and leaned the selection of the device towards the technical aspects of the developed criteria (Winkler & Moser, 2016). Including non-technical people in the panel could have resulted in a different outcome, but since the cohort of the panel will be the potential user of the technology for the specific purpose of

supporting exotic disease investigation, a high level of competence was one crucial criterion for recruiting participants. Anonymity also shielded the individuals from the influence of personal biases, higher status or hierarchy and dominant personality, typically present in focus group discussions (Powell, 2003). The scoring of the different platforms could have also been influenced by the multicriteria used, primarily driven by the requirements and scope of the project and the subjectivity of scoring. Nevertheless, a participatory and collective opinion generated by several independent experts provided validity in the decision-making compared to when done by an individual alone (Shen et al., 2010).

In succeeding chapters, the development of a field-ready multiplex assay for the detection of FMDV and BVDV on the T-COR 8™ is presented (Chapter 5), including the assessment of simple sample processing alternatives to the standard nucleic acid extraction protocols (Chapter 3), followed by the assessment of the analytical and diagnostic (Chapter 6 and 7) performance of the test.

2.5. References

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Chapter 3. Assessing and optimising sample preparation methods for RT-qPCR performance in the field

3.1. Introduction

One major challenge in adopting a field deployable nucleic acid detection method as a pen-side test is the availability of a simple but robust sample processing technique (Dineva et al., 2007; King et al., 2010). "Simple" in this context means that the method has few practical steps without requiring bulky equipment or cold chain management of reagents and can be performed with a limited amount of operator training. A simplified sample preparation process would reduce the turnaround time of the test, which is essential for faster diagnosis and clinical decision-making in the field. Ideally, such preparation methods should be appropriate for DNA and RNA test formats.

Sample preparation is critical for optimum efficiency and sensitive identification of nucleic acid (NA) targets by PCR-based methods (Bustin et al., 2009). This involves right sample acquisition, handling, and extraction of DNA, RNA, or both. Extraction protocols work by releasing NA through cell lysis, separating NA from other inorganic and non-organic debris, and protecting NA from nucleases (Steward & Culley, 2010). The primary goal is the optimal yield of quality NA without any PCR assay inhibitors, so these can be used as a template in a PCR or RT-PCR reaction (Schrader et al., 2012). Most commercial extraction methods consist of several steps and require equipment such as a micro-centrifuge, a vortex, and a heating block (Dineva et al., 2007). The process takes at least 30-60 minutes to complete depending on the protocol and sample numbers. Most laboratory-based NA extraction, however, is not readily deployable and considerably challenging to perform in field settings. For this reason, a more streamlined sample processing approach is desirable for pen-side PCR testing.

Simple and fast preparatory techniques to obtain PCR-ready templates from clinical samples that can potentially be adopted for field use have been reported (Dineva et al., 2007; Howson et al., 2017). One example is the successful amplification of viral RNA target directly from virus in the cell culture supernatant (Pastorino et al., 2005; Wambura, 2006) or from serum (Deregt et al., 2002; Howson et al., 2017; Ravaggi et al., 1992) without prior NA

extraction. It was previously demonstrated that sample dilution (Deregt et al., 2002) or pre-heating (Ravaggi et al., 1992), when applied to crude samples, reduced the effect of inhibitors during direct BVDV-1 PCR testing. PCR testing, without prior NA purification, was also used for screening bacterial DNA (Fode-Vaughan et al., 2001), as well as for detecting specific genes in murine solid tissues (Kobayashi et al., 2003; Panaccio et al., 1993) and human blood (McCusker et al., 1992; Mercier et al., 1990). Similarly, there is a growing recognition of the use of commercially available sample dilution buffers to treat cell cultures which can be used as templates to detect target RNA successfully without extraction (Shatzkes et al., 2014). Such proprietary buffers (e.g., Cells to Ct kits, Thermo Fisher Scientific) are formulated to lyse cells and stabilise RNA within only 2-5 min after addition to cell lysates. Depending on the desired test sensitivity and turn-around time, these protocols have their application, although no validations have been done for diagnostic purposes (Thatcher, 2015).

Several studies have shown the applicability and appeal of simple extraction-free PCR template preparatory techniques, but this cannot be applied universally to all sample types. Inhibitory substances unique to a sample type could challenge the efficient amplification of any virus target present, especially when RNA extraction is not performed (Yan et al., 2020). Even when using commercial DNA and RNA purification kits in the laboratory, validation and optimisation procedure need to be conducted to ensure the efficiency of NA extraction for a given sample type.

The main aim of the work presented in this chapter was to evaluate the suitability of four sample processing methods, including three extraction-free approaches and a bead-based nucleic acid extraction method for a pen-side RT-qPCR assay. More specifically, to compare the analytical sensitivity of RT-qPCR when these simple techniques were applied to serum and oral swab samples for diagnosing FMDV, BVDV-1 and BVDV-2.

3.2. Materials and methods

3.2.1. Collection of samples

Serum and oral swab sample matrices were used in preparing a mock-infected test panel. Blood (n = 30) and oral swabs (n = 60) were conveniently collected at a cattle slaughter plant (Wellington region) immediately after stunning and bleeding the animal. Samples were

transported within 24 hours in chilled condition to AHL-MPI at Wallaceville, Upper Hutt, New Zealand.

Whole blood was collected in a 10 mL Vacutainer red top blood collection tube (Becton, Dickinson, and Company) mid-stream from the jugular vein. Tubes with clotted blood were centrifuged at 1,500 x *g* for 3 min, and sera were collected by aspiration, poured into 5 mL screw-capped tubes, and stored at -80°C. Oral swabs were collected by rubbing a dry rayon Dryswab® (Thermo Fisher Scientific) around the oral mucosa and tongue surfaces, saturating it with oral fluids. The swab tip was inserted immediately into a vial with 2 mL virus transport media (VTM) (Fort Richard Laboratories) to approximate a 1:10 suspension. Upon receipt in the laboratory, oral swabs were stored immediately at -80°C.

3.2.2. Preparation of virus stock

BVDV-1 and equine rhinitis A virus (ERAV) were used to spike serum and oral swabs to create a test panel for evaluating the sample preparation methods. ERAV was used as a surrogate for FMDV, given the similarity between their biological and physicochemical properties (Li et al., 1996; Varrasso et al., 2001). The virology section at AHL-MPI provided a cytopathic BVDV-1 (Bovax TVL vaccine strain passage 4 in bovine lungs) isolate. The ERAV (isolate 393/76, passage 23: 18 passages in equine foetal kidney cells and 5 passages in Vero cells) was kindly provided by Dr Magda Dunowska from Massey University.

BVDV-1 was propagated in monolayers of bovine lung cells (BL 12, working cell stock 37, passage 19) generated from primary foetal bovine lung at AHL-MPI, and ERAV in Vero cells (ATCC CL 81, working cell stock 5 passage 165), inside a biosafety cabinet II. Briefly, a 25 cm^2 tissue culture Nunc flask (Thermo Fisher Scientific) was seeded with 1×10^5 cells. Upon achieving at least 80 % confluency, cells were inoculated with 0.5 mL of either BVDV-1 or ERAV. The inoculum was adsorbed to the cell monolayer for 30 min at 37°C in a humidified incubator with 5% CO_2. After incubation, 10 mL of pre-warmed maintenance medium, MEM-Earle's salts supplemented with 10% (v/v) adult bovine serum (ABS), was added into the virus flasks. These flasks were incubated for 5 to 7 days or until a 90% cytopathic effect (CPE) was observed. Cytoplasmic vacuolation, rounding of cells, cell death and detachment of the cell monolayer were seen in the BVDV-1 infected cell culture. The CPE of ERAV infected cells was mostly cell rounding and detachment of the cell monolayer. The virus was released from the

infected cells with one freeze-thaw cycle at -80°C for 1 hour and a 37°C water bath. The cell debris was pelleted by centrifugation for 10 min at 1500 × *g*, and the virus-containing supernatant was collected into 2.0 mL Nunc cryovials (Thermo Fisher Scientific) for storage at – 80°C.

Virus titrations were performed based on the method described by Hierholzer & Killington (1996), and titres expressed as tissue culture infective dose 50% ($TCID_{50}$). Virus stock was serially diluted from 10^{-1} to 10^{-8} in a 96-well tissue culture plate in duplicate using MEM-Earle's salts with 2% (v/v) ABS. Fifty microlitres of each dilution were transferred to a corresponding well in a new 96-well Nunc plate containing 50 µL of maintenance medium (MEM-Earle's salts with 2% ABS). BL 12 or Vero cell suspension (50 µL containing 1.5×10^3 cells) was then added to each well. The cell controls with media as inoculum were assigned to the last two columns of wells. Plates were incubated for four days for BVDV-1 and three days for ERAV at 37°C incubator in a humidified 5 % CO_2. Each well was scored as positive when viral CPE was present or negative if none was observed. The virus titre (Log_{10} $TCID_{50}$/mL) was derived and calculated according to the Spearman Kärber method (Hierholzer & Killington, 1996).

3.2.3. Preparation of test panels

Four mock "infected" test panels were prepared by spiking pooled cattle sera or oral swabs with either BVDV-1 or ERAV. Prior to use, the two matrices were screened for the presence of either virus using RT-qPCR adopted from Willoughby et al. (2006) (BVDV-1) or Quinlivan et al.(2010) (ERAV).

Initially, the stock solutions of viruses (section 3.2.2) were diluted in phosphate-buffered saline pH 7.2 (PBS) to generate working stocks containing 6.0×10^5 $TCID_{50}$/mL (BVDV-1) or 2.0×10^5 $TCID_{50}$/mL (ERAV). An aliquot (1 mL) of the working stock virus was then added to the respective panel matrix (serum or oral swab suspension) (10^{-1}) and serially 10-fold diluted (up to 10^{-6}) in the respective panel matrix by adding 1 mL of the diluted virus into 9 mL of the matrix. Each panel was aliquoted in nine sets (1 mL per dilution point) into 1.5 mL cryovials and stored at -80°C. Thus, the titres of standards in each panel ranged from 6.0×10^4 to 6.0×10^{-1} $TCID_{50}$/mL for BVDV-1 and from 2.0×10^4 to 2.0×10^{-1} $TCID_{50}$/mL for ERAV (Table 3-1). Although back titration was not performed to confirm the titre of the

panels, all experiments were conducted in equal footing by using panels with the same virus titre.

Table 3-1. Description of the four panels that were used for optimisation of sample preparation for RT-qPCR testing. Each panel consisted of a series of ten-fold dilutions of a virus (equine rhinitis A virus (ERAV), or bovine viral diarrhoea type 1 (BVDV-1) spiked into a matrix (oral swab or serum) to a desired titre, expressed as tissue culture infective dose 50% (TCID50)/mL.

Panel	**Virus**	**Matrix**	**Standards ($TCID_{50}$/mL)**
1	ERAV	Oral swab	2×10^4
			2×10^3
			2×10^2
			2×10^1
			2×10^0
			2×10^{-1}
2	BVDV-1	Oral swab	6×10^4
			6×10^3
			6×10^2
			6×10^1
			6×10^0
			6×10^{-1}
3	ERAV	Serum	2×10^4
			2×10^3
			2×10^2
			2×10^1
			2×10^0
			2×10^{-1}
4	BVDV-1	Serum	6×10^4
			6×10^3
			6×10^2
			6×10^1
			6×10^0
			6×10^{-1}

3.2.4. Verification of BVDV-1 and ERAV RT-qPCR

Following kit instructions, BVDV-1 and ERAV RNA were extracted from infected cell culture lysates (section 3.2.2) using the QIAamp Viral RNA kit (Qiagen). Briefly, 140 µL cell culture lysate was mixed with 560 µL lysis buffer (AVL) and 5.6 µL carrier RNA; and incubated for 10 min at room temperature. After brief centrifugation, 560 µL ethanol (99%) was added, and the content was mixed by vortexing. The solution was transferred into a spin column and centrifuged at 6000 × *g* for 1 min. This step was repeated until all the lysate passed through the spin column. The initial wash was done with 500 µL of buffer AW1 at 6000 × *g,* followed by a second wash (500 µL of buffer AW2) at 20,000 × *g* centrifugation. After each washing step, the filtrate was discarded. RNA was eluted with 60 µL of supplied elution buffer.

The primers and probes for both BVDV-1 (Willoughby et al., 2006) and ERAV (Quinlivan et al., 2010) one-step RT-qPCR targeted the untranslated (UTR) 5' end region (Table 3-2). To establish optimal conditions for RT-qPCR performance, standard curves were created from serially diluted (7 log dilution) viral RNA in RNase-free water (RFW) and amplified using three different master mixes. The PCR mixes were designated as master mix 1 - Taqman® Fastvirus 1-Step MasterMix (Thermo Fisher Scientific), master mix 2 - SuperScript™ III Platinum™ One-step qRT-PCR (Thermo Fisher Scientific), and master mix 3 - UltraPlex™ 1-StepToughMix (Quantabio). Viral RNA standards were tested in duplicate in two different runs using virus-specific RT-qPCR reaction and cycling conditions as specified in Table 3-3 and Table 3-4. PCR testing was carried out in the CFX96 PCR (BioRad) machine.

A standard curve of each run was generated through the BioRad CFX Manager 3.1 software, plotting the log dilution factor against the corresponding quantification cycle (Cq) value. Amplification efficiency and the linear regression line or the correlation of determination (R^2) were derived from the slope of the standard curve automatically by the software. Acceptable PCR efficiency (*E*) ranged between 90% and 110%, and R^2 >0.98 was considered acceptable. *Efficiency* of 100% indicates that the number of copies of PCR product doubles at each cycle. This is rarely achieved due to various factors including presence of inhibitory substances, primer design, reagents concentrations, dilution errors in preparing standards, and random pipetting errors (Ruijter et al., 2021; Svec et al., 2015). *Efficiency* <100% is commonly attributed to poor primer design and sub-optimal reagent concentrations, and

E>100% is commonly due to inhibitory factors that decrease the ΔCq between standard dilutions, thus flattening the plot and reducing the slope.

Table 3-2. BVDV-1 (Willoughby et al., 2006) and ERAV (Quinlivan et al., 2010) primers and probe sequences that were used for RT-qPCR assays. Both sets of primers targeted the 5' UTR region of the respective virus.

Target	Primers / Probe	Sequence	Amplicon size
BVDV 1, BVDV2	BVDV1_2 F	CCA TGC CCT TAG GAC TAG C	92 & 112 bp
	BVDV1_2R	TGA CGA CTA CCC TGT ACT CAG G	
	BVDV1MGB probe	FAM-AAC AGT GGT GAG TTC GT-NFQ-MGB	
	BVDV2MGB probe	VIC-ACT AGC GGT AGC AGT GAG-NFQ-MGB	
ERAV (NC_003982)	ERAV 468F	CCA GGT AAC CGG ACA GCG	118 bp
	ERAV 569R	GGC AGC GCT ACC ACA GG	
	ERAV 508b MGB probe	FAM-CAT TGC TCT GGA TGG TGT-MGB	

To assess the ability of the two viral-specific RT-qPCRs to amplify viral RNA from crude samples without previous RNA extraction, infected cell culture lysates were also used as a template. BVDV-1 (6.0×10^5 $TCID_{50}$/mL) and ERAV (2.0×10^5 $TCID_{50}$/mL) cell culture lysates were serially diluted (10-fold) in PBS. Six dilutions of each template were tested in triplicate using 5 µL of each dilution per reaction. PCR testing was carried out in the CFX96 PCR machine with the same cycling conditions as those used to amplify purified RNA (Table 3-3 and Table 3-4). The analytical sensitivity and intra-run variability were examined in these PCR runs.

3.2.5. PCR template preparation and RT-qPCR analysis

Each of the four panels (section 3.2.3) was subjected to one of the four simple sample preparation protocols or a reference extraction method (Table 3-5). Prepared templates were tested with either BVDV-1 (panels 2 and 4) or ERAV RT-qPCR (panels 1 and 3). Unless otherwise stated, the general PCR parameters and conditions used were those described in Table 3-3 and Table 3-4. PCR templates prepared using Protocols 1, 2, 4 and 5 (reference method) were tested on the CFX96 thermocycler using 96-well clear PCR plates. The pre-heating step in Protocol 3 was carried out in the T-COR 8™ portable real time PCR machine, also referred to as T-COR. To mimic pen-side testing, templates prepared using Protocol 3 were analysed on the T-COR using machine-specific PCR reaction tubes. For each PCR run,

non-template control (NTC), serum or oral swab negative control and positive RNA control were included in the analysis.

3.2.5.1. Protocol 1. Sample dilution without RNA purification

Standards from each panel (1 – 4) (section 3.2.3) were diluted 1:5, 1:10 and 1:20 with RNAse free water (RFW) and used as a template for the virus-specific RT-qPCR reactions. Both assays were run using three different master mixes and PCR conditions (Table 3-3 and Table 3-4). The performance of the three RT-qPCR chemistries was compared to determine their ability to amplify viral RNA in the presence of PCR inhibitors expected to be present in the un-extracted samples. Pre-diluted samples in each panel were tested in five replicates with virus-specific primers appropriate for the panel. Each test was run twice at different times.

In a separate experiment, the potential effect of template volume (2.5 µL or 5 µL) on the sensitivity of the assay was determined. Three selected standards (6.0×10^3, 6.0×10^2 and 6.0×10^1 $TCID_{50}$/mL) from panel 4 (BVDV-1 spiked serum) were diluted 1:5 and 1:10 in RFW and 2.5 µL or 5 µL of each dilution was then used as a template in the BVDV-1 RT-qPCR using master mix 2 conditions (Table 3-3).

Table 3-3. Bovine viral diarrhoea virus-1 (BVDV-1) one step RT-qPCR reaction parameters and cycling conditions (AHL-MPI protocol). Master mix 1 - Taqman® Fastvirus 1-Step MasterMix (Thermo Fisher Scientific), Master mix 2 - SuperScript™ III Platinum™ One-step qRT-PCR (Thermo Fisher Scientific), Master mix 3 - UltraPlexTM 1-StepToughMix (Quantabio).

Master mix 1		Master mix 2*		Master mix 3	
4x Mastermix 1	*5 µL*	2x Mastermix 1	*12.5 µL*	4× Mastermix 3	*5 µL*
10 µM BVDV-1_2F	*500 nM*	10 µM BVDV-1_2F	*500 nM*	10 µM BVDV-1_2F	*500 nM*
10 µM BVDV-1_2R	*500 nM*	10 µM BVDV-1_2R	*100 nM*	10 µM BVDV-1_2R	*500 nM*
10 µM MGBPro	*100 nM*	10 µM MGBPro	*100 nM*	10 µM MGBPro	*100 nM*
template	*5 µL*	template	*2.5 or 5 µL*	Template	*5 µL*
		RT-Taqman enzyme	*1*	-	-
RNase free water	*Up to 20 µL*	RNase free water	*Up to 25 µL*	RNase free water	*Up to 20 µL*
Hold (1 cycle)	50°C, 5 min		50°C, 15 min		50°C, 5 min
Hold (1 cycle)	95°C, 20 sec		95°C, 2 min		95°C, 20 sec
(40 cycles)					
Denature	95°C, 3 sec		95°C, 15 sec		95°C, 3 sec
Anneal	60°C, 30 sec		60°C, 30 sec		60°C, 30 sec

* 2.5 µL template was used compared with 5 µL template; normal volume in all other assay runs.

Table 3-4. Equine rhinitis A virus one step RT-qPCR reaction parameters and cycling conditions were modified from those described by Quinlivan et al. (2010). The PCR efficiency and analytical sensitivity were verified and shown in Figure 3-1 and Table 3-7. Master mix 1 - Taqman® Fastvirus 1-Step MasterMix (Thermo Fisher Scientific), Master mix 2 - SuperScript™ III Platinum™ One-step qRT-PCR (Thermo Fisher Scientific), Master mix 3 - UltraPlex™ 1-StepToughMix (Quantabio).

Master mix 1		Master mix 2		Master mix 2	
4× Master mix 1	*5 µL*	2x Master mix 2	*12.5 µL*	4× Master mix 3	*5 µL*
10 µM ERAV 468F	*625 nM*	10 µM ERAV 468F	*500 nM*	10 µM ERAV 468F	*625 nM*
10 µM ERAV569R	*625 nM*	10 µM ERAV569R	*500 nM*	10 µM ERAV569R	*625 nM*
10 µM ERAV MGBPro	*125 nM*	10 µM ERAV MGBPro	*100 nM*	10 µM ERAV MGBPro	*125 nM*
Template	*5 µL*	template	*5 µL*	template	*5 µL*
		RT-Taqman enzyme	*1*	-	-
RNase free water	*Up to 20 µL*	RNase free water	*Up to 25 µL*	RNase free water	*Up to 20 µL*
Hold (1 cycle)	50℃, 5 min		50℃, 15 min		50℃, 5 min
Hold (1 cycle)	95℃, 20 sec		95℃, 2 min		95℃, 20 sec
(40 cycle)					
Denature	95℃, 3 sec		95℃, 15 sec		95℃, 3 sec
Anneal	60℃, 30 sec		60℃, 30 sec		60℃, 30 sec

Table 3-5. Sample preparation protocols tested. PCR templates prepared using Protocol 1, 2 and 3 did not undergo RNA purification before RT-qPCR analysis. PCR templates prepared using Protocol 4 and the reference method (Protocol 5) comprised purified total nucleic acids and total RNA, respectively. Protocol 1 had several variations including three different chemistries and dilutions.

Protocols
1. Sample dilution (1:5, 1:10, and 1:20) with no nucleic acid extraction using:
Master mix 1. Taqman® Fastvirus 1-Step Master Mix*
Master mix 2. Superscript™ III Platinum™ One-step qRT-PCR*
Master mix 3. UltraPlex™ 1-StepToughMix**
2. iScript treatment (iScript™ RT-qPCR Sample Preparation Reagent) ***
Superscript™ III Platinum™ One-step qRT-PCR
3. Pre-heating of the sample prior to direct amplification
Superscript™ III Platinum™ One-step qRT-PCR
4. Bead-based manual extraction (Genesig Easy DNA/RNA Extraction kit) ****
Superscript™ III Platinum™ One-step qRT-PCR
5. RNA purification using QIAamp Viral RNA kit*****(reference method)
Superscript™ III Platinum™ One-step qRT-PCR

**Thermo Fisher Scientific; **Quantabio; ***BioRad; ****Genesig, Primer Design; *****Qiagen*

3.2.5.2. Protocol 2. iScript treatment

A commercial sample preparation reagent, iScript™ RT-qPCR Sample Preparation Reagent (BioRad), which lyses cell cultures so that the lysates can be used directly as PCR templates, was tested first using panel 4 (BVDV-1 spiked serum). Each of the five BVDV-1 standards (6.0×10^0 to 6.0×10^4 $TCID_{50}$/mL) was tested in two sets of pilot experiments. The standards were diluted 1:5, 1:10 and 1:20 (first experiment) or 1:4, 1:2, 3:4 (second experiment) with iScript buffer and incubated for 1 min at room temperature. Five and 2.5 microlitres of the iScript treated standards were then used as templates and tested in duplicate with the BVDV-1 RT-qPCR assay (Table 3-3) using master mix 2. The 2.5 µL template volume followed the commercial buffer recommendation of ≤ 10 % lysate in the total PCR reaction.

No further testing was done using other panels (1, 2 and 3) after unfavourable results were obtained from the pilot test using panel 4.

3.2.5.3. Protocol 3. Pre-heating of samples

Initial experiments to determine the effect of pre-heating samples prior to direct amplification were conducted using the BVDV-1 (panels 2 and 4) and ERAV (panel 3) spiked standards. Because of the low capacity of the T-COR (eight wells) to conduct experiments, three standards with low virus titres were selected from each panel. This was to demonstrate strategically the capability of the sample processing method to detect the virus using virus-specific PCR even at low concentrations. Three BVDV-1 standards (6.0×10^3, 6.0×10^2 and 6.0×10^1 $TCID_{50}$/mL) from panels 2 and 4 were diluted 1:5 in PBS in duplicate. Dilution of the standards was followed by a heating step to destabilise the capsid proteins and release viral RNA. The heating conditions were 1 min at 80°C followed by 2 min at 95°C and 1 min at 42°C. The duration of heat treatment was a modification to the method previously described of 95°C for 4 min and an ice bath for 3-5 min (Bachofen et al., 2013). The T-COR device only allowed 80°C as the initial high temperature setting instead of 95°C for rapid heating, and the 42°C was used to lower the temperature of standards.

A 20 µL aliquot of each diluted BVDV-1 standard was deposited in a T-COR-specific tube for heat treatment. An aliquot (5 µL) of the heated sample was added into the reaction mix (n = 2) containing master mix 2 and analysed with the BVDV-1 one step RT-qPCR (Table 3-3) on the T-COR. Separate aliquots of the diluted samples without heat treatment were also

tested using a 5 µL template in a separate run. Non-template control and BVDV-1 RNA positive control were included in each run.

Three ERAV standards from panel 3 (2.0×10^3, 2.0×10^2 and 2.0×10^1 $TCID_{50}$/mL) were processed as above and tested for ERAV using one step RT-qPCR with master mix 2 parameters and conditions (Table 3-4). Diluted ERAV serum samples without the heating step were also tested. NTC and positive control were included in each run.

To show repeatability of the pilot testing, a second round of experiments was also performed using freshly prepared panels 1, 2, 3 and 4 (section 3.2.3). For each diluted standard in this round, one portion was heat treated, another portion was tested directly without heating, and the last portion was used for RNA extraction using the QIAamp Viral RNA kit (Qiagen) as described in section 3.2.4. Heated, unheated and purified RNA samples were tested for BVDV-1 or ERAV using RT-qPCR master mix 2 parameters and conditions (Table 3-3 and Table 3-4).

3.2.5.4. Protocol 4. Paramagnetic bead-based method

Selected BVDV-1 standards from panel 4 (6.0×10^2 and 6.0×10^1 $TCID_{50}$/mL) and panel 2 (6.0×10^1 and 6.0×10^0 $TCID_{50}$/mL) were subjected to NA extraction using the Genesig Easy DNA/RNA Extraction kit (Genesig) according to the kit's insert. Selected ERAV concentrations from panel 3 (2.0×10^3, 2.0×10^2 and 2.0×10^1 $TCID_{50}$/mL) were also prepared in the same manner. Modifications were done to ensure that the amount of the starting material and elution volume was comparable to that used in reference Protocol 5. The standards from panel 1 were not subjected to bead-based protocol due to the limited availability of reagents in the trial kit.

Briefly, 140 µL of the sample was mixed with 60 µL of RFW and lysed for 15 min with 200 µL lysis buffer and 20 µL proteinase K at room temperature. A binding buffer (500 µL) containing magnetic beads was added to each tube, the content was vortexed for 15 seconds, and the tubes were incubated for 15 min at room temperature to allow the binding of released NA to the beads. Using a DynaMag™-2 Magnet (Thermo Fisher Scientific), the beads were separated from the solution, and the liquid was aspirated by pipetting. Washing was done twice with two different (500 µL) wash buffers. Each time the beads were re-suspended, mixed by manual shaking, and then separated from the solution using a magnetic rack before

removing the liquid. Beads were washed with 80% ethanol, then air dried for 10 min, followed by NA elution with 60 µL of the supplied elution buffer. The eluent was transferred to a fresh microfuge tube and used as a template for RT-qPCR. RNA was also extracted from the same set of samples using the same procedure but with a shorter lysis time of 5 min. Purified RNA was then tested in either BVDV-1 or ERAV one-step RT qPCR using master mix 2 (Table 3-3 and Table 3-4).

3.2.5.5. Protocol 5. Reference extraction method

A spin-column filter-based manual RNA extraction method, the QIAamp Viral RNA kit (Qiagen), was used as the reference method. Five replicates of each standard from two BVDV-1 and two ERAV panels (section 3.2.3) were purified according to the manufacturer's instruction (section 3.2.4). Purified RNA was then tested at neat, 1:5, or 1:10 dilution with BVDV-1 or ERAV one step RT-qPCR as described in Table 3-3 and Table 3-4 using master mix 2.

3.2.6. Data analysis

For both BVDV-1 and ERAV RT-qPCR, samples with a Cq value of ≤ 40 were considered positive using automatic cycle threshold determination by the BioRad CFX Manager 3.1 software. An RT-qPCR run was considered valid if the positive control generated the expected Cq value (22-23 ±3) and no signal was seen in the negative and NTC controls. The RT-qPCR results obtained using different sample preparation methods were compared to the PCR test results obtained with the reference extraction method. The comparison was based on analytical sensitivity using Cq values. Selection of the optimal sample preparation protocol for field use was based on the following criteria: detection sensitivity compared to the reference extraction method, level of complexity and length of the protocol. The Cq mean, standard deviation (SD) of the Cq mean, % coefficient of variance (CV) and 95% confidence interval (CI) of the summarised data were calculated using Microsoft® Excel® for Microsoft 365 MSO.

3.3. Results

3.3.1. RT-qPCR performance

The BVDV-1 and ERAV RT-qPCR amplification curves using three master mixes and purified RNA as a template were linear over a range of 10^{-1} to 10^{-7} dilutions of viral RNA. The R^2 for all standard curves ranged from 0.998 to 1.00. The mean efficiencies (Figure 3-1) obtained for the BVDV-1 RT-qPCR were 108.1% for master mix 1, 102.3% for master mix 2, and 106.8% for master mix 3. The mean efficiencies of the ERAV RT-qPCR were 102.7%, 99.0%, and 102.5 % for master mixes 1, 2 and 3, respectively. Analytical sensitivity of BVDV-1 and ERAV RT-qPCR were between 10^{-6} and 10^{-7} viral RNA dilution.

The analytical sensitivity of BVDV-1 and ERAV-specific RT-qPCR assays using an un-purified sample was determined by testing a 10-fold dilution series of either BVDV-1 or ERAV in PBS without prior RNA extraction. All replicates, except for the BVDV-1 RT-qPCR using Fastvirus mix (2/3), showed positive Cq value at the highest virus dilution tested at 6.0×10^{-1} $TCID_{50}$/mL for BVDV-1 (Table 3-6) and 2.0×10^{-1} $TCID_{50}$/mL for ERAV (Table 3-7). Non template controls produced negative results.

3.3.2. PCR template preparation

The four sample processing protocols (protocols 1-4, Table 3-8) considered for field PCR testing were assessed against a reference laboratory RNA extraction method (Protocol 5). The estimated time to process up to five samples using Protocols 1-3 was approximately 5 to 7 min. The bead-based method (Protocol 4) took 15-25 min to extract the total NA. Reference Protocol 5 required 30-min to extract RNA from five samples. Extra equipment was needed in Protocol 3 for the pre-heating step (T-COR) and in Protocol 4 to separate the beads (magnetic rack). Test controls used in all PCR runs generated the expected results.

3.3.3. Protocol 1. Sample dilution with no purification

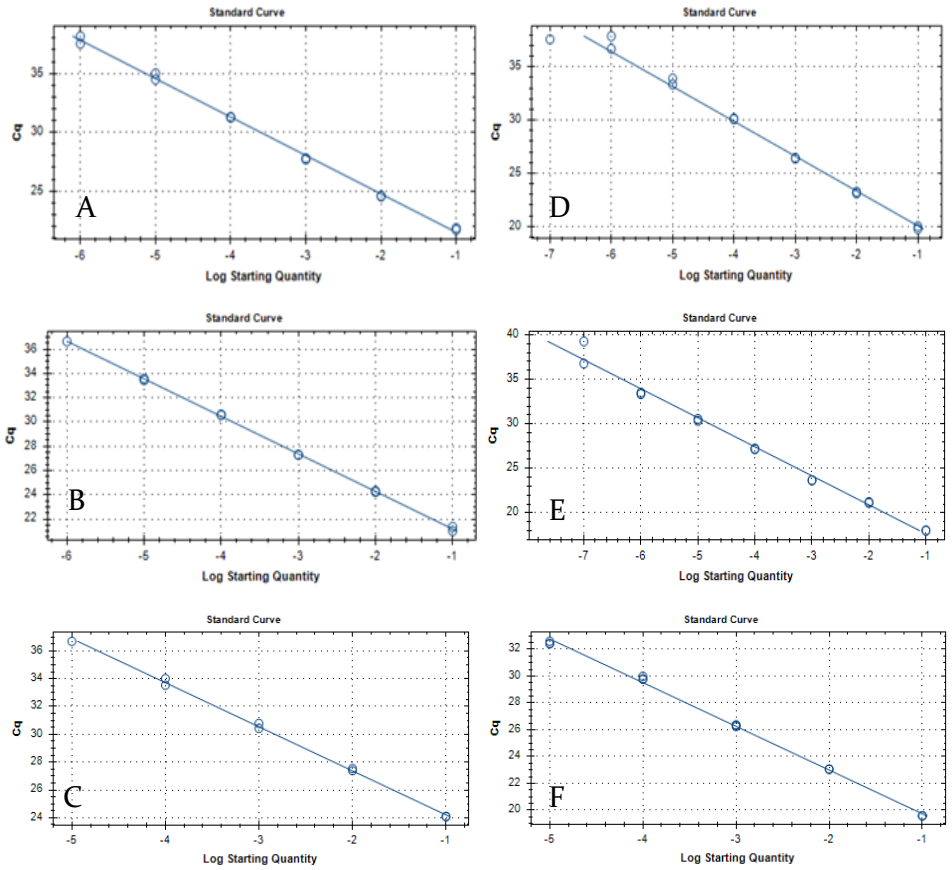
Detection of viral NA in diluted standards from each of the four panels is shown in Table 3-9, Table 3-10, Table 3-12, and Table 3-13. The detection limit was about 2 logs higher

for diluted standards used without NA purification compared to the detection of purified viral NA from the same standards.

BVDV-1 RNA was most consistently detected in standards from panels 2 (oral suspension) and 3 (serum) at 1:5 and 1:10 dilutions (Table 3-9 and Table 3-10). At those dilutions, standards with pre-dilution titres between 6.0×10^4 and 6.0×10^1 $TCID_{50}$/mL were positive for BVDV-1 using all three master mixes in some or all of the replicates. Standards containing higher levels of BVDV-1 (6.0×10^4 $TCID_{50}$/mL and 6.0×10^3 $TCID_{50}$/mL) tested positive in BVDV-1 RT-qPCR in all replicates using all three master mixes at all three dilutions tested. The detection of BVDV-1 in standards containing lower levels of BVDV-1 (6.0×10^2 $TCID_{50}$/mL and 6.0×10^1 $TCID_{50}$/mL) was more variable. The sensitivity of BVDV-1 detection seemed to be slightly lower for panel 4 (serum) than for panel 2 (oral swabs). Overall, the 1:5 sample dilution combined with UltraPlex 1-Step Toughmix produced the best results in terms of sensitivity. However, testing using Fastvirus 1-Step MasterMix had the lowest intra-assay variability, as indicated by the least Cq mean CV (oral swab = 1.63% and serum = 1.65%) across all standard dilutions. Initial BVDV-1 PCR testing of a neat serum test panel produced no reliable results (data not shown). The use of two template volumes (2.5 µL and 5 µL) of diluted (1:5 and 1:10) standards from panel 4 did not affect the results (Table 3-11).

Table 3-6. Analytical sensitivity of BVDV-1 RT-qPCR assay using serial dilution of virus in PBS without RNA extraction. The dilution series was tested in triplicate with 5 µL template using cycling parameters in Table 3-3. Reaction with quantification cycle value (Cq) of < 40 was considered deemed positive. The Cq value represents the mean of three replicates. SD = standard deviation, CV = coefficient of variance. All replicates showed positive Cq value at the highest virus dilution tested except for the PCR using Fastvirus mix (2/3). Very low levels of target DNA in the sample are governed by Poisson sampling variation, which leads to some replicates receiving less than one copy of target, therefore showing no amplification or Cq (Ruiz-Villalba et al., 2021).

BVDV-1	**Superscript III**				**Fastvirus mix**				**Toughmix**			
$TCID_{50}$/mL	*Cq*	SD	CV %	no. of positive*	*Cq*	SD	CV %	no. of positive*	*Cq*	SD	CV %	no. of positive*
6.0×10^5	22.85	0.871	3.81	3/3	24.75	0.720	2.91	3/3	23.02	0.107	0.46	3/3
6.0×10^4	23.90	0.099	0.41	3/3	26.83	0.142	0.53	3/3	26.58	0.119	0.45	3/3
6.0×10^3	27.15	0.708	2.61	3/3	29.57	0.052	0.18	3/3	29.84	0.100	0.34	3/3
6.0×10^2	31.5	0.116	0.37	3/3	32.39	0.297	0.92	3/3	32.76	0.164	0.50	3/3
6.0×10^1	33.88	0.292	0.86	3/3	34.89	0.566	1.62	3/3	35.28	0.165	0.47	3/3
6.0×10^0	35.41	0.187	0.53	3/3	35.42	0.409	1.15	3/3	36.04	0.310	0.86	3/3
6.0×10^{-1}	35.93	0.339	0.94	3/3	37.30	0.036	0.10	2/3	37.68	0.928	2.46	3/3
NTC	-	-	-	-/-	-	-	-	-/-	-	-	-	-/-



		Chemistry	Efficiency, %	R^2	Slope	y-intersect
BVDV-1 RT-qPCR	A	Superscript™ III Platinum™ one step	102.3	0.998	3.27	21.49
	B	Taqman® Fastvirus 1-Step Master Mix	108.1	1.000	3.14	21.17
	C	Ultraplex™ 1 step ToughMix	106.8	0.998	3.17	21.036
ERAV RT-qPCR	D	Superscript™ III Platinum™ one step	99.0	0.999	3.35	15.32
	E	Taqman® Fastvirus 1-Step Master Mix	102.7	0.990	3.26	14.393
	F	Ultraplex™ 1 step ToughMix	102.5	0.998	3.26	16.465

Figure 3-1. Standard curve analysis of the BVDV-1 (A, B, C) and ERAV (D, E, F) RT-qPCRs using three different master mixes. Representative standard curves were generated by the CFX96 Manager software. Viral RNA was serially diluted in RNAse free water over a range of 10^{-1} to 10^{-7} and 5 µL template was tested using virus specific RT-qPCR as described in Table 3-3 (BVDV-1) and Table 3-4 (ERAV).

Table 3-7. Analytical sensitivity of ERAV RT-qPCR using serial dilution of virus in PBS without RNA extraction. The dilution series was tested in triplicate with 5 µL template using cycling parameters in Table 3-4. Reaction with quantification cycle value (Cq) of < 40 was considered deemed positive. The Cq value represents the mean of three replicates. SD = standard deviation, CV = % coefficient of variance.

ERAV	Superscript III				Fastvirus mix				Toughmix			
$TCID_{50}$/mL	*Cq*	SD	CV	no. of positive*	*Cq*	SD	CV	no. of positive*	*Cq*	SD	CV	no. of positive*
2.0×10^5	24.62	0.312	1.27	3/3	25.63	0.387	1.51	3/3	24.78	0.143	0.58	3/3
2.0×10^4	25.23	0.090	0.36	3/3	27.84	0.157	0.56	3/3	27.45	1.45	5.28	3/3
2.0×10^3	27.26	0.066	0.24	3/3	30.35	0.206	0.68	3/3	31.82	0.335	1.05	3/3
2.0×10^2	29.87	0.187	0.63	3/3	31.51	0.191	0.61	3/3	33.46	0.489	1.46	3/3
2.0×10^1	31.79	0.401	1.26	3/3	32.25	0.057	0.18	3/3	34.44	0.661	1.92	3/3
2.0×10^0	34.39	0.440	1.28	3/3	33.83	0.273	0.81	3/3	35.46	0.653	1.84	3/3
2.0×10^{-1}	35.21	0.049	0.14	3/3	35.23	0.595	1.69	3/3	37.55	0.970	2.58	3/3
NTC	-	-	-	-/-	-	-	-	-/-	-	-	'-	-/-

Table 3-8. Summary of assessed sample processing methods for field RT-qPCR testing.

	Protocol summary	Reagents & consumables needed	Equipment needed	No. of steps	Estimated processing time, 1-5 samples
1	Sample dilution with no RNA extraction	nuclease-free water or PBS; 1.5 mL microfuge tube	microfuge tube rack; pipettes (2-20 µL and 20-200 µL)	4	<5 min
2	iScript treatment	iScript™ RT-qPCR Sample Preparation Reagent*; nuclease-free water or PBS; 1.5 mL microfuge tube	microfuge tube rack; pipettes (2-20 µL and 20-200 µL)	5	<5 min
3	Sample dilution and pre-heat treatment	nuclease free water or PBS; 1.5 mL microfuge tube	TCOR™-8 thermocycler; microfuge tube rack; pipettes (2-20 µL and 20-200 µL)	5	7 min
4	Paramagnetic bead-based extraction	Genesig Easy DNA/RNA Extraction kit	microfuge tube rack; pipettes (2-20 µL, 20-200 µL, 100-1000 µL). magnetic device	33	15-25 min
5	Reference method	QIAamp Viral RNA kit	microcentrifuge; microfuge tube rack; pipettes (2-20 µL, 20-200 µL, 100-1000 µL)	34	30 min

*iScript™ RT-qPCR Sample Preparation Reagent (BioRad) is stable at room temperature for a short period but should be stored at 4°C.

Table 3-9. Results of RT-qPCR detection of bovine viral diarrhoea disease virus type 1 (BVDV-1) in serum using three master mixes. The templates (n=10 per dilution) were prepared either by dilution of the sample in water or by dilution of extracted RNA (Qiagen Viral RNA kit, positive control). Negative serum and non-template controls were negative in all runs. $TCID_{50}$/mL – tissue culture infective dose 50 per millilitre, Cq – quantification cycle, SD – standard deviation, CV – % coefficient of variance, CI – confidence interval, n/a – not applicable.

BVDV-1 titre in serum ($TCID_{50}$/mL)	**QIAamp Viral RNA Mini Kit (Qiagen)**				**TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific)**				**Superscript® III One-Step RT-PCR System (Thermo Fisher Scientific)**				**UltraPlex 1-Step Toughmix (Quantabio)**			
sample dilution in water	# of pos	*Cq* mean	*SD*	CV	# of pos	*Cq* mean	*SD*	CV	# of pos	*Cq* mean	*SD*	CV	# of pos	*Cq* mean	*SD*	CV
6.0×10^4																
Neat	8	24.53	0.24	0.99												
1:5	10	25.82	0.18	0.93	10	32.41	0.50	1.56	10	32.95	0.56	1.70	10	29.85	1.43	4.81
1:10	10	26.80	0.24	1.15	10	32.39	0.47	1.45	10	32.56	0.59	1.81	10	30.46	1.75	5.75
1:20					10	32.35	0.54	1.68	10	32.70	0.75	2.30	10	30.93	1.88	6.06
6.0×10^3																
Neat	10	27.71	0.15	0.55												
1:5	10	29.13	0.25	0.83	10	36.17	1.09	3.01	10	35.58	0.77	2.17	10	32.44	1.71	5.27
1:10	10	30.07	0.30	0.92	10	35.36	0.46	1.29	10	35.42	0.81	2.30	10	32.87	1.45	4.41
1:20					10	35.63	0.58	1.62	10	36.38	1.46	4.00	10	33.62	1.66	4.95
6.0×10^2																
Neat	8	31.11	0.21	0.68												
1:5	10	32.74	0.41	1.32	5	37.53	0.70	1.86	7	37.94	0.95	2.49	10	36.32	1.39	3.83
1:10	10	33.70	0.46	1.31	3	38.29	0.29	0.76	5	37.05	0.38	1.03	10	36.00	1.74	4.83
1:20					0				4	37.03	1.47	3.98	8	36.83	1.40	3.81
6.0×10^1																
Neat	7	34.32	0.23	0.66												
1:5	10	36.10	1.22	3.21	1	38.78	n/a	n/a	1	38.89	n/a	n/a	3	37.45	2.43	6.48
1:10	10	36.64	0.56	1.76	1	38.40	n/a	n/a	1	38.19	n/a	n/a	2	38.79	1.57	4.05
1:20					0				0				0			
6.0×10^0																
Neat	10	37.34	0.43	1.08												
1:5	9	38.78	0.53	1.64	0				0				0			
1:10	6	39.49	0.48	1.12	0				0				1	35.62	n/a	n/a
1:20					0				0				1	36.71	n/a	n/a
6.0×10^{-1}																
Neat	2	39.75	0.35	0.11												
1:5	2	39.29	0.04													
mean % CV				1.12				1.65				2.42				4.93
95% CI				0.75-1.49				1.12-2.18				1.66-3.18				4.33-5.53

Table 3-10. Results of RT-qPCR detection of bovine viral diarrhoea disease virus type 1 (BVDV-1) in oral swab using three master mixes. The templates (n=10 per dilution) were prepared either by dilution of the sample in water or by dilution of extracted RNA (Qiagen Viral RNA kit, positive control). Negative serum and non-template controls were negative in all runs. $TCID_{50}$/mL – tissue culture infective dose 50 per millilitre, Cq – quantification cycle, SD – standard deviation, CV – % coefficient of variance, CI – confidence interval, n/a – not applicable.

BVDV-1 titre in oral swab ($TCID_{50}$/mL)	**QIAamp Viral RNA Mini Kit (Qiagen)**				**TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific)**				**Superscript® III One-Step RT-PCR System (Thermo Fisher Scientific)**				**UltraPlex 1-Step Toughmix (Quantabio)**			
sample dilution in water	# of pos	*Cq* mean	*SD*	CV	# of pos	*Cq* mean	*SD*	CV	# of pos	*Cq* mean	*SD*	CV	# of pos	*Cq* mean	*SD*	CV
6.0×10^4																
Neat	10	24.17	0.16	0.67												
1:5	10	26.61	0.18	0.69	10	29.41	0.11	0.36	10	29.78	0.33	1.09	10	27.29	0.39	1.41
1:10	10	27.62	0.09	0.32	10	30.55	0.19	0.62	10	30.19	0.25	0.84	10	28.24	0.41	1.46
1:20					10	31.72	0.38	1.19	10	30.52	0.25	0.80	10	29.36	0.36	1.21
6.0×10^3																
Neat	10	27.65	0.16	0.59												
1:5	10	30.07	0.08	0.28	10	32.06	0.23	0.72	10	32.84	0.25	0.77	10	30.26	0.39	1.28
1:10	10	31.08	0.15	0.47	10	33.32	0.41	1.24	10	33.46	0.35	1.06	10	31.44	0.44	1.40
1:20					10	34.46	0.44	1.27	10	33.83	0.22	0.65	10	32.74	0.46	1.40
6.0×10^2																
Neat	10	31.03	0.13	0.41												
1:5	10	33.26	0.10	0.29	10	35.17	0.80	2.29	10	35.76	0.90	2.52	10	34.61	1.69	4.87
1:10	10	34.37	0.16	0.46	10	37.06	0.97	2.62	10	35.87	0.45	1.26	7	31.80	5.13	16.13
1:20					8	37.70	1.08	2.87	10	36.78	0.92	2.51	6	28.23	4.26	15.11
6.0×10^1																
Neat	10	34.37	0.25	0.73												
1:5	10	36.88	0.42	1.15	2	37.38	0.65	1.74	4	38.65	1.92	4.96	5	26.99	2.34	7.17
1:10	10	37.96	0.55	1.45	2	38.56	0.61	1.59	4	38.07	0.45	1.19	1	27.26	n/a	n/a
1:20					2	39.27	1.19	3.03	7	38.77	0.72	1.85	1	27.76	n/a	n/a
6.0×10^0																
Neat	10	37.73	0.68	1.81												
1:5	7	39.46	0.87	2.21	1	38.99	n/a	n/a	0				0			
1:10	7	39.93	0.33	0.83	0				0				2	33.48	0.60	1.80
1:20					0				0				0			
6.0×10^{-1}																
Neat	2	38.84	0.10	0.26												
1:5	0															
mean % CV				0.79				1.63				1.63				4.84
95% CI				0.48-1.10				1.06-2.20				0.85-2.41				0.90-8.78

Table 3-11. RT-qPCR analysis of BVDV-1 serum panel indicating that template volume (2.5 vs 5 µL) seemed had no influence in the detection sensitivity of viral RNA when samples without prior RNA purification were used as templates. Samples were tested in duplicate.

Virus conc, TCID50/mL	**RT-qPCR, *Cq***			
BVDV-1	2.5µL template		5.0 µL template	
	1:5 dilution	1:10 dilution	1:5 dilution	1:10 dilution
6.0×10^3	34.69 / 34.74	35.50 / 36.52	35.57 / 35.04	35.54 / 34.82
6.0×10^2	38.31 / 37.65	37.69 / 36.26	36.26 /37.28	0 /38.01
6.0×10^1	0 / 38.09	0	0	0

ERAV RNA detected using diluted standards from panels 1 (oral swabs) and 3 (serum) as templates in RT-qPCR was similar to that described above for BVDV-1 using panels 2 and 4. Viral RNA was detected in all 10 replicates when serum (Table 3-12) or oral swab (Table 3-13) standards with titres of 2.0×10^4 $TCID_{50}$/mL and 2.0×10^3 $TCID_{50}$/mL were used as templates at all three dilutions tested with all three master mixes. The detection of ERAV in a 2.0×10^2 $TCID_{50}$/mL standard was variable, with only some replicates testing positive for ERAV RNA. As with the BVDV-1 virus, the use of 1:5 dilution of the sample combined with UltraPlex 1-Step Toughmix master mix produced the most consistent positive results with the lowest Cq (Table 3-12 and Table 3-13) for both panels 1 and 3. The Fastvirus 1-Step MasterMix however had the lowest intra-assay variability among the master mixes used as shown by the lowest Cq mean CV (oral swab = 1.20% and serum = 2.89%) in all virus dilutions.

Across all reactions, a considerable shift of Cq to higher values was apparent when crude samples were used compared to purified RNA prepared from the same sample.

3.3.4. Protocol 2. iScript treatment

A commercially prepared sample preparation buffer designed for lysing cell cultures and the direct use of the lysates in RT-qPCR was assessed with standards from panel 4. Pilot RT-qPCR testing for BVDV-1 RNA of virus-spiked serum samples diluted with various concentrations of iScript reagent (BioRad) generated no Cq values for any of the samples (results not shown).

Table 3-12. Results of RT-qPCR detection of equine rhinitis A virus (ERAV) in serum using three master mixes. The templates were prepared either by dilution of the sample in water or by dilution of extracted RNA (Qiagen Viral RNA kit, positive control). Negative serum and non-template controls were negative in all runs. $TCID_{50}$/mL – tissue culture infective dose 50 per millilitre, Cq – quantification cycle, SD – standard deviation, CV – % coefficient of variance, CI – confidence interval, n/a – not applicable.

ERAV titre in serum ($TCID_{50}$/mL)	**QIAamp Viral RNA Mini Kit (Qiagen)**				**TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific)**				**Superscript® III One-Step RT-PCR System (Thermo Fisher Scientific)**				**UltraPlex 1-Step Toughmix (Quantabio)**			
sample dilution in water	# of pos	*Cq* mean	*SD*	CV	# of pos	*Cq* mean	*SD*	CV	# of pos	*Cq* mean	*SD*	CV	# of pos	*Cq* mean	*SD*	CV
2.0×10^4																
Neat	10	21.11	0.11	0.50												
1:5					10	32.36	0.48	1.48	10	32.9	1.33	4.03	10	27.21	1.13	4.15
1:10	10	24.66	0.21	0.85	10	32.98	0.30	0.90	10	33.05	0.79	2.39	10	27.46	1.31	4.77
1:20					10	33.38	0.17	0.52	10	33.02	0.78	2.36	10	28.58	1.41	4.94
2.0×10^3																
Neat	10	24.46	0.19	0.77												
1:5					10	35.13	0.47	1.33	10	35.16	1.16	3.30	10	30.46	1.48	4.85
1:10	10	28.03	0.24	0.87	10	36.09	0.51	1.42	10	36.51	1.31	3.58	10	31.78	1.08	3.40
1:20					10	36.52	1.11	3.03	10	36.61	1.30	3.56	10	31.18	1.59	5.09
2.0×10^2																
Neat	10	27.35	0.23	0.83												
1:5					4	37.46	0.58	1.55	3	38.17	0.68	1.77	7	32.67	1.54	4.72
1:10	10	31.09	0.26	0.82	3	37.76	0.02	0.04	5	38.31	0.57	1.49	6	33.49	1.25	3.75
1:20					5	37.56	0.61	1.62	2	38.32	0.14	0.35	4	32.33	1.45	4.47
2.0×10^1																
Neat	10	29.83	0.21	0.70												
1:5					0				1	38.49	n/a	n/a	1	33.00	n/a	n/a
1:10	10	33.43	0.40	1.19	2	38.08	0.04	0.11	1	39.08	n/a	n/a	0			
1:20					0				0				1	31.7	n/a	n/a
2.0×10^0																
Neat	10	31.85	0.24	0.74												
1:5					1	38.16	n/a	n/a	0				0			
1:10	10	35.46	0.49	1.37	0				0				0			
1:20					0				0				1	34.45	n/a	n/a
2.0×10^{-1}																
Neat	10	33.88	0.33	0.97												
1:5																
1:10	10	37.33	0.46	1.23												
mean CV				0.90				1.20				2.54				4.46
95% CI				0.74-1.06				0.57-1.83				1.62-3.46				4.01-4.91

Table 3-13. Results of RT-qPCR detection of equine rhinitis A virus (ERAV) in oral swab using three master mixes. The templates were prepared either by dilution of the sample in water or by dilution of extracted RNA (Qiagen Viral RNA kit, positive control). Negative serum and non-template controls did not generate amplification curve in all runs. $TCID_{50}$/mL – tissue culture infective dose 50 per millilitre, Cq – quantification cycle, SD – standard deviation, CV – % coefficient of variance, CI – confidence interval, n/a – not applicable.

ERAV titre in oral swab ($TCID_{50}$/mL)	**QIAamp Viral RNA Mini Kit (Qiagen)**				**TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific)**				**Superscript® III One-Step RT-PCR System (Thermo Fisher Scientific)**				**UltraPlex 1-Step Toughmix (Quantabio)**			
sample dilution in water	# of pos	*Cq* mean	*SD*	CV	# of pos	*Cq* mean	*SD*	CV	# of pos	*Cq* mean	*SD*	CV	# of pos	*Cq* mean	*SD*	CV
2.0×10^4																
Neat	10	20.58	0.24	1.17												
1:05					10	29.98	1.19	3.96	10	30.71	1.29	4.20	10	27.60	1.15	4.17
1:10	10	24.00	0.27	1.11	10	31.30	1.14	3.64	10	31.10	1.72	5.53	10	28.97	1.05	3.63
1:20					10	32.09	1.10	3.42	10	32.17	1.21	3.77	10	29.57	1.12	3.78
2.0×10^3																
Neat	10	24.05	0.11	0.45												
1:05					10	33.88	0.80	2.37	10	34.28	1.43	4.18	10	29.82	2.37	7.96
1:10	10	27.35	0.14	0.50	10	34.47	0.70	2.03	10	35.16	1.35	3.83	10	32.35	1.83	5.67
1:20					10	35.42	1.24	3.50	10	36.03	1.30	3.61	10	32.50	1.93	5.93
2.0×10^2																
Neat	10	31.71	0.22	0.68												
1:05					6	36.71	1.44	3.92	10	37.08	0.84	2.26	3	33.20	0.71	2.13
1:10	10	35.46	0.41	1.16	3	36.79	0.92	2.50	7	37.69	1.30	3.46	3	33.06	1.26	3.81
1:20					7	37.12	1.31	3.53	7	38.00	2.83	7.46	0			
2.0×10^1																
Neat	10	33.11	0.14	0.43												
1:05					0				4	38.75	1.12	2.88	2	34.46	1.46	4.23
1:10	10	36.57	0.59	1.61	2	36.80	0.02	0.04	2	39.64	0.28	0.70	0			
1:20					1	36.62	n/a	n/a	3	39.14	0.78	1.98	0			
2.0×10^0																
Neat	10	27.15	0.16	0.61												
1:05					0				1	39.15	n/a	n/a	0			
1:10	10	30.77	0.15	0.49	0				1	38.28	n/a	n/a	0			
1:20					0				0				0			
2.0×10^{-1}																
Neat	10	29.77	0.14	0.48												
1:05																
1:10	10	33.29	0.24	0.73												
mean CV				0.79				2.89				3.65				4.59
95% CI				0.55-1.03				2.03-3.75				2.56-4.74				3.29-5.89

3.3.5. Protocol 3. Pre-heating of samples

In a pilot experiment, the mean Cq for duplicates of selected standards (6.0×10^3, 6.0×10^2 and 6.0×10^1 $TCID_{50}$/mL) from panel 4 (serum) were lower (29.30, 32.30, 35.60) compared to the mean Cq for duplicates of the same standards without heat treatment (35.20, 37.75, 37.10). The mean Cq for selected (6.0×10^3 and 6.0×10^2 $TCID_{50}$/mL) standards from panel 2 (oral swabs) that were heat treated were similar (34.70 and 37.20) to the mean Cq obtained with the un-heated standards (34.05 and 36.00). Results from the pre-heated ERAV serum standard experiment were inconsistent (results not shown).

A repeat of the protocol 3 (section 3.3.5) experiment further confirmed that pre-heating of the virus-spiked sera (panels 3 and 4) improved the sensitivity of detection by RT-qPCR for both BVDV-1 and ERAV (Table 3-14). This was particularly obvious for pre-heated BVDV-1-spiked sera, which produced Cq values comparable to those obtained with the purified RNA (Table 3-14).

Heat treatment of the virus-spiked oral swabs (panels 1 and 2) provided no apparent advantage, as the Cq values obtained with heat-treated samples were similar to the Cq values obtained with the untreated samples for both BVDV-1 and ERAV (Table 3-14).

3.3.6. Protocol 4. Bead-based extraction method

BVDV-1 and ERAV RNA from a paramagnetic bead-based extraction protocol (Genesig DNA/RNA kit) were amplified from all selected standards from panel 3 and 4 (sera). BVDV-1 RNA was also amplified from selected standards from panel 2 (oral swabs) (Table 3-15) with a Cq range between 31.45 and 38.17 (6.0×10^0 and 6.0×10^1 TCID50/mL virus titre).

In a separate experiment, a reduction of lysis time from 15 min to 5 min using panel 4 (BVDV-1 spiked sera) did not affect the Cq values and overall detection limit of the target prepared using the bead-based extraction method (Table 3-16).

Table 3-14. Quantification cycle (Cq) values obtained using selected standards from panels 2 and 4 (BVDV-1) and from panels 1 and 3 (ERAV), with or without heat treatment. RT-qPCR runs were conducted in the T-COR 8™ real time PCR machine using Table 3-3 (BVDV-1) and Table 3-4 (ERAV) conditions. Positive control comprised RNA purified with QIAamp Viral RNA kit (Protocol 5). RNA was diluted 1:5 in water before using 5 µL as a template. NTC = non-template control.

Virus titre, $TCID_{50}$/mL	**Panel 3 and 4 (serum)**			**Panel 1 and 2 (oral swabs)**		
	Heat treated, *Cq (mean)*	No heat treatment, *Cq (mean)*	Extracted RNA Cq *(mean)*	Heat treated, *Cq (mean)*	No heat treatment, *Cq (mean)*	Extracted RNA, Cq *(mean)*
BVDV-1						
6.0×10^3	28.1 / 28.4 (28.25)	33.8 / 34.5 (34.15)	29.0 / 28.7 (28.85)	31.3 / 31.1 (31.2)	30.8 / 31.1 (30.95)	29.5 / 28.9 (29.2)
6.0×10^2	31.1 /31.6 (31.35)	36.4 / 36.9 (36.65)	31.4 / 31.2 (31.3)	34.1 / 34.8 (34.45)	33.4 / 30.2 (31.8)	32.2 / 32.5 (32.35)
6.0×10^1	33.6 / 33.1 (33.35)	37.7 / 0	33.5 / 33.9 (33.7)	35.4 / 35.5 (35.45)	35.1 / 34.9 (35.0)	34.7 / 34.2 (34.45)
Pos control	30.5	30.8	31.3	31.5	31.3	31.3
NTC	0	0	0	0	0	0
ERAV						
2.0×10^3	31.2 / 32.0 (31.6)	38.1 / 0	26.9 / 27.0 (26.95)	34.8 / 34.3 (34.55)	35.9 / 35.5 (35.7)	26.5 / 26.9 (26.7)
2.0×10^2	34.0 / 33.4 (33.7)	0/ 0	29.2 / 29.1 (29.15)	0 / 36.4	36.2 / 36.4 (36.3)	28.9 / 29.6 (29.25)
2.0×10^1	34.1 / 34.7 (34.4)	0 / 0	29.6 / 29.7 (29.65)	0 / 0	0 / 36.3	30.4 / 30.4 (30.4)
Pos control	25.46	26.1	25.4	25.4	29.5	25.5
NTC	0	0	0	0	0	0

3.3.7. Protocol 5. Reference extraction method

All standards from panels 1 and 3 (spiked with ERAV) and 2 and 4 (spiked with BVDV) were positive for the respective virus in RT-qPCR when purified RNA was tested neat and at 1:10 dilution (Table 3-9, Table 3-10, Table 3-12, and Table 3-13).

*Table 3-15. Mean Cq values (two runs) obtained following amplification of RNA extracted using a bead-based protocol (Genesig Easy DNA/RNA extraction kit) as compared with RNA extracted using QIAamp Viral RNA kit (control). Positive RNA control had the expected Cq value and none in the non-template control. Cq – quantification cycle, SD – standard deviation, CV – % coefficient of variance. *Extraction with QIAamp Viral RNA Mini Kit (Qiagen). ND = not done.*

Virus titre, $TCID_{50}$/mL	Panels 3 and 4 (serum)						Panels 1 and 2 (oral swabs)					
	Genesig Easy DNA/RNA extraction			Extracted RNA* control			Genesig Easy DNA/RNA extraction			Extracted RNA* control		
	mean Cq	SD	% CV	mean Cq	SD	% CV	mean Cq	SD	% CV	mean Cq	SD	% CV
BVDV-1												
6.0×10^1	34.23	0.01	0.04	31.09	0.16	0.52	31.45	0.06	0.20	34.37	0.12	0.36
6.0×10^0	37.62	0.77	2.05	34.32	0.05	0.14	38.17	0.4	1.06	37.73	0.02	0.05
ERAV												
2.0×10^3	27.37	0.02	0.08	24.05	0.09	0.39	ND					
2.0×10^2	29.72	0.05	0.17	31.71	0.01	0.05						
2.0×10^1	31.25	0.05	0.16	33.11	0.12	0.36						

*Table 3-16. Comparison of RT-qPCR Cq values of samples lysed in two different time (15 and 5 min) in the Genesig DNA/RNA Extraction kit. Cq mean were quantification cycle values of three replicates. Cq – quantification cycle, SD – standard deviation, CV – % coefficient of variance. *Extraction with QIAamp Viral RNA Mini Kit (Qiagen).*

BVDV-1 concentration, $TCID_{50}$/mL	15 min lysis			5 min lysis			Extracted RNA*		
	mean Cq	SD	% CV	mean Cq	SD	% CV	mean Cq	SD	% CV
6.0×10^2	34.23	0.218	0.64	34.6	0.434	1.25	32.74	0.167	0.51
6.0×10^1	37.51	0.960	2.56	38.08	1.06	2.79	36.1 0	0.595	1.65

3.4. Discussion

Nucleic acid extraction is an established step for the sensitive detection of viral targets in PCR assays. Although regarded as routine in molecular laboratories, such procedures are challenging to perform in field settings. One of the limitations is the sample preparation step. This typically involves extraction of NA, which takes time and provides many opportunities for cross-contamination between samples when performed outside a controlled laboratory environment. In this study, the suitability of three extraction-less template preparation protocols and a bead-based extraction method were assessed for their suitability for field PCR testing using 4 test panels, each comprising various dilutions of either BVDV-1 or ERAV spiked into serum or oral swab samples and compared using virus-specific RT-qPCR assays. Performance evaluation was based on the observed detection limit, compared to a reference extraction method as judged by Cq values.

The positive threshold for the BVDV-1 and ERAV PCRs was selected at ≤40 Cq to increase the sensitivity of the assays when applied to unextracted samples. Cq is the cycle number when the exponential phase of a PCR amplification curve crosses a fluorescence threshold (Caraguel et al., 2011). This value is inversely related to the starting target concentrations, such that the higher the initial concentration of the target in the sample, the lower the Cq and vice versa (Bustin et al., 2009). Optimally, a Cq ≤35 corresponds to ten copies or more of the target in the sample and is generally used to classify the samples as positive for the target of interest (Ruiz-Villalba et al., 2021). However, the fundamental property of Poisson distribution governs the high variability of Cq value in replicates with low concentrations (<10 copies). As a result, in a sample containing 1-4 copies, about 36% to 2% of the reactions will not contain the target and, thus, the sample could be misclassified as false negative (Ruiz-Villalba et al., 2021). In this study, highly diluted viral RNA (10^{-9}) of BVDV-1 and ERAV produced late Cq values (around 40) in only one of two replicates (data not shown) in the virus-specific RT-PCR. A similar study by Goris et al. (2009) also demonstrated the uncertainties in detecting FMDV (SAT 1 ZIM 25/89) in highly diluted virus-spiked blood sample (i.e., dilution 10^{-6}). For instance, even if the FMDV-spiked sample had high Cq (> 40), the probability of re-testing positive using two FMDV RT-qPCRs was at least 95% (Goris et al., 2009). Willoughby et al. (2006) and Quinlivan et al. (2010) also did not report any artefactual signals due to primer dimers in later cycles of the two assays (BVDV-1 and ERAV) when used for testing. Since Poisson variation requires high number of replicates (seven) with no Cq value to prove that a sample is genuinely negative (99% confidence level), therefore, a Cq ≤40 was considered positive (Ruiz-Villalba et al., 2021) all throughout experiments described in this thesis.

Overall, the two preparations by simple dilution without extraction (protocol 1 and 3) were selected for developing the multiplex pen-side PCR test. Heating (protocol 3) or no heating (protocol 1) of the template prior to testing enabled the detection of BVDV-1 and ERAV genomes at a level comparable to that using a bead-based method (protocol 4). Although the analytical sensitivities of protocols 1 and 3 were 1-2 log lower than the sensitivity of the virus-specific PCRs with purified RNA used as a template (protocol 5), both protocols (1 and 3) had 28 fewer steps than RNA purification methods (Protocol 4 and 5), making such simple methods palatable for the adaptation to the pen-side PCR format. Interestingly, the effect of heat-treatment on the analytical sensitivity was not consistent between assays. Heating of serum template enhanced BVDV-1 detection, while only had minimal effect in

improving the detection ERAV. These observations might affect the multiplex assay operational performance and are evaluated further in succeeding chapters (Chapter 5, 6 and 7).

Viral RNA could be amplified from samples without prior extraction steps. Neat unextracted samples contain PCR inhibitors (e.g., nucleases, proteins, polysaccharides, and heme pigments) that interfere with a PCR reaction (Schrader et al., 2012). A simple dilution step tends to minimise such effects, allowing the amplification of viral NA from crude samples. Although attempts to amplify BVDV-1 in undiluted mock-infected serum failed, probably due to the presence of PCR inhibitors, both BVDV-1 and ERAV 5' RNA was detected when diluted mock-infected serum samples were used as templates with all dilution ratios and PCR master mixes tested. Others reported the amplification of BVDV-1 RNA directly from crude cell lysates (Gilbert et al., 1999) and diluted blood or serum (Deregt et al., 2002). Similarly, influenza virus and New Castle disease virus (NDV) were detected by virus-specific RT-qPCR when diluted influenza virus-spiked saliva (Song et al., 2009), diluted NDV cell culture supernatant or diluted allantoic fluid (Wambura, 2006) were used as templates without prior NA extraction. More recently, sample dilution of serum with nuclease-free water (1:10) helped the detection of FMDV in a field-based RT-qPCR, with 26/28 positive samples (Howson et al., 2017), supporting the potential use of extraction-free template preparation for PCR field testing.

BVDV-1 and ERAV 5' RNA was detected in diluted and un-extracted mock-infected samples with viral titres as low as 6.0×10^2 and 2.0×10^2 $TCID_{50}$/mL, respectively. Such a level of sensitivity may be acceptable to confirm the presence of the virus in clinical samples from diseased animals where the virus is expected to be abundant especially during the acute phase of infection. The estimated viral load of a viremic 70-day old PI calf was $10^{4.7}$ to $10^{6.3}$ $TCID_{50}$/mL (Brock et al., 1998) and a mean of $10^{4.5}$ $TCID_{50}$/mL for a 7-year-old PI cow (Bedeković et al., 2012). At this viral load level in PI cows, the detection of BVDV-1 can be enabled by the three sample preparation protocols (1, 3 and 4), as shown in our data. In parallel, remarkably high FMDV RNA levels in serum ($10^{4.5}$ to $10^{7.5}$ copies/mL) and in oral swabs (10^5 to $10^{5.7}$ copies/mL) were reported 1-5 days after the development of lesions in experimentally inoculated cattle (Arzt et al., 2010). Howson et al. (2017) demonstrated the detection of FMDV from 1 to 2-day old FMD lesions using diluted samples as templates in a "pen-side" RT-qPCR test (Tetracore).

Heating of diluted virus-spiked sera for direct use as templates in RT-qPCR improved the sensitivity of detection of both BVDV-1 and ERAV. The addition of the heating step may have destabilised the integrity of the viral capsids (Hewitt et al., 2009), making viral RNA available for the RT-qPCR assay, even in the presence of ribonuclease activity (Steward & Culley, 2010). In addition, heating may have helped to denature nucleases and other protein PCR inhibitors, especially in sera. Heat-treated sera and plasma from viraemic calves were successfully used as templates in high throughput testing for BVDV-1 by RT-qPCR (Bachofen et al., 2013). In that study, heating of diluted serum (1:5 in PBS) for four min at 95°C allowed the detection of BVDV-1 RNA in 84/86 positive sera by RT-PCR. The 86 sera were previously positive with a commercial antigen capture enzyme linked immunosorbent assay.

The effect of heating on the ability to detect viral RNA appeared to be sample-dependent. Heating provided no benefit for detecting viral RNA in virus-spiked oral swabs, in contrast to virus-spiked serum samples. Variable response to heat treatment suggests that heating may be more helpful for the preparation of complex sample matrices in comparison with less complex samples. Serum proteins, such as immunoglobulins and hemoglobulin, could function as a protective shield against viral RNA during heating. In contrast, heating simpler matrices, such as cell culture supernatant or oral swab suspension, could at once affect the viral RNA integrity (Pastorino et al., 2005). In fact, some authors reported poorer detection of viral RNA in heated versus non-heated samples. For example, Pastorino et al. (2005) reported that the sensitivity of Chikungunya virus detection was higher when non-heated infected cell culture lysate was used as a template than when it was heated. Zou et al. (2020) supported such premise that even a short 30 min water bath treatment at 56°C decreased the PCR detection rate of SARS-CoV-2 in throat swab samples, particularly those with low viral load. Thus, it can be postulated that the effect of heating crude clinical samples on the sensitivity of viral detection by PCR varies depending on the sample type and possibly the thermotolerance of the virus involved.

The observed sensitivity of virus-specific RT-qPCR with a template prepared by dilution of mock samples with or without heating was 1-2 logs lower than with the RNA template (Protocol 5). This reduction in sensitivity can be attributed to two possible reasons. Firstly, inhibitors could still be present in the samples following dilution and heating, which may have influenced the efficiency of PCR reactions. Second, the 5 µL template of the diluted crude sample (1:5 and 1:10) contained less target viral RNA than the template prepared from the same undiluted standard using the reference viral extraction method. In the latter, RNA

was extracted from 140 µL of the sample and eluted in 60 µL buffer. Thus, a 5 µL purified RNA template was more concentrated than the crude template.

Although commercial sample preparation buffers have been used successfully to produce PCR-ready cell lysate without extraction (Ho et al., 2013; Shatzkes et al., 2014; Van Peer et al., 2012), experiments conducted in this work to apply iScript (BioRad) cell lysis reagent in virus-spiked serum to detect BVDV-1 were unsuccessful. One possible reason is that the iScript reagent was designed to work only in washed cell culture pellets devoid of inhibitors present in serum. The undisclosed composition of this commercial reagent due to proprietary reasons also hindered designing more appropriate experiments (Shatzkes et al., 2014).

The sensitivity and simplicity of using diluted sera or oral swabs, with or without heat treatment, as templates in virus specific RT-qPCRs, suggest that such approaches can be used for field PCR testing. Based on the data presented in this chapter, sample dilution with or without heating was selected for integration into the multiplex FMDV/BVDV RT-qPCR assay and for further validation. The main limitation of this study is that clinical samples were mock-infected with a surrogate virus (ERAV) rather than with FMDV. Hence the suitability of the described protocols for detecting FMDV in clinical samples needs to be experimentally confirmed. Field validation of the protocols described in the current chapter is reported in Chapter 6 (for FMDV) and Chapter 7 (for BVDV).

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Chapter 4. Development of a universal positive control for detecting pathogens that cause vesicular lesions in infected animals.

4.1. Introduction

Diagnostic assays require appropriate controls to support the integrity of results and ensure a quality-assured testing program (Burkardt, 2000). Controls and quantification calibrators in qPCR assays may include a positive template control (PC), a negative template control (NC), a no template control (NTC), and an internal amplification control (IC) (Bustin et al., 2009). For a PCR run to be considered valid and acceptable, the quantification cycle (Cq) value of the PC should be within the predicted range; and the NC and NTC should have no positive signal. The PC is tested as a separate reaction to check variations in the amplification efficiency of the PCR assay over time. A positive Cq in NC or NTC may be attributed to cross-contamination, and any positive sample within such a run may be a false positive (Hoorfar et al., 2004; Madi et al., 2015). The presence of amplifiable nucleic acid in the template is indicated by a positive signal in the IC reaction (Sachadyn & Kur, 1998). This helps rule out false-negative results due to inhibitors or poor nucleic acid quality.

A PC is a well-characterised analyte-positive template with an established Cq value. A PC for endemic pathogen assays is readily accessible and typically comprises nucleic acids extracted from clinical specimens or *in-vitro* grown pathogens. However, positive controls for exotic pathogens may require a higher biosafety containment level, especially for high-impact pathogens such as FMDV (Madi et al., 2015). Such pathogens, even inactivated, are usually problematic to source and bring into New Zealand due to strict import regulations.

An alternative to infectious material as a PC source is synthetic nucleic acid polymers (DNA or RNA) containing the target genome fragment of interest. One option is an artificial RNA transcript produced by *in vitro* transcription of a target DNA construct inserted into a plasmid. Another option is to use an MS2 bacteriophage encapsulated recombinant RNA containing the target region and packaged in *E. coli* bacteria, also known as armoured RNA (Paloske et al., 1998). The produced phage particles are highly resistant to RNases and stable

at room temperature (RT) for an extended period (Rueckert & Cary, 2009; Yu et al., 2008). Similarly, a surrogate plant virus, the cowpea mosaic virus (CPMV), can be engineered to contain viral targets (e.g., FMDV 3D and 5' UTR region) that are inserted into one of the two RNA molecules of the CPMV genome (Madi et al., 2015). The recombinant CPMV can be propagated in plants without the ability to cause productive infection in the plant host. One advantage of these recombinant PC options compared to the conventional infectious source is that several target genes can be included in one particle, simplifying any control system by serving as a PC for nucleic acid extraction and amplification steps. However, armoured RNA and the engineered CPMV are more expensive and technically challenging to produce than the synthetic DNA or RNA constructs.

Specific primers and probes detect the IC target in a separate reaction or in the same reaction multiplexed with the target pathogen assay. An endogenous housekeeping or reference gene, such as 18s RNA or beta-actin, is a commonly used IC target. However, endogenous genes are usually abundant, so amplicons are still generated even in the presence of PCR inhibitors. Such limitation can be overcome using exogenous IC, which is either spiked to the neat sample at the beginning or during the lysis step of the nucleic acid extraction procedure. Examples of commercially available synthetic nucleic acid ICs are Vetmax™ Xeno™ Internal positive control (Thermo Fisher Scientific) or qPCR Extraction control (Meridian Bioscience). Bacteriophages, including MS2 (Blaise-Boisseau et al., 2010; Ninove et al., 2011) and Qß (Ninove et al., 2011), also provide versatile exogenous ICs for extraction efficiency and PCR amplification set-up. These are non-pathogenic organisms to both humans and animals. In addition, bacteriophages are very stable in various conditions, and their genomes are less susceptible to degradation by nucleases than other nucleic acids. Commercial assays for bacteriophages, however, are uncommon and may require the development and validation of unique primers and probe detection systems as a singleplex assay or for incorporation into multiplex PCR set-up.

With the ultimate objective of developing a pen-side PCR assay for FMDV that can be carried out in the field, important criteria in designing PC and IC include safety (e.g., controls should not be the source of new infections) and thermal stability. Ideally, one control could be used to detect several similar pathogens. Thus, the first aim of the work presented in this chapter was to develop a universal synthetic RNA PC for vesicular disease detection assays that target FMDV, BVDV-1, and BVDV-2. The second aim was to develop and validate an exogenous Qß IC PCR that can be incorporated into any multiplex assay.

4.2. Materials and methods

The general approach in producing the universal synthetic control started with identifying the desired viral fragments and combining these into one long construct *in silico*. The designed DNA construct was then commercially produced and provided as an insert within pMA-T plasmid. The plasmid was amplified by transformation into chemically competent *Escherichia coli* cells. After plasmid purification, the plasmid was either linearised or the insert was amplified by PCR, and then *in vitro* transcribed into RNA. The synthetic RNA was purified to remove unwanted DNA contaminants. The synthetic RNA copy numbers were then estimated based on the length (nt) of the target insert and the measured RNA concentration. The synthetic RNA was then used to produce RNA standards with known quantity of the target RNA.

4.2.1. Synthetic RNA PC design

The universal synthetic PC was designed to include the nucleic acid targets for the following vesicular disease pathogens: FMDV, BVDV-1, BVDV-2, swine vesicular disease virus (SVDV), vesicular exanthema of swine virus (VESV), Seneca Valley virus A (SVV), and vesicular stomatitis virus (VSV) New Jersey (NJ) and Indiana (Ind) strains. Along with these viruses, two candidate IC targets (18S rRNA and Qß) were included in the construct. The control was designed to work with the assays (Table 4-1) currently used by AHL-MPI (New Zealand) for exotic disease diagnosis.

Gene fragments were obtained from complementary DNA sequences of eight RNA viral genomes and two IC organisms using Geneious® R10 software (https://www.geneious.com). Nucleotide (nt) substitutions between 1 to 8 bases were introduced to each gene fragment to serve as a unique signature of each target. Gene fragments of two pathogens (e.g., BVDV-1 and BVDV-2; and VSV NJ and Ind) were combined into one construct by substituting nt bases at primer or probe binding sites.

The edited gene fragments were then concatenated into a 2 kb long nt fragment to be used as a template for the control RNA transcript production. The concatenated DNA fragment is referred to as "R3+" in reference to the three main viral RNA targets of interest (R3: FMDV, BVDV-1 and BVDV-2). The "+" sign refers to the remaining RNA targets in the

concatenated DNA (listed in Table 4-1). The plasmid containing the concatenated insert was synthesised commercially (GeneArt Gene Synthesis, Thermo Fisher Scientific) and supplied as a recombinant pMA-T 4,394 bp long plasmid vector (Figure 4-1).

Table 4-1. Virus-specific reference assays that were incorporated into the design of the universal synthetic RNA control (R3+). Foot and mouth disease virus (FMDV), bovine viral diarrhoea virus type 1 and type 2 (BVDV-1, BVDV-2), swine vesicular disease virus (SVDV), vesicular exanthema of swine virus (VESV), Seneca Valley virus A (SVV), vesicular stomatitis virus (VSV) New Jersey (NJ) and Indiana (Ind) strains, and Q Beta bacteriophage (Qß). UTR= untranslated region, IRES = internal ribosomal entry site, rRNA = ribosomal RNA, NP = nucleoprotein.

Assay	Reference
Eukaryotic 18S rRNA qPCR	Sales et al., 2002
VSV NJ and Ind conventional PCR	Hofner et al., 1994
BVDV-1 & 2 5'UTR RT-qPCR	Willoughby et al., 2006
FMDV 3D RT-qPCR	Callahan et al., 2002
FMDV IRES RT-qPCR	Shaw et al., 2007
SVDV 1 and 2 RT-qPCR	Reid et al.,2003
SVDV 2C gene RT-qPCR	McMenamy et al., 2011
SVV 3D gene RT-qPCR	Fowler et al., 2017
VES polymerase gene RT-qPCR	Reid et al., 2007
VSV Ind NP gene RT-qPCR	AAHL unpublished
VSV NJ NP gene RT-qPCR	AAHL unpublished
Qß coat protein gene RT-qPCR	this chapter

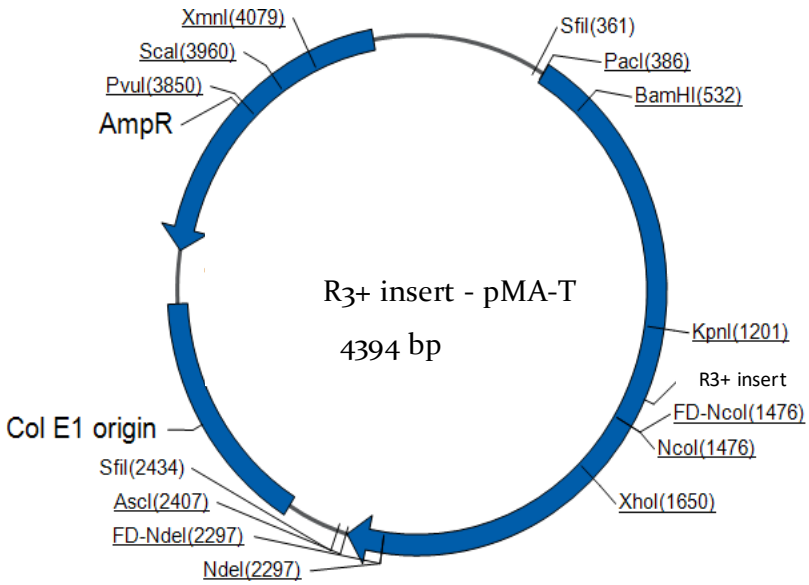


Figure 4-1. Illustration of the pMA-T plasmid with the inserted synthetic construct (GeneArt Gene Synthesis, Thermo Fisher Scientific).

4.2.1.1. TOPO TA Cloning

The R3+ insert was amplified by PCR using the pMA-T plasmid vector as a template and then re-inserted into a T-tailed linearised PCR® II TOPO plasmid vector using the TOPO TA cloning kit (Thermo Fisher Scientific). The R3+ was amplified in a PCR reaction containing 1 µM of each forward (GTAAAACGACGGCCAG) and reverse (CAGGAAACAGCTATGAC) M13 primers, 1 µL pMX plasmid, 12.5 µL 2× Kapa2G Fast HotStart ReadyMix (Kapa Biosystems) using the Veriti thermocycler (Thermo Fisher Scientific). The cycling conditions were 95°C for 5 min initial denaturation followed by 40 cycles of 95°C for 15 sec (denaturation), 50°C for 15 sec (annealing) and 72°C for 90 sec (extension). A final extension at 72°C for 3 min was added to ensure the sufficient addition of 3' adenine overhangs necessary for the cloning procedure. The successful amplification of the target was confirmed using an FMDV 3D PCR assay (Callahan et al., 2002) with standard validated conditions (Appendix 1). The PCR product (25 µL) was then subjected to gel electrophoresis through a 1.5% agarose gel in TAE buffer containing 0.01% GelRed™ (Biotium) for 80 mins at 100v. The amplicon, with an expected size of 2,221 bp, was recovered from the gel with Zymoclean™ Gel DNA Recovery kit (Zymo Research) following the kit protocol and eluted with 10 µL PCR grade water.

Cloning was performed according to the kit-supplied procedure. Briefly, ligation was done by mixing the gel-purified amplicon (4 µL), salt solution (1 µL) and PCR® II TOPO vector (1 µL) gently into a 1.5 mL nuclease-free microfuge tube. The ligation reaction was incubated for 30 min at RT and placed on ice before transferring 2 µL into newly thawed TOP10 chemically competent *Escherichia coli* cells. The transformants were incubated on ice for 30 min, followed by 30 sec in a 42°C water bath. The vial of the heat-shocked cells was immediately placed back in the ice bath. From here, pre-warmed Super Optimal broth with Catabolite repression (SOC) medium (250 µL) was added to the cells, and the tube was incubated while shaking horizontally (200 rpm) for 1 hour at 37°C. Two volumes (15 and 30 µL) of the transformed *E. coli* were then spread onto separate pre-warmed Luria Bertaini (LB) agar plates containing 50 µg/mL ampicillin each. The LB plates were left overnight in a 37°C incubator. The following day, colonies were picked and individually inoculated to a 2 mL pre-warmed LB broth with 50 µg/mL ampicillin. The vials were incubated overnight at 37°C with shaking and used for plasmid purification when they reached a density corresponding to McFarland turbidity standard 4. The LB broth was screened for the presence of the insert by directly analysing a diluted liquid culture for the presence of four targets (FMDV 3D gene, BVDV-1, and BVDV-2 and Qß) with a multiplex RT-qPCR. First, the broth was diluted 1:40 in

nuclease free water in a 1.5 mL microfuge tube and heated at 95°C in a dry heating block. After heating, the diluted broth was centrifuged at 10,000 × *g* for 5 min, and 3 µL of the supernatant was used as a template in the multiplex reaction using PCR conditions described in Appendix 4.

After screening, half (1 mL) of the LB broth was used for plasmid purification, and the other half was stored at -80°C for future use either in ceramic beads (500 mL) (Cryobank™) or mixed (400 µL) with an equal volume of 50% glycerol. An aliquot of 100 µL of the cultured broth was used to scale up the production of the recombinant plasmid upon confirmation of the insert by sequencing.

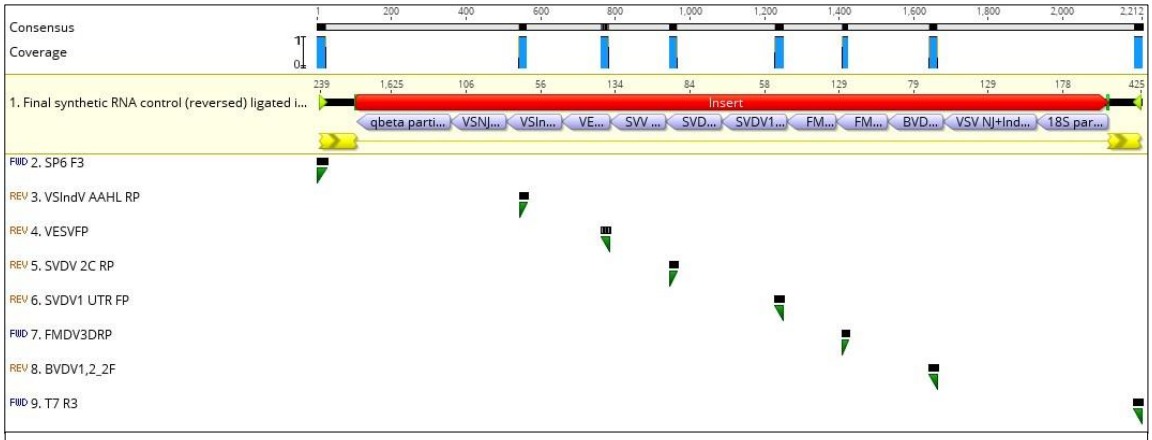
4.2.1.2. Plasmid purification

The plasmid was purified using PureLink Quick Plasmid Miniprep (Thermo Fisher Scientific) according to the manufacturer's protocol. One millilitre LB culture broth in a 5 mL tube was centrifuged at 12,000 × *g* for 10 min. The liquid medium was removed, and the *E. coli* pellet was resuspended in a 250 µL resuspension buffer containing RNase A. After making a homogeneous solution, 250 µL lysis buffer (L7) was added. The capped tube was mixed by inverting gently, and then the solution was incubated at RT for 5 min. After incubation, 350 µL precipitation buffer (N4) was added to the solution, and the capped tube was shaken vigorously. The homogeneous suspension was centrifuged at 12,000 × *g* for 10 min. The supernatant was loaded into a spin-column in a 2 mL wash tube and centrifuged at 12,000 × *g* for a minute. After discarding the lysate in the wash tube, the spin-column was washed with 700 µL wash buffer (W9) containing ethanol and the column was centrifuged again at 12,000 × *g* for a minute. Seventy-five µL TE buffer was added into the spin column in a clean 1.5 mL microfuge tube and incubated for 1 min at RT. The purified plasmid was eluted by centrifugation of the spin column at 12,000 × *g* for 2 min. The recovered plasmid was stored at 4°C or -20°C before use.

4.2.1.3. Confirmation of plasmid insert

The presence of the nucleic acid insert was confirmed with conventional PCR using vector primers SP6 as the forward (CCAAGCTATTTAGGTGACACTATAG) and T7 as the reverse (TGTAATACGACTCACTATAGGG) primer. The PCR mix consisted of 12.5 µL 2× Kapa2G Fast HotStart ReadyMix (Kapa Biosystems), 500 nM of each primer, 1 µL purified

plasmid and nuclease-free water to make up a 25 µL reaction volume. The PCR and gel purification steps were performed as described in section 4.2.1.1.



Primer set	sequence	size
SP6/VESF	CCAAGCTATTTAGGTGACACTATAG / GAYGACGGTGTYTACATYGTYC	781
VSIndV (AAHL) NP R / SVDV 1 UTR F	AGCCCATCCATGAGCTTTTTT / CGAGAAACCTAGTACCACCATGAA	712
SVV A 3D R / BVDV F	GAGCCAACATAGAAACAGATTGC / CCATGCCCTTAGTAGGACTAGC	895
FMD 3D R / T7	ACTGGGTTTTACAAACCTGTGA / TGTAATACGACTCACTATAGGG	825

Figure 4-2. Locations of four primer sets used to amplify overlapping regions of the R3+ insert to confirm its sequence and insertion site in the PCR® II TOPO vector.

In addition, four overlapping regions of the insert were amplified with primer sets shown in Figure 4-2. The reaction mix consisted of 25 µL 2X Phusion High Fidelity PCR master mix (NEB), 500 nM of each primer, 1 µL plasmid and nuclease free water to make up a 50 µL reaction volume. The cycling conditions used on the Veriti thermocycler (Thermo Fisher Scientific) were an initial denaturation of 98°C at 30 s, 35 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 15 s and extension of 72°C for 15 s; and a final extension of 72°C for 4 min. Gel purification was done using the parameters described in section 4.2.2.2.

The amplicons (2,203 bp) were sequenced at Ecogene. The edited chromatograms were mapped to the concatenated R3+ construct (section 4.2.1) using Geneious® R10 software (https://www.geneious.com).

4.2.1.4. In vitro transcription

In vitro transcription of the recombinant plasmid was performed using either a linearised plasmid or the PCR product of the amplified insert as a template. Prior to restriction enzyme digestion, the plasmid was purified with Zymo Genomic DNA Clean &

Concentrator (Zymo Research) using the kit instructions. The concentration of the cleaned plasmid was measured with a Qubit 4 fluorometer (Thermo Fisher Scientific). The digestion reaction was prepared by combining 1 µg plasmid DNA and 5 µL 10× NEBuffer (NEB) in a 1.5 mL microfuge tube first before adding 1 µL or 10 units of Xbal restriction enzyme and nuclease free water to reach a total reaction volume of 50 µL. The mixture was mixed by pipetting or flicking. Quick centrifugation to collect the liquid to the bottom of the tube was followed by 37°C incubation for 1 hour. Immediately before *in-vitro* transcription, the Xbal enzyme was denatured at 65°C for 20 min. In parallel, the R3+ synthetic insert was amplified with SP6/T7 primer pair using the PCR parameters and conditions described for the Phusion High Fidelity PC R master mix (NEB) in section 4.2.1.3.

The linearised plasmid or the PCR product was *in-vitro* transcribed with SP6 RNA polymerase (NEB) using the manufacturer's instructions. Depending on the initial concentration of the starting DNA template, the transcription reaction consisted of 1-4 µg linearised plasmid or PCR amplicon, 2-8 µL 10× RNAPol Buffer (NEB), 0.5 µL Ribonucleotide Solution Mix (NEB) for a final concentration of 0.5 mM each, 0.5-2 uL Murine RNase Inhibitor (NEB), 2-4 µL SP6 RNA Polymerase at 20 units/µL (NEB) and RNase-free water to a total volume between 20 and 80 µL. The linearised plasmid was usually transcribed in a smaller reaction volume (20 µL) than the PCR product (80 µL). The mixture was then incubated for an hour at 40°C.

4.2.1.5. DNAse treatment of the RNA transcript

The sequence of an *in vitro* transcribed RNA transcript was verified with a partially validated multiplex RT-qPCR assay targeting FMDV 3D, BVDV-1, BVDV-2 and Qß, using the conditions described in Appendix 4. To examine the presence of DNA contamination in the *in vitro* transcribed R3+ RNA, a no reverse transcriptase PCR reaction was included in the run. The reverse transcriptase was denatured in the ready-to-use TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) by subjecting PCR reagent to a 95°C in a heat block for 5 mins before using it in the no-reverse transcriptase PCR reaction.

The transcribed R3+ RNA was then treated with DNAse to remove remnant DNA using TURBO™ DNAse (Thermo Fisher Scientific) according to the kit's protocol. *In vitro* transcribed RNA (10 µg) was mixed with 50 µL RNAse free water and 0.1 volume of 10× TURBO DNAse buffer before the addition of 1 µL TURBO™ DNAse. The mixture was incubated at

37°C for 30 min. After incubation, 5 µL DNAse Inactivation Reagent was added to the solution. During a 5 min incubation at RT, the tube was flicked 2-3 times to ensure dispersion of the DNAse Inactivation Reagent. The tube was then centrifuged at 10,000 × *g* for 15 min, and the supernatant was transferred to a new RNAse-free 0.5 mL tube. DNase treatment was repeated if remnant DNA was still present after the first treatment.

The DNAse-treated R3+ transcript was purified with RNACLEAN® XP (Beckman Coulter) beads following the manufacturer's instructions. In a 1.5 mL RNase-free microfuge tube, 50 µL transcribed RNA was combined with 90 µL RNACLEAN® XP beads and mixed by pipetting. At the end of 5 min incubation at RT, the tube was placed onto a DynaMag™-2 Magnet (Thermo Fisher Scientific) rack for 5 min to separate the beads. The cleared solution was slowly aspirated and discarded. The beads were washed by incubating with 1 mL 70% ethanol for 30 s at RT. The ethanol was aspirated out, and the washing was repeated twice. After the last wash, the reaction tube was air dried for 10 min with an opened cap. The purified RNA was eluted by re-suspending the beads with 50 µL RNase-free water. The eluent was transferred to a new microfuge tube after separating the beads onto the magnetic tube rack.

To examine the effect of remnant DNA on PCR performance with regards to Cq value, a quantified (section 4.2.1.6) R3+ RNA control ($(10^0–10^7$ copies/reaction) was tested with the multiplex RT-qPCR described in Appendix 4. A no reverse transcription (-RT) control was included in the run, where the RT enzyme was omitted from the reaction mix.

4.2.1.6. Quantification of the synthetic RNA standard

The copy numbers of the R3+ RNA control was estimated according to the following formula (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr):

$$number\ of\ copies = \frac{amount\ (ng) * 6.022 \times 10^{23} (Avogrado's\ number)}{length\ (bp) * 1 \times 10^{9} * 340\ (average\ mass\ of\ ssRNA)}$$

This was based on the predicted transcript length of 2,203 nt bases (amplicon length) or 5989 nt (linearised plasmid length) and the mean RNA concentration (ng/µL) of two Qubit 4 (Thermo Fisher Scientific) fluorometer readings.

4.2.2. Qß IC PCR assay

4.2.2.1. Assay design

Primers and probe targeting the major protein coat gene of the Qß genome (Table 4-2) were created using Primer3 in Geneious® R10 software (https://www.geneious.com) based on GenBank accession number FJ483843. The following criteria were used for designing the primer pair: 40-60% GC, G or C at the 3' end, 65°C-75°C melting temperature with less than 5°C difference within the pair (https://www.thermofisher.com/blog/behindthebench/pcr-primer-design-tips/), and a product size of between 18 and 30 bases. The specificity of the Qß primers and probe was assessed by the level of similarity to unrelated sequences available in GenBank using Primer-BLAST (Ye et al., 2012). The Qß probe was labelled with Texas red dye with a black hole quencher 2 (BHQ-2, Eurogentec).

Table 4-2. Qß primers and probe targeting the major protein coat gene.

Primers / Probe	Sequence, 5" to 3'	Base Position	Amplicon size	Reference
Qß Forward	CAA ACG GTT CTT GTG ACC CA	1574-1594	93	GenBank FJ483843
Qß Reverse	AAG CTC GTT CCT CAT CGG TA	1666-1646		
Qß Probe	Texas red -TCG CCA GGC ATA TGC TGA CGT -BHQ-2	1602-1623		

4.2.2.2. Assay Optimisation

The Qß bacteriophage or Qß was supplied commercially (Attostar). The RNA template was extracted from 140 µL diluted Qß suspension (1:10 in RNAse free water) with QIAamp Viral RNA manual kit (Qiagen) following the manufacturer's instructions. Four replicates of each primer concentration (400, 500, 600, 700, 800, and 900 nM) were used for optimisation with a fixed probe concentration of 250 nM. Primer concentration that produced the lowest Cq value and high relative fluorescence unit (RFU) was selected and tested with four probe concentrations (100, 150, 200, and 250 nM). In preparation for inclusion in a multiplex PCR format, the initially selected primer concentration was further compared to the next best-performing primers at lower concentrations using the selected probe concentration.

The PCR reaction mix consisted of 5 µL Qß RNA template (diluted at 10^{-4}), 5 µL TaqMan™ Fast Virus 1-Step Master Mix (Thermo Fisher Scientific), various final

concentrations of forward and reverse primers, various concentrations of the probe in a total reaction volume of 20 µL. The optimisation was conducted on the CFX96 Thermocycler (BioRad). The reverse transcription step was carried out at 50°C for 5 min, followed by the initial denaturation at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s and annealing/extension at 60°C for 30 s. Annealing temperature gradient was also conducted with the optimised primer and probe concentrations using a pre-set (56.1, 57.8, 60, 61.8, and 63.3 °C) annealing temperature conditions on the CFX96 machine. A non-template control (NTC) was included for each PCR optimisation experiment. Results from the PCR runs were analysed using the CFX96 PCR Manager (BioRad) software with a baseline threshold set automatically.

The efficiency and analytical sensitivity of the Qß RT-qPCR were assessed using ten-fold dilutions of the Qß RNA template (10^{-1} to 10^{-7}) and repeated using the newly developed universal R3+ transcript (1.0×10^{0} to 1.0×10^{8} copies/reaction) (section 4.2.1). In both instances, the standards were assayed in four replicates. The linear regression function of the CFX96 software was used to examine efficiency.

4.3. Results

4.3.1. Designing the universal R3+ PC

Twelve gene fragments (Table 4-3), including targets from eight vesicular disease-causing viruses (FMDV, VSV NJ, VSV Ind, SVDV, SVV, BVDV-1, BVDV-2, and VESV), and two amplification IC targets (18S and Qß) were concatenated into a 2,016 nt long R3+ construct (Figure 4-3). The length of each control fragment and the expected amplicon of each PCR assay are shown in Table 4-3. and Table 4-5.

Individual gene fragments comprising target sequences showing primer and probe binding sites, as well as modified bases to distinguish each control fragment from the corresponding wild-type virus, are shown in Table 4-4. For example, unique GCTA bases were used to substitute ATCC and TGAC at position 7500-7503 and 514-517 in the FMDV 3D gene (construct 4), and internal ribosomal entry site or IRES (construct 5) fragments, respectively. The same four bases (GCTA) were also introduced to the SVV (construct 8) and VESV

(construct 9) targets, replacing nt between 7076-7079 (AGCT) and 5198-5201 (CAGT) respectively.

Table 4-3. Individual fragments of eight pathogen genomes incorporated into the R3+ control.

	Target construct	*Minimum*	*Maximum*	*Length*	*Reference*	*Base position*
1	Eukaryotic 18S rRNA	1	193	193	AF176811	57-249
2	VSV NJ and Ind L gene	194	443	250	KT429217.1	7111-7361
3	BVDV-1 & 2 5'UTR	444	593	150	NC_001461	91-210
4	FMDV 3D	594	732	139	AJ251473.1	462-593
5	FMD IRES	733	864	132	AJ251473.1	7441-7579
6	SVDV1 and2 5'UTR	865	1,038	174	AY876011.1	181-270 and 380-464
7	SVDV 2C	1,039	1,188	150	KT285005.1	3941-4090
8	SVV 3D gene	1,189	1,338	150	EU271766.1	6997-7146
9	VES Pol gene	1,339	1,466	128	U76874.2	5129-5256
10	VSV Ind NP gene	1,467	1,616	150	NC_001560	430-579
11	VSV NJ NP gene	1,617	1,766	150	M31846.1	586-735
12	Qß coat protein gene	1,767	2,016	250	FJ483843	1422-1678

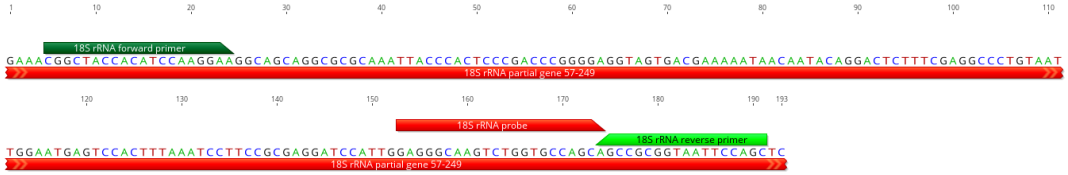
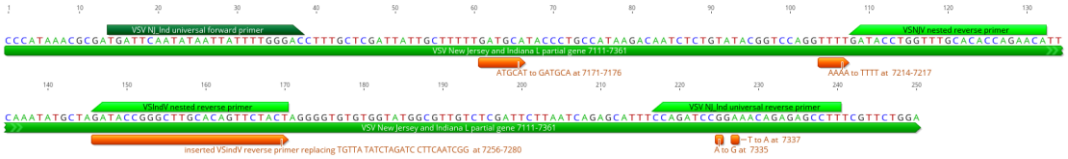
Since BVDV-1 and BVDV-2 assays use the same forward and reverse primers, the BVDV-1 5' UTR fragment (construct 3) was changed to accommodate the BVDV-2 probe binding site by inserting a 30 nt bases (AGGAGGGGACTAGCGGTAGCAGTGAGTTCA) between position 170 and 171. The unique GCTA bases were positioned at positions 172-175, which replaced the AAGC.

Similarly, the VSV L gene segment (construct 2) of a VSV NJ strain was modified with a complementary sequence for the Ind strain nested reverse primer (GATACCGGGCTTGCACAGTTCTACT) at positions 7256-7280. Unique bases were introduced at positions 7171-7176 (ATGCAT to GATGCA) and 7214-7217 (AAAA to TTTT). In addition, two nt at positions 7335 and 7337 were substituted to match the VSV universal reverse primer. Two separate nucleoprotein gene segments from VSV NJ (construct 11) and Ind (construct 10) strains were combined to form one fragment within the R3+. Construct 11 had a single unique nt substitution at position 654 (G to T), while four nt bases (GGCA) were replaced with ATGC at positions 496-499 in construct 11.

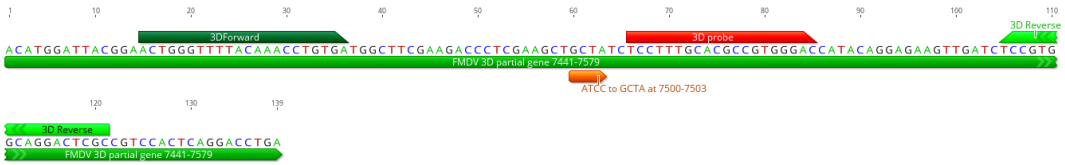
Two SVDV construct, the 5'UTR fragment (construct 6) and 2C gene (construct 7), were included in the R3+ control. Construct 6 was a concatenation of two fragments comprising nt positions 181-270 and 380-464, where each fragment held a set of qPCR primer and probe binding sites. The unique bases inserted for the first fragment were ATAT, replacing GCGC at positions 215-218, while ATCT was used to substitute CCAG at positions 435-438. The SVDV construct 7 had nt substitution from TTGGGCGT to ACTAAGTA at positions 4033-4040. No alterations were made to the 18S rRNA and Qß IC fragment (constructs 1 and 12).

Figure 4-3. Concatenated viral genomic targets used for producing the R3+ artificial RNA transcript. Refer to Table 4-3 and Table 4-4 for details of each target construct. Red= 18s gene derived segment, white= segment derived from 5'UTR (untranslated region), green = segment derived from polyprotein genes.

Table 4-4. The 12 individual gene fragments that were concatenated into one construct for insertion into a plasmid (pMA-T). The concatenated construct served as a template for generation of the RT-qPCR positive control RNA. Primer binding sites (green and light green labels), probe binding sites (red labels), modifications in nucleotide bases to differentiate the control from the wild-type virus; and deletions, substitution, or insertions of nucleotides to combine two PCR targets in one construct (orange labels) are shown. The gene constructs are: 1. eukaryotic 18S rRNA; 2. Vesicular stomatitis virus New Jersey and Indiana strain - L gene; 3. Bovine viral diarrhoea virus type 1 and 2 5'UTR; 4. foot and mouth disease virus 3D gene; 5. foot and mouth disease virus 5' UTR; 6. swine vesicular disease virus 5'UTR; 7. swine vesicular disease virus 2C gene; 8. Seneca Valley virus 3D gene; 9. vesicular exanthema of swine polymerase gene; 10. vesicular stomatitis virus Indiana strain nucleoprotein gene; 11. vesicular stomatitis virus New Jersey strain nucleoprotein gene, 12. Qß phage.

	Individual gene fragment
1	
	eukaryotic 18S rRNA
2	
	vesicular stomatitis virus New Jersey and Indiana strain - L gene
3	
	bovine viral diarrhoea virus type 1 and 2 - 5'UTR

4

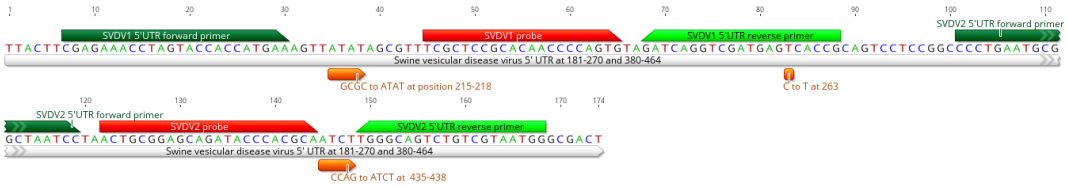


foot and mouth disease virus 3D gene

5

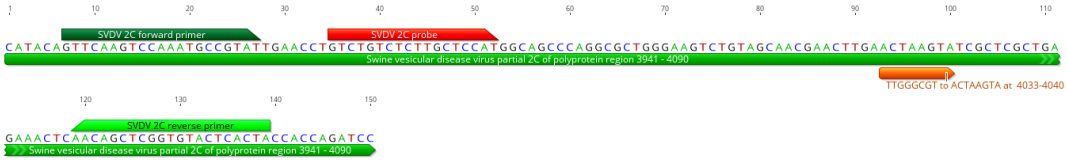
foot and mouth disease virus 5' UTR

6



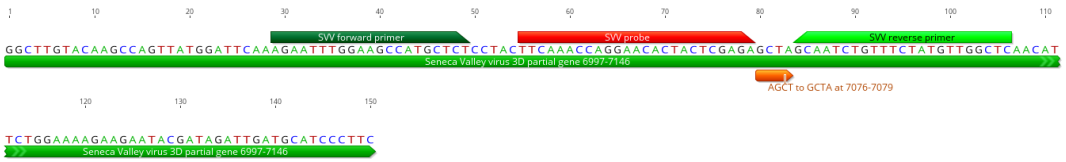
swine vesicular disease virus 5'UTR

7



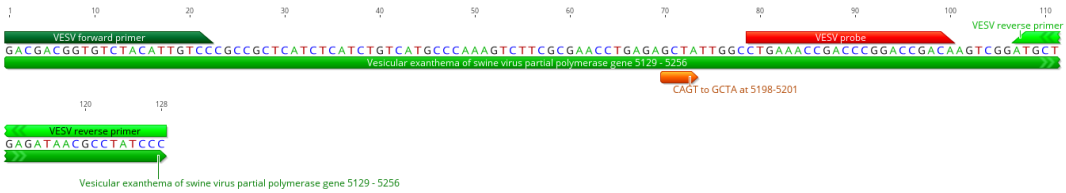
swine vesicular disease virus 2C gene

8



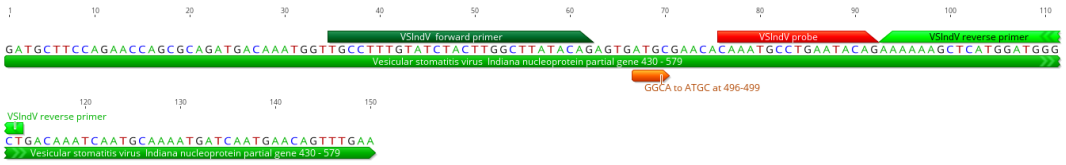
Seneca Valley virus 3D gene

9



vesicular exanthema of swine polymerase gene

10

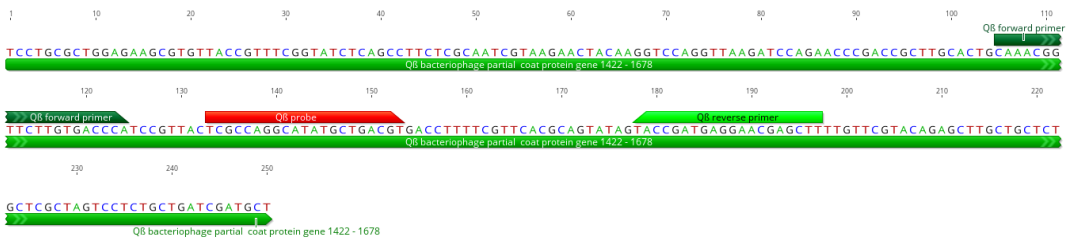


vesicular stomatitis virus Indiana strain nucleoprotein gene

11

vesicular stomatitis virus New Jersey strain nucleoprotein gene

12



Qß phage coat protein gene

Table 4-5. Reverse transcription PCR assays (primers and probes) for which the R3+ RNA transcript can be used as a universal positive control.

	Target	Primers and probes sequence (5' to 3')	R3+ base position	Amplicon size, bp	Reference
1	Eukaryotic 18S ribosomal RNA	F - CGGCTACCACATCCAAGGAA R - GCTGGAATTACCGCGGCT Probe - TGCTGGCACCAGACTTGCCCTC	5-24 174-191 153-174	187	Sales et al., 2002
2	Vesicular Stomatitis Virus (VSV) New Jersey and Indiana - L gene	Universal F - TGATTCAATATAATTATTTTGGGAC NJ nested R - TGTTCTGGTGTGCAAACCAGGTATC Ind nested R - AGTAGAACTGTGCAAGCCCGGTATC Universal R - AAGGCTCTCTGTTTCCGGATCTGG	207-231 301-325 339-363 410-433	NJ nested - 119 Ind nested - 157 primary PCR - 227	Hofner et al, 1994
3	Bovine viral diarrhoea virus 1 & 2 5'UTR (untranslated region)	F - CCATGCCCTTAGTAGGACTAGC R - TGACGACTACCCTGTACTCAGG BVDV-1 probe AACAGTGGTGAGTTCGT BVDV-2 probe ACTAGCGGTAGCAGTGAG	459-480 559-580 498-514 532-549	BVDV-1 and 2 - 122	Willoughby et al.,2006
4	Foot and mouth disease virus (FMDV) 3D gene	F - ACTGGGTTTTACAAACCTGTGA R - GCGAGTCCTGCCACGGA Probe – TCCTTTGCACGCCGTGGGAC	608-629 659-678 698-714	107	Callahan et al., 2002
5	FMDV 5'UTR - IRES (internal ribosome entry site)	F - CACYTYAAGRTGACAYTGRTACTGGTA R - TAACAHGHGWCACTYGGRATCTG Probe - CCTCGGGGTACCTGAAGGGCATCC	746-773 820-842 796-819	97	Shaw et al., 2004
6	Swine vesicular disease virus (SVDV) 5'UTR	F1 - CGAGAAACCTAGTACCACCATGAA R 1- CGGTGACTCATCGACCTGATC Probe1 - TCGCTCCGCACAACCCCAGTG F2 - CCCTGAATGCGGCTAATCC R 2- CCATTACGACAGACTGCCCA Probe 2 - ACTGCGGAGCAGATACCCACGCA	871-894 932-952 909-929 965-983 1013-1032 986-1008	SVDV1 and 2 - 82	Reid et al.,2004
7	SVDV 2C gene	F - GTTCAAGTCCAAATGCCGTAT R - TAGTGAGTACACCGAGCTGTT MGB Probe - GTCTGTCTCTTGCTCCAT	1045-1065 1157-1177 1073-1090	133	McMenamy et al., 2011
8	Seneca valley virus 3D gene	F - AGAATTTGGAAGCCATGCTCT R - GCAATCTGTTTCTATGTTGGCTC Probe - TTCAAACCAGGAACACTACTCGAGA	1217-1237 1272-1294 1243-1267	78	Fowler et al., 2017
9	Vesicular exanthema of swine polymerase gene	F - GAYGACGGTGTYTACATYGTYC R - GGGAYDGGCGTTATYTCAGCRT Probe - CTGAARCCGACYCGGACCGACA	1339-1360 1445-1466 1417-1438	128	Reid et al., 2007
10	VSV Ind nucleoprotein (NP) gene	F - TGCCTTTGTATCTACTTGGCTTATACAG R - AGCCCATCCATGAGCTTTTTT MGB Probe - CAAATGCCTGAATACAG	1501-1528 1559-1579 1542-1558	79	AAHL unpublished
11	VSV NJ NP gene	F - GCATGAACGTGCTCCAATCA R - GCTGCACAGTCCTTGAATCTTG MGB Probe - ATACGGAACCATAGTCT	1665-1684 1703-1724 1686-1702	60	AAHL unpublished
12	Qß coat protein gene	F - CAAACGGTTCTTGTGACCCA R - AAGCTCGTTCCTCATCGGTA Probe - TCGCCAGGCATATGCTGACGT	1871-1890 1899-1919 1944-1963	93	in-house

4.3.2. Generation of the R3+ control plasmid

The R3+ insert from the pMA-T vector was subcloned into a pCR II TOPO vector. A 2,221 bp PCR product (Figure 4-4) of the R3+ was inserted into a pCR II TOPO vector (Figure 4-5). A positive result confirmed the presence of R3+ in the TOPO plasmid in the FMDV 3D RT-qPCR (result not shown).

Only four white colonies were present on six LB agar plates of transformed TOP10 chemically competent *E. coli* cells, three of which were successfully grown in individual 5 mL screw-capped vials with LB broth (2 mL) containing 50 µg/mL ampicillin. Rapid screening of the three broth cultures with a multiplex RT-qPCR showed the presence of FMDV, BVDV-1, BVDV-2 and Qß targets (Figure 4-6). However, purified plasmids from only 2 out of 3 PCR-positive broths had the insert of the expected size of approximately 2,203 bp (lanes 3, 4 and 5 in Figure 4-7) when screened for the presence of R3+ construct using vector primers SP6 and T7.

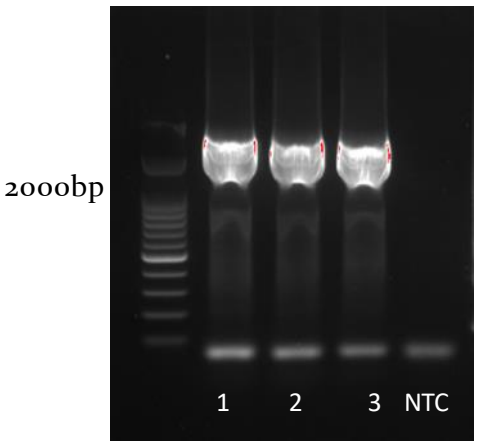


Figure 4-4. M13 primers amplicons (Lane 1-3) containing the R3+ insert after PCR analysis of the pMA-T vector. The PCR product was prepared for TOPO TA cloning.

4.3.3. Confirmation of the sequence of the R3+ insert

Sequencing of the 2.2 kbp amplicon with primers Sp6 and T7 PCR did not produce the complete sequence of the entire fragment (results not shown). Thus, conventional PCR was performed using four primer sets to confirm the sequence of the R3+ insert. Figure 4-8 shows the mapped sequence of the four amplicons against the original construct, demonstrating an intact R3+ insert in the plasmid.

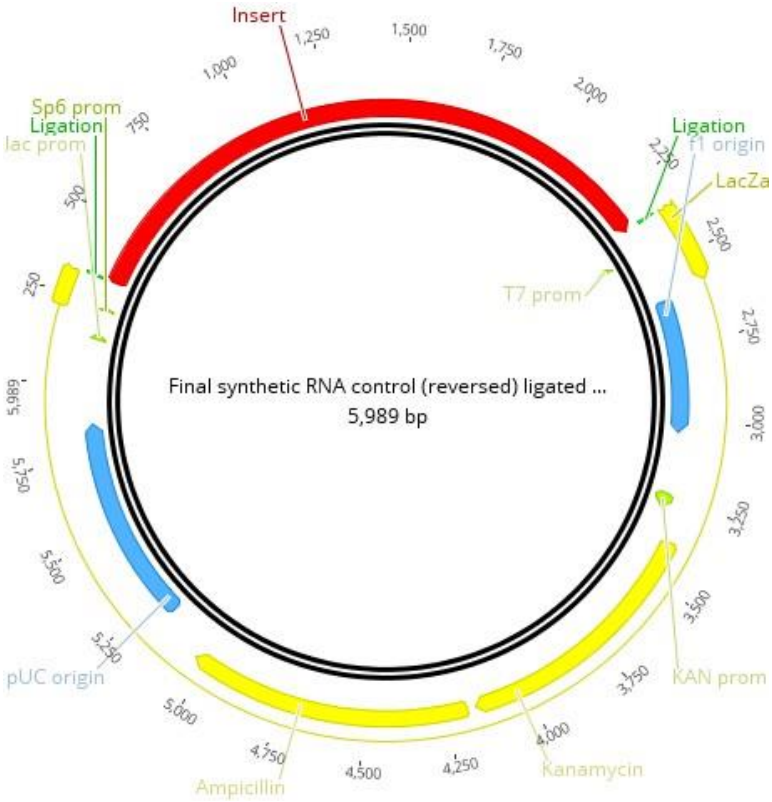


Figure 4-5. A diagram showing the R3+ insert (red band) within the pCR TOPO vector prepared using Geneious® R10 software (https://www.geneious.com).

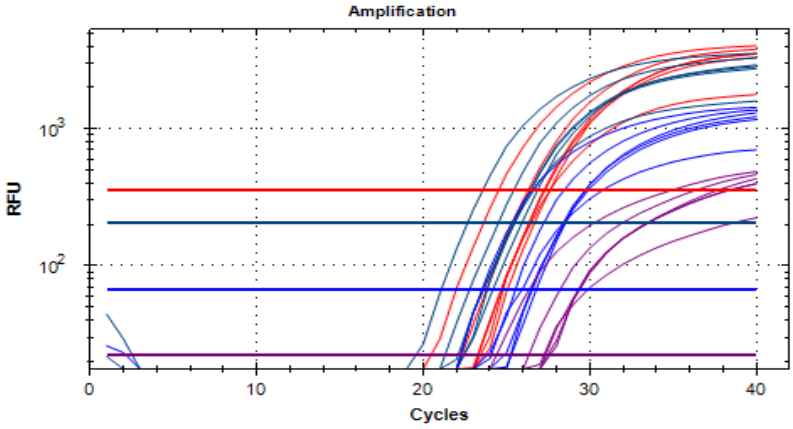


Figure 4-6. Rapid screening of three transformed E. coli colonies showing the amplification of four control targets (dark blue- FMDV, light blue-BVDV-1, red – Qβ, purple – BVDV-2) using a multiplex RT-qPCR described in Appendix 4.

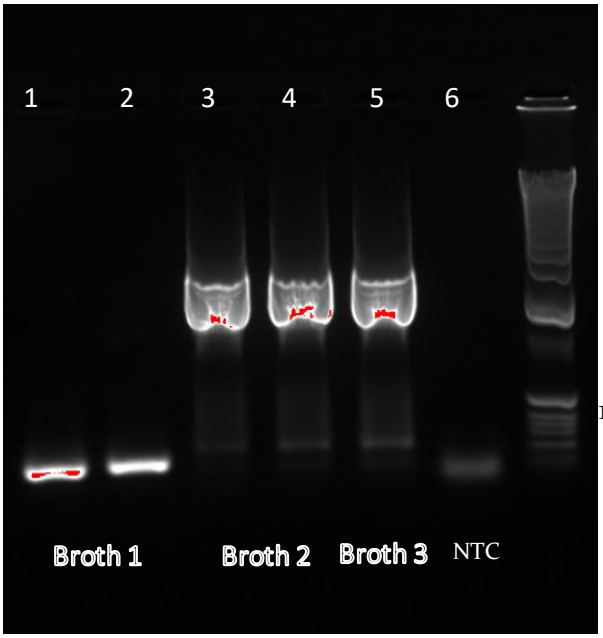


Figure 4-7. Bands of approximately 2000 bp were generated, indicating the presence of the R3+ insert (2203 bp) from E. coli colony 2 (Lane 3 and 4) and colony 3 (lane 5) after PCR amplification using vector primers SP6 and T7. Broth was LB (Luria Bertaini) broth.

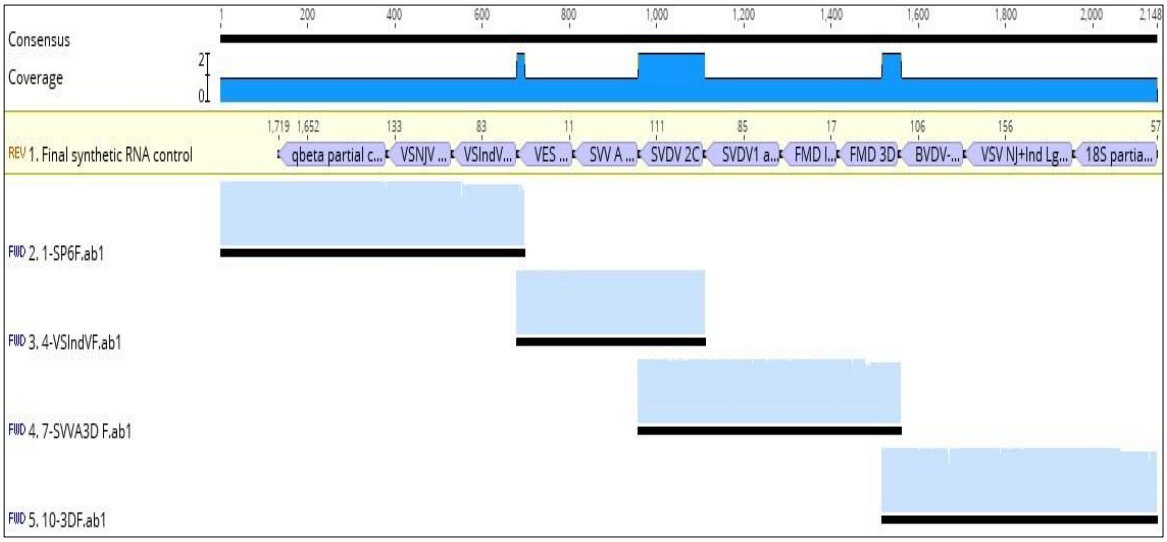


Figure 4-8. Four overlapping sequences of four PCR amplicons (refer to Figure 4-2) demonstrated that the intact artificial construct was inserted into pCR II TOPO plasmid.

4.3.4. *In vitro* transcription

The *in vitro* transcribed R3+ RNA from either the linearised plasmid (5989 bp) or Sp6/T7 amplicon (2203 bp) contained the four viral RNA targets based on the results of the multiplex RT-qPCR (Figure 4-9). A no reverse transcriptase multiplex RT-qPCR control was positive, indicating residual DNA's presence in the R3+ transcribed RNA. Treatment of the R3+ transcript with DNAse twice and subsequent purification with RNA using Clean XP beads resulted in the elimination of most of the residual DNA, as the Cq value of "no-RT" positive control was considerably higher (>30) than the Cq value of no-RT reaction using RNA template before DNAse treatment. A difference of ≥ 10 Cq value between the normal multiplex RT-qPCR and "no RT" control PCR run (Table 4-6) was also observed, which was equivalent to the approximately 1000-fold difference in concentration of the target sequence assuming 100% PCR efficiency.

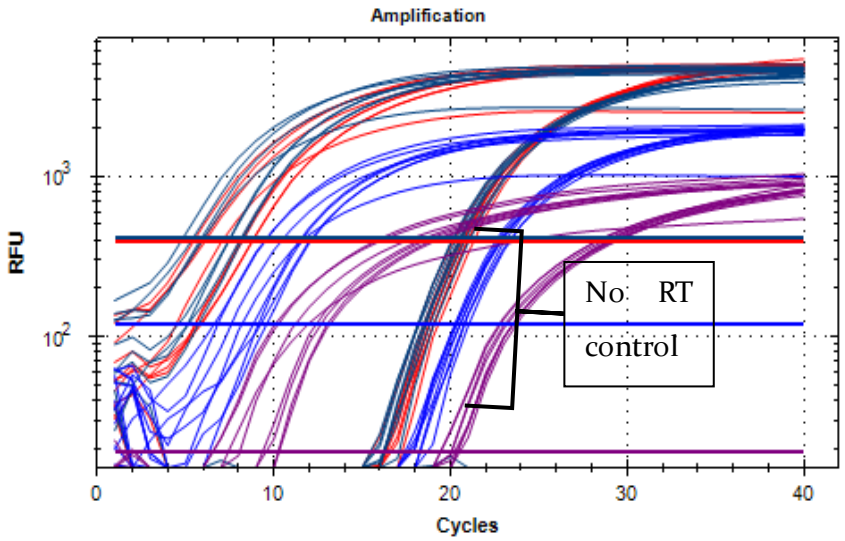


Figure 4-9. Four targets (FMDV - dark blue, BVDV 1 - light blue, BVDV- 2 – purple, and Qβ - red, present in the R3+ RNA was amplified using a provisional multiplex RT-qPCR assay described in Chapter 4. A no reverse transcriptase (no RT) control showed the presence of remnant DNA in the newly transcribed R3+.

*Table 4-6. Comparison of quantification cycle (Cq) value of twice DNAse treated R3+ RNA (10^0 – 10^7 copies/reaction) with a no reverse transcriptase (no RT) control run using a provisional mRT-qPCR (FMDV, BVDV-1, BVDV-2 and Qß) described in section 5.3.1 in Chapter 5. The amount of RNA and DNA in the R3+ template was measured with two Qubit readings and copy numbers was estimated using the copy number calculator (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr). The Cq values obtained in reactions without the RT step were more than 10 units higher than the Cq values obtained with the same template with the RT step. Enclosed in parentheses was the standard deviation (n=3). * One Cq only.*

R3+ RNA copies per reaction	R3+ DNA copies per reaction	FMDV		BVDV-1		BVDV-2		QB	
		RT-qPCR	no RT	RT-qPCR	no RT	RT-qPCR	no RT	RT-qPCR	no RT
10^7	5248	17.01 (0.144)	29.58 (0.438)	21.11 (0.078)	34.29 (0.831)	23.06 (0.068)	37.47 (0.995)	18.28 (0.062)	29.29 (0.265)
10^6	524	20.3 (0.052)	35.98 (0.564)	24.44 (0.001)	0	26.51 (0.024)	0	21.36 (0.069)	35.03 (0.664)
10^5	52	23.98 (0.790)	0	28.1 (0.765)	0	30.03 (0.709)	0	25.24 (0.799)	37.77*
10^4	0.52	25.33 (0.102)	0	29.13 (0.149)	0	31.13 (0.150)	0	26.54 (0.085)	38.17*
10^3		28.21 (0.038)	0	31.8 (0.146)	0	33.69 (0.007)	0	29.22 (0.012)	0
10^2		30.72 (0.344)	0	33.82 (0.333)	0	35.85 (0.629)	0	31.32 (0.095)	0
10^1		32.36 (0.359)	0	36.83 (0.115)	0	38.84 (0.498)	0	32.98 (0.404)	0
10^0		35.92 (1.885)	0	37.98*	0	39.87*	0	35.18 (0.054)	0

4.3.5. Qß RT-qPCR optimisation

The Qß primer concentration of 700 nM was selected for further probe optimisation, although the difference in the mean Cq values obtained with 700 nM and with lower primer concentrations tested were negligible (Table 4-7). Similarly, the 200 nM probe was selected from four probe concentrations tested using primers at 700 nM, with no apparent differences in Cq mean (Table 4-8).

The selected primer concentration (700 nM) was also compared to a lower concentration of 500 nM using the optimal probe concentration (200 nM) to determine the effect of limiting the Qß primers for the Cq value. Both Qß primer concentrations (500 nM and 700 nM) performed similarly (Table 4-9), which indicated that either concentration could be used for the subsequent multiplex PCR development. The annealing temperature was determined to be optimal between 57.9°C (26.83 mean Cq, 0.039 SD) to 60°C (26.93 mean Cq, 0.104 SD) (Table 4-10) with the selected primer (500 nM) and probe (200 nM) concentrations.

Table 4-7. Quantification cycle (Cq) mean of amplification curves obtained in Qβ-specific RT-qPCR using five primer concentrations with a fixed concentration of the probe (200 nM). A Qβ RNA diluted at 10^{-4} was used as a template. Standard deviation (SD) was calculated using Microsoft® Excel®.

	Qβ RNA 10^{-4}	
Primer concentration	Cq mean (n=4)	Cq SD
A 900/900 nM	28.61	0.07
B 800/800 nM	29.37	0.09
C 700/700 nM	29.02	0.07
D 600/600 nM	29.43	0.08
E 500/500 nM	29.42	0.06
F 400/400 nM	29.44	0.12

Table 4-8. Quantification cycle mean (Cq mean) of amplification curves obtained in Qβ-specific RT-qPCR using four probe concentrations and a fixed concentration of primers (700 nM each). Amplification conditions are specified in section 4.2.2.2. The Qβ RNA diluted at 10^{-4} was used as a template. Standard deviation (SD) was calculated using Microsoft® Excel®.

	Qβ RNA 10^{-4}	
Probe, nM	Cq mean (n=4)	Cq SD
250	29.21	0.069
200	29.20	0.018
150	29.28	0.061
100	29.25	0.053

Table 4-9. Quantification cycle mean (Cq mean) of amplification curves obtained in Qβ-specific RT-qPCR using two primer concentrations and the probe concentration of 200 nM. Amplification conditions are specified in section 4.2.2.2. The Qβ RNA diluted at 10^{-4} was used as a template. Standard deviation (SD) was calculated using Microsoft® Excel®.

	Qβ RNA 10^{-4}	
Primer conc, nM	Cq mean (n=4)	Cq SD
700/700	27.85	0.179
500/500	27.4	0.058

Table 4-10. Quantification cycle mean (Cq mean) obtained in QB-specific RT-qPCR with 500 nM Qß primers, 200 nM probe and different annealing temperatures. The standard Qβ RNA diluted at 10^{-4} was used as a template. Amplification conditions are specified in section 4.2.2.2.

	Qß RNA 10^{-4}	
Annealing T°	Cq mean	Cq SD
63.3	27.27	0.339
61.8	27.02	0.05
60.0	26.93	0.104
57.9	26.83	0.039
56.1	27.03	0.024

4.3.6. Qß RT-qPCR performance

Using a selected primer concentration of 500 nM and 200 nM concentration of the probe, the Qß PCR assay was linear (R^2=0.999) over a range of serially diluted Qß RNA (10^{-1}–10^{-7}) (Figure 4-10). The mean amplification efficiency of the two standard curves was 102.3 %, within the acceptable range of 90-110 % (Figure 4-10).

Intra-assay variability of two runs (Qß RNA dilutions 10^{-1} to 10^{-8}) was within a mean Cq SD of 0.240 (CV = ≤ 2.13 %) and 0.190 (1.43%) across all dilution points (Table 4-11). The inter-assay reproducibility of two runs had a Cq mean CV between 0-1.15%, with an overall mean of 0.38%.

The Qß RT-qPCR had a linear dynamic range between 1.0 × 10^0 to 1.0 × 10^8 copies/reaction using the R3+ standard and an observed detection limit between 1-10 copies (Table 4-12). Blastn searches to examine the *in-silico* specificity of the Qß primers showed homology to Qß bacteriophage sequences.

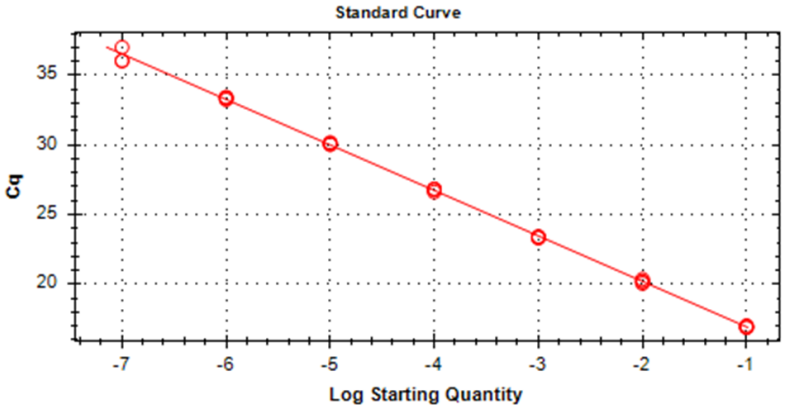


Figure 4-10. Representative amplification plot of the Qß RT-qPCR assay using seven dilution points (10^{-1} - 10^{-7}) of Qß RNA (n = 4). The mean of two runs was: efficiency = 102.3 %, R^2 = 0.999, slope = -3.27.

Table 4-11. Intra-assay repeatability and inter-assay reproducibility of the Qß RT-qPCR. Each dilution of the Qß RNA template was tested in four replicates. All generated Cq values except the 10-8 dilution (2/4) of run 2. Mean quantification cycle (Cq), standard deviation (SD), and coefficient of variation (CV) were calculated using Microsoft® Excel®.

	Run 1			Run 2			Inter-run variability		
Qß RNA	Cq Mean	Cq SD	CV, %	Cq Mean	Cq SD	CV, %	mean Cq	Cq SD	CV, %
10^{-1}	16.75	0.073	0.44	16.87	0.242	1.43	16.81	0.085	0.50
10^{-2}	20.04	0.135	0.67	20.04	0.065	0.32	20.04	0.000	0.00
10^{-3}	23.21	0.045	0.19	23.31	0.043	0.18	23.26	0.071	0.30
10^{-4}	26.58	0.089	0.33	26.61	0.076	0.29	26.60	0.021	0.08
10^{-5}	29.92	0.089	0.30	30.04	0.08	0.27	29.98	0.085	0.28
10^{-6}	33.21	0.070	0.21	33	0.216	0.65	33.10	0.148	0.45
10^{-7}	35.98	0.594	1.65	35.84	0.455	1.27	35.91	0.099	0.28
10^{-8}	38.56	0.823	2.13	37.94	0.312	0.82	38.25	0.438	1.15

Table 4-12. Analytical sensitivity of the Qβ phage RT-qPCR using the selected primer (500 nM) and probe (200 nM) concentrations. Amplification parameters are described in section 4.2.2.2. SD=standard deviation.

R3+ RNA transcript	**Qβ singleplex assay**	
copies/reaction	Cq mean (n=3)	SD mean
1×10^8	15.00	0.059
1×10^7	17.79	0.145
1×10^6	21.49	0.637
1×10^5	24.39	0.024
1×10^4	27.58	0.168
1×10^3	30.75	0.016
1×10^2	33.55	0.441
1×10^1	36.62	0.504
1×10^0	37.77	0.846

4.4. Discussion

This chapter describes the development of a synthetic RNA (R3+) that can be used as a positive control in RT-qPCR assays targeting eight viral pathogens. All eight viral pathogens can produce similar clinical signs in infected animals. Hence, clinical specimens from suspect cases may need to be tested for any combination of these viruses. The availability of a universal positive control has the potential to streamline such testing. However, the main

driver of the generation of this control in the context of the current project was the need for a suitable positive control for use in a pen-side multiplex RT-qPCR for simultaneous detection of FMDV, BVDV-1, and BVDV-2 as described in Chapter 5. Considering the limited reaction capacity of a portable PCR platform, the positive control was designed to satisfy two crucial measures of validity of a PCR run. The first measure was to ensure that the PCR could detect specific viral targets. The second measure was controlling for the viral nucleic acid extraction efficiency and PCR inhibitors' presence. To fulfil these requirements, a synthetic PC (R3+) transcript containing fragments of genomic RNA of viruses of interest (FMDV, BVDV-1, and BVDV-2), exogenous QB phage RNA and a house-keeping gene (18S ribosomal) RNA was built using the recombinant technology and *in vitro* transcription of the DNA construct. The R3+ construct also included other vesicular disease-causing viruses (SVDV, VESV, SVV, and VSV NJ and Ind strains) so that it could be used as a universal positive control for the detection of all eight pathogens in the standard singleplex laboratory-based RT-qPCR tests currently offered by AHL-MPI (Table 4-1).

Synthetic controls circumvent the handling of infectious viruses to generate positive control. The R3+ universal PC was designed and produced by artificially linking a series of virus fragments (Figure 4-3) based on published sequences. The concatenated fragments were then cloned into a plasmid (Figure 4-5). This PC approach has been used previously in various studies, including validating FMDV RT-qPCR (Vandenbussche et al., 2016) and developing PCR assays for emerging vector-borne diseases in the midwestern United States (Warang et al., 2021). Vandenbussche et al. (2016) used *in vitro* transcribed synthetic RNA (306 bp) with two partial FMDV regions (3D and 5' UTR) to optimise two RT-qPCR methods using 5'-tailed primers. Warang et al. (2021) employed commercially synthesised naked DNA and RNA fragments (gBlock, IDT) as PCs to develop PCRs for the Bourbon virus, Heartland virus, West Nile virus and *Trypanosoma cruzi*. During the onset of the COVID-19 pandemic, assay developers relied on the initially published SARS-CoV-2 genetic sequences to create synthetic transcripts of the orf 1 (Feng et al., 2020); and E and RdRp genes (Corman et al., 2020) for validating the COVID-19 assays. The utility of synthetic controls to rapidly introduce PCRs for new emerging diseases is highly beneficial in supporting biosafety and biosecurity of disease-free countries such as New Zealand (Whiley et al., 2010).

Changes to the viral sequences at selected nucleotides within the R3+ were designed to help distinguish each PC component from the wild-type virus (Table 4-4). For instance, the FMDV 3D fragment of R3+ had four changed nt (GCTA) at positions 7500-7503.

Inadvertent cross-contamination may happen during a PCR run. The ability to discriminate the FMDV 3D PC from the field virus by sequencing prevents the prohibitive cost of false positive classification. However, one drawback of a synthetic PC construct is that mutation in the primer binding sites may require a corresponding modification in the sequences of the PC to maintain specificity. Alterations can be done easily through *de novo* site-directed mutagenesis of the plasmid insert (Kodani & Winchell, 2012). The synthetic PC, similar to native nucleic acid templates, is also susceptible to nuclease activity. One transcription reaction of the R3+ insert, however, can produce enough PCs for thousands of PCR reactions, such that the R3+ can be aliquoted into smaller volumes to limit degradation. The PC is replaced when the observed Cq is not within the expected range.

A Qß phage RNA was included in developing the R3+ transcript to allow the use of QB phage as an exogenous IC. Such an approach can be used to control for the presence of PCR inhibitors (if QB RNA is added to the samples) or to control both for the presence of PCR inhibitors and extraction efficiency (if QB phage is added to the samples at the beginning of the nucleic acid extraction process). The developed singleplex Qß RT-qPCR had high efficiency (102 %), high linearity (R2 >0.999) and good intra and inter-assay reproducibility, showing that this assay was suitable for detecting a Qß RNA IC target. Unlike commercially produced synthetic nucleic acid transcripts, which are prone to nuclease degradation, the Qß bacteriophage RNA is protected by a coat protein, making it more stable when spiked to test samples. Also, although the endogenous IC (e.g., 18S ribosomal RNA) has the advantage of representing the sample host, endogenous gene mRNA is present in abundance such that it can be amplified even in the presence of PCR inhibitors. An established amount of exogenous Qß IC material with corresponding Cq value can efficiently validate individual reactions in the PCR process, increasing the veracity of a negative call.

In summary, in this study, a universal non-infectious R3+ PC was engineered that can be used in either singular or multiplex RT-PCR assays to detect several vesicular disease pathogens. The described procedures can also be used to custom-design synthetic PCs for other disease targets. Also, an optimised RT-qPCR assay for an exogenous Qß IC was developed. The exogenous Qß IC system is versatile because it can be used in any PCR assay to detect an RNA target. The R3+ PC, including Qß IC, was adopted as a control strategy for developing a pen-side multiplex RT-qPCR as presented in Chapters 5, 6 and 7.

4.5. References

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Chapter 5. Development of a multiplex RT-qPCR assay for detection of foot and mouth disease virus, bovine viral diarrhoea virus type 1 and type 2 in New Zealand cattle

5.1. Introduction

Foot and mouth disease is a highly contagious viral disease affecting cloven-hoofed animals, including farm animals and wildlife animals such as African buffaloes, impala, and gazelles (Thomson et al., 2003; Vosloo et al., 2002). The FMDV, an aphthovirus, causes disease of various severity in susceptible animals. Acute FMDV infection in cattle and pigs produces typical lesions of lethargy, fever, and vesicular lesions of the mouth, feet, and teats. In contrast, FMD in sheep, goats, and deer is typically mild. FMD-affected animals can also manifest other clinical signs, including salivation, pain, and lameness due to painful erosions of mucosal surfaces in the mouth or interdigital skin. Due to its contagiousness, FMD has high morbidity. However, other diseases can present with a similar clinical appearance to FMD, including vesicular stomatitis, bluetongue, and swine vesicular disease. These diseases when present, can confound the clinical diagnosis of FMD (Hindson et al., 2008). In New Zealand, two endemic diseases, the mucosal disease (MD) form of BVDV-1 infection and malignant catarrhal fever (MCF) due to OvHV-2 infection, may be confused with FMD. Animals affected by either of these two diseases also present with mucosal ulcerations of the lips, tongue, dental pads, gingival margins, and hard palate, akin to late FMD lesions (Baker, 1995), although the disease patterns differ from that of FMD. Some isolates of BVDV-2, although exotic to New Zealand, also induce ulcerations in the mouth and the hoof coronary band in cattle (Malacari et al., 2018).

Field testing of cattle with suspected FMD could support the rapid diagnosis of such a highly consequential disease in New Zealand. Pen-side testing eliminates the lag time of the transport of samples to the laboratory, thus shortening the sample-to-result turn-around time. A positive pen-side test reinforces clinical diagnosis, triggering immediate implementation of FMD incursion controls. Various reports demonstrated the potential application of the pen-side PCR test for rapid disease diagnosis of African swine fever (Daigle et al., 2021), FMD (Howson et al., 2017), and Bluetongue (Ambagala et al., 2015). However,

most molecular detection-based pen-side assays in the literature are in a single pathogen assay format and lack field validation data. The availability of a pen-side test that detects several pathogens can facilitate the diagnosis of a causative agent during an investigation of an outbreak of a disease with clinical signs common to infection with multiple agents, such as vesicular lesions.

qPCR assays generate faster results than conventional laboratory test methods (e.g., virus isolation, serology, microscopy) (Hoffmann et al., 2009). The use of a hybridisation probe, such as the 5' nuclease hydrolysis probe, allows the sensitive and specific identification of pathogens (Bhudevi & Weinstock, 2001). The hydrolysis probe or TaqMan is a short oligonucleotide with a reporter fluorescence dye at the 5' end and a quenching dye at the 3' end. During PCR amplification, the reporter dye is separated from the quenching dye due to the hydrolysis of the hybridised probe by *Taq* polymerase (Espy et al., 2006). The unquenched fluorescence dye emits a light signal measured after each amplification cycle by the PCR device. Since various fluorescence dyes emit different spectrums of colours, multiplexing PCR assays with several probes of different fluorophores can be used to detect several pathogens simultaneously in one reaction. Thus, the use of multiplex PCR assays can improve disease surveillance of related pathogens (Brault et al., 2015; Parodi et al., 2021) or diagnostic screening of systemic diseases with possible multiple causes, for example, avian respiratory disease complex (Nguyen et al., 2013), reproductive syndrome in pigs (Yue et al., 2009), or vesicular disease in multiple species (Fernandez et al., 2008).

Therefore, the aim of the work presented in this chapter was to develop a multiplex reverse transcription qPCR (mRT-qPCR) assay to support the clinical investigation of FMDV incursion in New Zealand. The pen-side multiplex assay included four targets: BVDV-1 as an endemic virus that is the main differential for FMDV infection in New Zealand, exotic BVDV-2, and Qß bacteriophage as an internal amplification control.

5.2. Materials and methods

Assay development and the demonstration of analytical performance were aligned with the WOAH's Manual for Assay development (WOAH, 2022) and the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009).

5.2.1. Multiplex assay design

Primers and probes homologous to the FMDV 3D polymerase gene (Callahan et al., 2002), as well as the 5' untranslated region (5' UTR) of BVDV-1 and BVDV-2 (Willoughby et al., 2006), were selected to form the multiplex PCR assay. The FMDV assay, which can detect all seven serotypes of FMDV, and both BVDV singleplex RT-qPCRs, have been validated and used as singleplex diagnostic assays at AHL-MPI (New Zealand) for the past five years (Appendix 1 and 2). A fourth primer pair amplifying a fragment of the Qβ major coat protein gene (Chapter 4) was incorporated into the multiplex format to serve as an internal amplification control.

Primers and probes are listed in Table 5-1**.** Each probe was labelled with a fluorophore and a quencher combination supported by the T-COR 8™ Real-time PCR Thermocycler (Tetracore), referred to as "T-COR" in this chapter.

5.2.2. Viruses, RNA standards, and clinical samples

Purified RNA from inactivated serotype O (O Manisa 10/6/10) FMDV (Pirbright Institute), BVDV-1 (Bovax TVL strain passage 4 in bovine lungs, AHL), BVDV-2 (97/730 passage 4 in bovine lungs, AHL), and Qβ bacteriophage(Qβ) (Attostar) were used for the initial verification and optimisation of singleplex assays.

A synthetic RNA transcript (R3+) described in Chapter 4 was used to generate standards for multiplex assay optimisation. The R3+ contained multiple target sequences and was designed as a universal RT-qPCR control for several targets, including FMDV, BVDV-1, BVDV-2, and Qβ internal control. Predetermined dilutions (2.0×10^{-1} to 2.0×10^{7} copies per µL PCR template) of R3+ were prepared in RFW immediately before use. The 5 µL R3+ template (1.0×10^{0} to 1.0×10^{8} copies) was used per reaction.

The analytical specificity panel consisted of seven serotypes of inactivated FMDV (A5 Allier, O Manisa, Asia 1 Shamir, C1 Noville Batch 1, SAT1/BOT1/68, SAT2/ZIM/5/81 and SAT3/ZIM/4/81) (Pirbright); vesicular stomatitis virus serotypes New Jersey (type NJ Batch 1) and Indiana (type Ind-1 Batch 1 (Pirbright); two isolates of infectious bovine rhinotracheitis virus (05-323/1 and 06-2553, AHL), BVDV-1 (C24v and Bovax, AHL), BVDV-2 (97/730 and

97/1273B, USA) and border disease virus (10-1118/2 and 91-5809, AHL); an orf virus (15-363, AHL) and bovine papillomavirus (79-6129, AHL); as well as selected bacterial pathogens endemic in New Zealand such as Theileria buffeli (906/12, AHL), Theileria chitose (906/12, AHL) and Mycoplasma bovis (ATCC 255). The panel also included a Qβ bacteriophage (Attostar 1) and a cattle serum and oral swab in VTM (1:10) that had previously tested negative for BVDV-1 in an antigen ELISA at AHL-MPI.

5.2.3. Viral RNA extraction

Viral nucleic acids were either extracted from a 140 µL sample using the QIAamp Viral RNA manual kit (Qiagen) or from a 200 µL sample using the Magjet Viral DNA and RNA Purification high throughput extraction kit (Thermo Fisher Scientific) on the KingFisher™ Flex (Thermo Fisher Scientific) purification system machine, following the manufacturer's instructions.

Table 5-1. Primers and probes with corresponding reporter dyes and quenchers that were used in the four-target multiplex RT-qPCR. The FMDV 3D hydrolysis probe had a 5' FAM (6-carboxyfluorescein) label with a non-fluorescent QSY quencher (Thermo Fisher Scientific). The BVDV-1 and BVDV-2 probes were labelled with Dragonfly orange (DFO, Eurogentec) and Cy5 (Eurogentec) reporter dyes, both with minor groove binders (MGB Eclipse, Eurogentec). The Qβ probe was labelled with 5" Texas Red dye and a 3' black hole quencher 2 (BHQ-2, Eurogentec). The primers and probes for FMD 3D assay (Callahan et al., 2002) and BVDV assay (Willoughby et al., 2006) have been reported previously. The primers and a probe for a QB assay have been designed as part of the current study in Chapter 4.

Primers / Probe	Target	Sequence, 5" to 3.'	Base Position	Amplicon size	GenBank Accession
FMD forward primer (FP)	3D gene	ACT GGG TTT TAC AAA CCT GTG A	6769-6791	106	AF189157
FMD reverse primer (RP)		GCG AGT CCT GCC ACG GA	6875-6858		
FMD probe		FAM-TCC TTT GCA CGC CGT GGG AC-QSY	6820-6840		
BVDV1_2 FP	5' UTR region	CCA TGC CCT TAG GAC TAG C	76-97	92	NADL genome
BVDV1_2 RP		TGA CGA CTA CCC TGT ACT CAG G	176-198		
BVDV1MGB probe	BVDV 1	DFO-AAC AGT GGT GAG TTC GT-MGB Eclipse	145-161		
BVDV2MGB probe	BVDV 2	Cy5-ACT AGC GGT AGC AGT GAG-MGB Eclipse	109-126	122	
Qβ FP - 1,574	Qβeta phage major protein coat gene	CAA ACG GTT CTT GTG ACC CA	1574-1594	93	FJ483843
Qβ RP 1,666		AAG CTC GTT CCT CAT CGG TA	1666-1646		
Qβ Probe 1,602		Texas red -TCG CCA GGC ATA TGC TGA CGT - BHQ-2	1602-1623		

5.2.4. Portable PCR machine

The T-COR is a portable, field-ready PCR device of about 4.5 kg in weight that can be operated from a battery for up to four hours (Figure 5-1). This field PCR machine was selected based on the criteria outlined in Chapter 2. The T-COR has eight independently programmable amplification wells that can be used for simultaneous runs using different protocols. It is compatible with the following dyes: FAM, DFO, Texas Red and Cy5, which allow the simultaneous amplification of multiple targets in a single reaction tube. While the PCR assay runs, the amplification curve can be viewed in real-time. Quantification cycle (Cq) values are shown in tabular format via the built-in software after a run is finished. A linear format of the amplification curve is also displayed, where the intersection point of the perpendicular line and the exponential phase of the amplification curve represents the cycle threshold. A logarithmic view of the amplification curve is unavailable in the T-COR output.

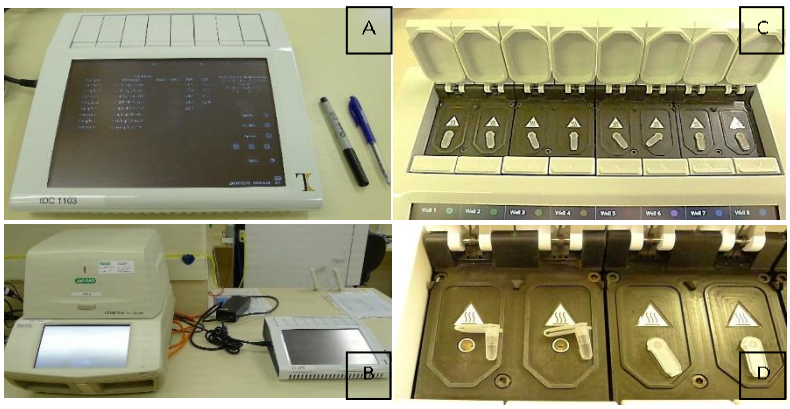


Figure 5-1. The T-COR 8™ Real-time PCR Thermocycler (Tetracore) (A) and a standard laboratory PCR machine (CFX96, BioRad), (B). The TCOR 8™ has eight independent wells capable for running multiple PCR protocols, (C, D) simultaneously. Each well has four specific channels for detecting FAM, Texas Red, DFO and Cy5 dyes in a multiplex assay.

5.2.5. RT-qPCR optimisation

Re-optimisation of the three RT-qPCR tests (FMDV, BVDV-1 and BVDV-2) that had been originally developed for use by the MPI diagnostic laboratory as singleplex assays on the CFX96 machine was performed due to the change of the PCR master mix and labels for the probes. The optimisation was focused on establishing satisfactory primer and probe concentrations and was initially carried out using the CFX96 PCR thermocycler (BioRad Laboratories), also referred to as "CFX96". After each assay had been optimised, these were combined into a multiplex RT-qPCR (mRT-qPCR) for simultaneous detection of all four

targets. Finally, the mRT-qPCR assay was transferred to the T-COR for verification and validation.

Each PCR optimisation experiment included a no-template control (NTC).

5.2.5.1. Singleplex assay optimisation

Optimisation of the three singleplex RT-qPCR assays was conducted according to the Real-time PCR Applications Guide (Anonymous, 2006) using the 96-well plate format of the CFX96 machine. The plate format enabled the evaluation of multiple conditions simultaneously, which would have been impossible to do using the eight reaction wells of the T-COR. Each reaction consisted of a 5 µL 4× Taqman® Fast Virus 1-step master mix (Thermo Fisher Scientific); varying concentrations of primers and probes; 5 µL viral RNA template, and RFW to a final volume of 20 µL. Samples were tested in quadruplicate. The cycling parameters followed the Taqman® Fast Virus 1-step master mix manufacturer's recommendation: 5 min at 50°C for the reverse transcription (RT) step, 20 s at 95°C RT for inactivation/initial denaturation, and 40 cycles of 3 s at 95°C (denaturation) and 30 s at 60°C (annealing/extension).

An annealing temperature of 60°C that had been determined during the original optimisation of each diagnostic assay used at AHL-MPI (FMDV, BVDV-1, BVDV-2, and Qβ) (Appendix 1 and 2) was used with no further optimisation through the temperature gradient. The PCR results were analysed using the Bio-Rad CFX Manager 3.1 software with an auto-calculated baseline threshold function.

Suitable primer and probe concentrations were selected based on the lowest Cq value, good replicate repeatability, and a robust or reasonable fluorescence intensity expressed as relative fluorescence unit (RFU) by the CFX96 software. After determining the primer and probe concentrations, a standard curve was constructed utilising ten-fold dilutions of a viral RNA template to evaluate the PCR performance. An optimised, robust, and reproducible qPCR was expected to have evenly spaced amplification curves, a linear regression line or a coefficient of determination (R^2) > 0.98, high amplification efficiency (90-110%) and good repeatability among replicates. Amplification efficiency (*E*) was obtained based on the formula: $E = 10^{(-1/slope)}-1$ (Bio-Rad CFX Manager 3.1 software). The standard deviation (SD) of

the mean Cq for each standard was used to express the intra-assay variability with the target value of ≤0.5.

5.2.5.1.1. FMDV PCR

Seven concentrations of both forward and reverse 3D primers (800, 700, 600, 500, 400, and 300 nM) were evaluated using 300 nM of FAM labelled probe. This initial probe concentration was based on the reference FMDV RT-qPCR assay (Appendix 1). After selecting the best primer concentration, probe concentrations of 150, 200, 250, and 300 nM were examined with the optimal primer concentration. The selection of FMDV RNA dilution was based on the Cq obtained (around Cq = 26) in a previous run using the original AHL assay (Appendix 1). An FMDV RNA template at 10^{-4} dilution (section 5.2.2) was used in all optimisation experiments.

5.2.5.1.2. BVDV-1 and BVDV-2 PCR

The forward and reverse primers targeting sequences common to BVDV-1 and BVDV-2 were first optimised with the BVDV-2 MGB probe with Cy5 fluorophore. Four primer pair concentrations (700, 600, 500, 400, and 300 nM) were assessed using the original AHL assay's 100 nM BVDV-2 probe concentration (Appendix 2). This was followed by BVDV-2 probe optimisation using 100, 150, 200, 250, and 300 nM with primers at the optimal concentration selected. A BVDV-2 RNA was used as a template at 10^{-3} dilution with an approximate Cq of 31 (based on earlier analysis using the original assay as described in Appendix 2).

The BVDV-1 primers (700, 600, 500, and 400 nM) were titrated using a 100 nM DFO labelled MGB probe. The BVDV-1 optimal primer concentration was then used to assess the probe at 100, 150, 200, 250, and 300 nM using BVDV-1 RNA at 10^{-3} dilution as a template. The BVDV-1 RNA had a Cq of 30 at this dilution when analysed with the original AHL assay (Appendix 2).

5.2.5.2. Multiplex RT-qPCR optimisation

The four optimised singleplex assays were assembled into a multiplex format, and the performance of mRT-qPCR was assessed using a mixture of viral RNA targets as a template (Table 5-2). The multiplex PCR was performed in parallel with the four singleplex assays in

the same 96-well plate, with all samples tested in quadruplicate. The amount of each target RNA in the mixture was expected to produce a Cq value between 25 and 30 based on prior testing in the relevant singleplex assay.

The reaction mixes for the singleplex RT-qPCR assays were the same as that for the multiplex assay, except that RFW was used in place of the "absent" primers and probes. Cycling conditions and parameters for the mRT-qPCR were the same as for the singleplex assays (section 5.2.5.1). The performance of the multiplex assay was considered optimal if the Cq value of the singleplex and multiplex RT-qPCR for the same target was similar (Anonymous, 2006).

A 10× serial dilution of R3+ was used as standard to create the standard curve. The standard curve was constructed to compare amplification efficiency and intra-assay repeatability between the singleplex and multiplex PCR assays for each target. Efficiency and intra-assay repeatability were analysed as described in section 5.2.5.1.

Table 5-2. Each viral RNA was diluted to the concentration that produced the pre-set Cq values in the initial RT-qPCR runs using AHL diagnostic assays (Appendix 1 and Appendix 2). The RNA mixture was initially used to assess the performance of the multiplex RT-qPCR.

Viral RNA template	Volume, µL	Final dilution	Expected Cq
QB	20	10^{-5}	31
FMDV	20	10^{-5}	29.68
BVDV-1	20	10^{-3}	28.34
BVDV-2	20	10^{-2}	29.06
TE buffer	120		
total volume	200		

5.2.5.3. mRT-PCR optimisation on the T-COR

The optimised mRT-qPCR assay was transferred to the T-COR, and the assay's performance was initially determined using the R3+ standards. The R3+ standards (1.0×10^1 to 1.0×10^7 copies/reaction) were tested in singleton in three runs, utilising seven out of eight reaction wells of the portable PCR machine. One well was always used for the NTC. A standard curve was created from the three-experiment data, and the mRT-qPCR efficiency was calculated using the formula described in section 5.2.5.1. However, because the efficiency of the optimised multiplex PCR on the CFX96 machine was higher than that on the T-COR, further optimisation of the singleplex assays was conducted on the portable device.

Due to the limited number of reaction wells in T-COR (n=8), re-optimisation of primers and probe concentrations was done in a stepwise approach according to the results of each experiment. In triplicate, two R+ standards (1.0×10^5 and 1.0×10^4 copies/reaction) were used in all optimisations runs. The PCR reactions were set up according to the parameters described in section 5.2.5.1 but with slight changes in the denaturation time from 3 s to 10 s, and the number of cycles was increased from 40 to 45. Each PCR reaction consisted of 5 µL of 4× Taqman® Fast Virus 1-step master mix, primers, and probes at various concentrations, 5 µL R3+ template, and RFW to a final volume of 20µL.

5.2.5.3.1. FMDV mRT-PCR T-COR optimisation

Following the manufacturer's recommendation for primer concentrations to be used with Taqman® Fast Virus 1-step master mix, four combinations of forward/reverse FMDV-specific primers (900/900, 900/400, 400/900, and 400/400 nM) were tested with a fixed concentration (250 nM) of the probe. This was followed by testing three probe concentrations (300, 250 and 150 nM) with the selected optimal concentration of primers.

5.2.5.3.2. BVDV-1 mRT-PCR T-COR optimisation

The following forward/reverse primer combinations (900/900, 900/400, 400/900, 400/400, 300/300, and 250/250 nM) were tested with the probe concentration of 250 nM for the BVDV-1 assay. Three BVDV-1 probe concentrations (250, 100, and 50 nM) were then tested with two selected combinations of primer concentrations (300/300 and 250/250 nM).

5.2.5.3.3. BVDV-2 mRT-PCR T-COR optimisation

Three concentrations of the BVDV-2 probe (150, 100, and 50 nM) were examined using the optimal concentrations of the forward and reverse primer selected during the optimisation of the BVDV-1 reagents (section 5.2.5.3.2).

5.2.5.3.4. Qβ T-COR optimisation

Only two Qβ primers (forward/reverse)/probe concentrations (250/250/100 nM and 150/150/50 nM) were assessed. The goal was to find the lowest Qβ primer concentration that would still produce a clear positive signal even if the RFU was comparatively low. The

advantage of limiting the primer concentration for the exogenous internal control was to minimise competition for reagents during the co-amplification of pathogen-specific PCR products.

5.2.6. Validation of the mRT- qPCR on the T-COR

The mRT-qPCR performance was assessed by determining the assay's analytical sensitivity, linearity, precision, and repeatability compared with the same parameters for each singleplex assay. Linearity was assessed based on the analysis of the standard curves (calculated reaction efficiency and R^2 values) generated from the standards. Analytical sensitivity was evaluated based on the lowest levels of the detectable target from the same standard curves.

Precision was evaluated by calculating intra-assay variability based on the distribution of Cq values for replicates of each standard in a single PCR run (section 5.2.6.2). Repeatability between runs was assessed by calculating inter-assay variability by comparison of the Cq values obtained for the same standards in 8 separate PCR runs (section 5.2.6.2). The intra- and inter-assay variability were expressed as CV, which was calculated as $CV = (SD/Cq\ mean) \times 100$.

Initial side-by-side comparisons between each optimised singleplex and the multiplex assay were performed on the T-COR using three replicates of one R3+ standard (1.0×10^6 copies/reaction). The Cq values were then compared and analysed for intra-assay variation and repeatability.

Amplification efficiencies of both singleplex and multiplex assays were examined using five R3+ standards (1.0×10^3 to 1.0×10^7 copies/reaction). The standards were tested in singleton four times in each singleplex RT-qPCR assay and three times in the multiplex mRT-qPCR assay. PCR runs were performed within a day to minimise inter-run variation. The optimised assays were expected to have a linear standard curve and acceptable amplification efficiency, as defined in section 5.2.5.1.

5.2.6.1. Analytical sensitivity and specificity

Analytical sensitivity of the singleplex and multiplex RT-qPCR assays was assessed using seven R3+ standards in singleton (1.0×10^0 to 1.0×10^6 copies/reaction) in three separate runs.

The mRT-qPCR specificity was determined by testing a panel of 15 RNA viruses, 7 DNA viruses and three bacteria (section 5.2.2). All samples, including RNA from cattle serum and oral swabs, were tested in duplicate for the analytical specificity trial.

5.2.6.2. Intra-assay and inter-assay variation

High, medium, and low copy numbers of R3+ standards (1.0×10^7, 1.0×10^5 and 1.0×10^3 copies/reaction) were tested in five replicates (one run per standard) on the T-COR. Intra-assay variation was assessed based on Cq values for each of the four targets described in section 5.2.5.1.

Two operators assessed inter-assay variation by testing five R3+ standards (1.0×10^4 to 1.0×10^8 copies/reaction) in singleton on different days for a total of eight runs. Inter-run repeatability was measured by calculating the coefficient of variation (CV) of the Cq values and the mean CV for each standard and target.

5.2.7. Data analysis

The Cq mean, SD of the Cq mean, % CV and 95% confidence interval (CI) of the summarised data were calculated using Microsoft® Excel® for Microsoft 365 MSO.

5.3. Results

5.3.1. Optimisation of primer and probe concentrations

A summary of Cq data for all singleplex optimisation experiments is shown in Table 5-3 and Table 5-4. The primer concentrations that produced the lowest Cq in the FMDV assay were 800 nM (Cq = 26.38), 700 and 600 nM (Cq = 26.49). The 700 nM primer concentration was selected for further evaluation with different FMDV probe concentrations. The 300 and 200 nM FMDV probe had similarly low Cq values (27.15 and 27.11), and the former was selected

for the optimised assay. For both BVDV-1 and BVDV-2 targets, a 500 nM primer concentration was chosen with a 100 nM probe concentration. The selection of the optimal Qβ phage primer and probe concentrations is described in Chapter 4 (500 nM of each primer and 200 nM probe).

Analysis of standard curves showed a strong linear correlation between the target concentration and the Cq value (average coefficient $R2 > 0.98$-0.99) in all singleplex RT-qPCR assays. Amplification efficiencies were FMDV= 105.8 %, BVDV-1=103.1 %, BVDV-2 = 109.7 %, and Qβ phage = 102.5% (Figure 5-2).

Table 5-3. Optimisation of primer concentrations (F= forward and R=reverse) of the three singleplex assays (foot and mouth disease virus = FMDV, bovine viral diarrhoea virus type 1 = BVDV-1, and BVDV type 2 = BVDV-2). The PCR was performed on the CFX96 machine according to the parameters and conditions described in section 5.2.5.1. Viral RNA (section 5.2.2) diluted to produce a predetermined Cq value was used as a template in all experiments. Results are presented as quantification cycle (Cq) values.

Primers, F/R nM	**FMDV RNA 10^{-4} Probe = 300 nM**		**BVDV-1 RNA 10^{-3} Probe = 100 nM**		**BVDV-2 RNA 10^{-3} Probe = 100 nM**	
	Cq mean, n=4	Cq SD	Cq mean, n=4	Cq SD	Cq mean, n=4	Cq SD
800/800	26.38	0.083	nt	nt	nt	nt
700/700	26.49	0.075	32.85	0.273	32.04	0.178
600/600	26.49	0.126	32.79	0.246	32.2	0.184
500/500	26.71	0.052	32.97	0.126	32.37	0.264
400/400	26.95	0.147	33.20	0.102	32.93	0.393
300/300	27.24	0.095	33.43	0.284	32.84	0.437

Table 5-4. Optimisation of probe concentrations of the three singleplex assays (foot and disease virus = FMDV, bovine viral diarrhoea virus type 1 = BVDV-1, and BVDV type 2 = BVDV-2) on the CFX96 machine using the selected optimal primer concentrations (Table 5-3). The PCR cycling conditions were described in section 5.2.5.1 with extracted viral RNA (section 5.2.2) used as a template. Results are presented as quantification cycle (Cq) values.

Probe concentration, nM	**FMDV RNA 10^{-4} Primers, F/R = 700 nM**		**BVDV-1 RNA 10^{-3} Primers, F/R = 500 nM**		**BVDV-2 RNA 10^{-3} Primers, F/R = 500 nM**	
	Cq mean, n=4	Cq SD	Cq mean, n=4	Cq SD	Cq mean, n=4	Cq SD
300	27.15	0.026	31.59	0.391	32.43	0.232
250	27.11	0.154	31.41	0.419	32.20	0.199
200	26.96	0.094	31.70	0.247	32.62	0.124
150	26.88	0.160	31.90	0.122	32.85	0.238
100	nt	nt	30.20	0.082	32.29	0.185

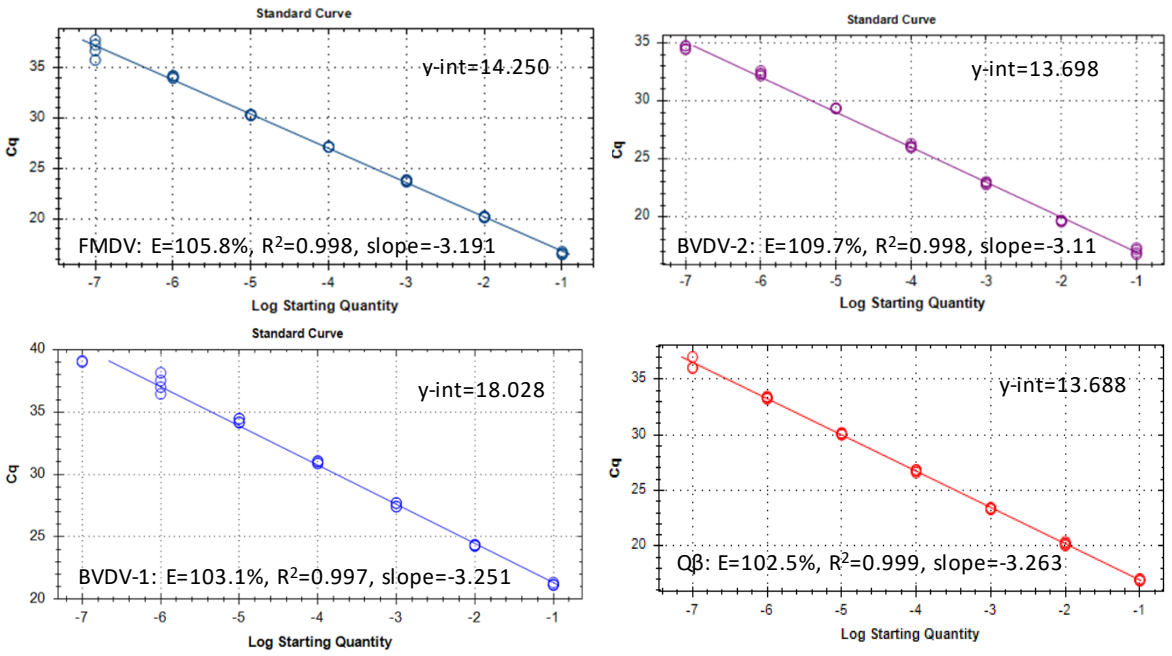


Figure 5-2. Standard curve analysis of the four singleplex assays (foot and mouth disease virus = FMDV, bovine viral diarrhoea virus type 1 = BVDV-1, and BVDV type 2 = BVDV-2) using the optimal primer and probe concentrations (Table 5-3 and Table 5-4). Ten-fold dilutions of relevant viral RNA (10^{-1} to 10^{-7}) were used as templates in quadruplicate. The PCR cycling conditions and parameters are detailed in section 5.2.5.1 and run were carried on the CFX96 machine.

The optimised singleplex assays (FMDV, BVDV-1, BVDV-2 and Qβ phage) were combined into the multiplex format on the CFX96 PCR machine. Using a mixture of RNAs from four targets as a template, simultaneous detection of the four targets was achieved by the mRT-qPCR (Figure 5-3). The mean Cq for each of the four targets in the mRT-PCR and the same target in the corresponding singleplex RT-qPCR were < 1 Cq different from each other (Figure 5-4). The repeatability of mRT-PCR was acceptable (Cq SD = < 0.5, CV = < 1%) for all targets (Figure 5-4).

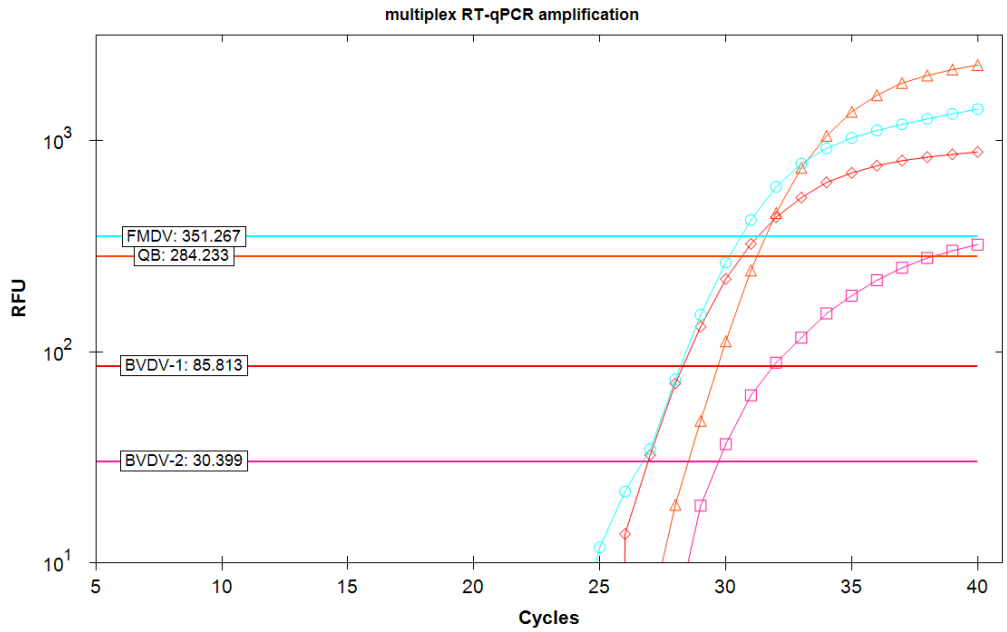
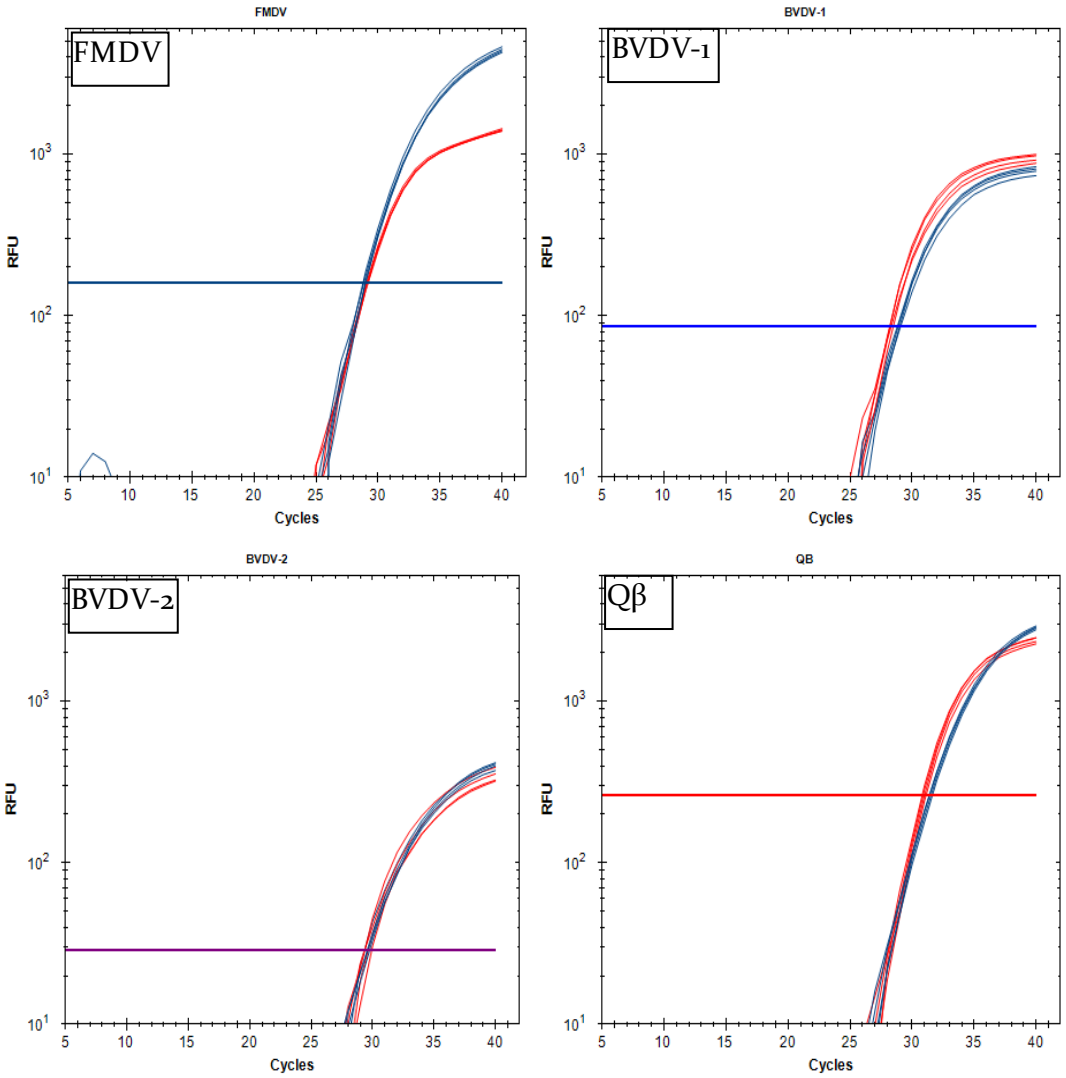


Figure 5-3. Verification of the multiplex RT-qPCR assay using the CFX96 machine. Amplification plots show the simultaneous detection of four targets (FMDV – ◯, BVDV-1 - ◇, BVDV-2 – □, and Qβ – △) after testing a mixed viral RNA template (Table 5-2). The horizontal lines are the baseline thresholds of each target determined automatically by the CFX96 software manager. RFU = relative frequency unit.

Efficiencies of the mRT-qPCR were within the 90% - 110% acceptable limits for all targets (FMDV = 100.1%, BVDV-1 = 97.1 %, BVDV-2 = 96.9% and Qβ = 101.0%) (Figure 5-5) when triplicates of the selected dilutions of the R3+ standard were assayed, which was comparable to the *E* of the corresponding singleplex RT-qPCRs (Figure 5-2). The final conditions of the optimised mRT-qPCR on the CFX96 PCR thermocycler are shown in Table 5-5. The NTC produced no Cq value in all optimisation reactions.



	Multiplex			Singleplex		
	Cq mean	SD	% CV	Cq mean	SD	% CV
FMDV	29.18	0.050	0.171	28.94	0.115	0.397
BVDV-1	28.23	0.116	0.411	28.9	0.133	0.460
BVDV-2	29.60	0.295	0.997	29.6	0.193	0.652
Qβ	31.04	0.132	0.425	31.56	0.117	0.371

Figure 5-4. Superimposed amplification curves of the multiplex (red) and singleplex (blue) RT-qPCR for the same targets using optimal primers and probe concentrations (section 5.3.1.) The template (Table 5-2) used was a mixture of viral RNAs (FMDV, BVDV-1, BVDV-2, and Qβ) and was analysed in quadruplicate on the CFX96 machine. Amplification plots overlapped during the exponential phase. The horizontal line in each graph indicates the threshold. The mean quantification cycle (Cq), standard deviation (SD) and % coefficient of variation (CV) for singleplex and multiplex RT-qPCR are shown underneath the plots. The Cq mean, SD of the Cq mean, and % CV were calculated using Microsoft® Excel® for Microsoft 365 MSO.

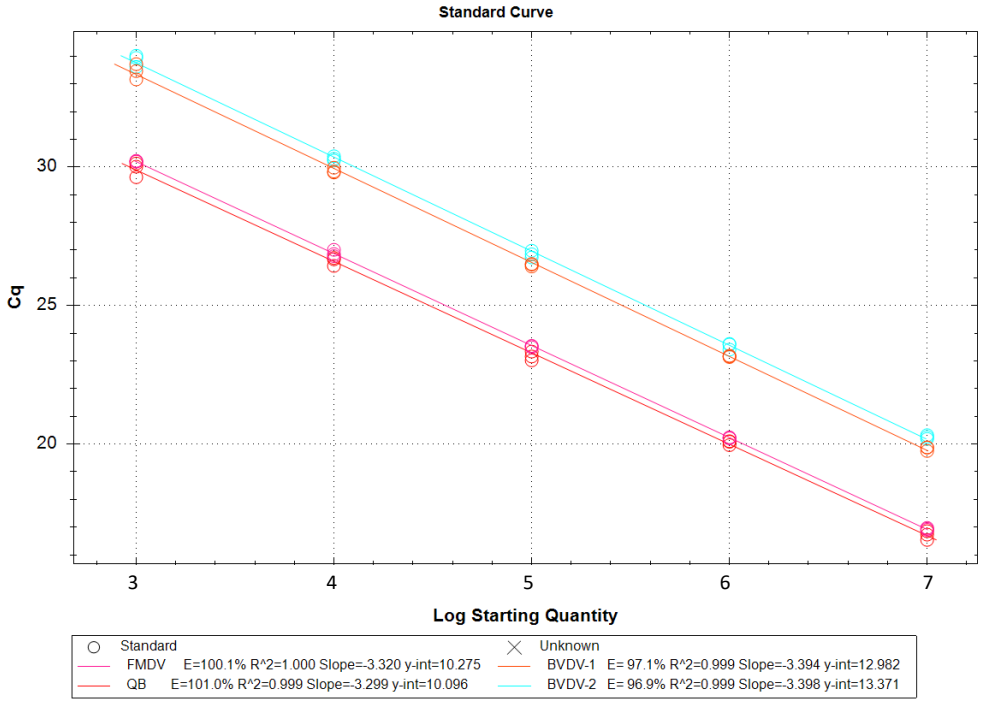


Figure 5-5. Standard curves were generated for each target within the multiplex RT-qPCR assay using the R3+ standards (1.0 × 10³ to 1.0 × 10⁷) shown as log quantity (x-axis). The PCR assay was performed on the CFX96 PCR machine using conditions described in ***Table 5-5****. Each dilution was tested in triplicate. PCR efficiencies (E) were all within the acceptable range (90-110%). The coefficient of determination (R^2) is ≥ 0.999 for each target.*

*Table 5-5. Optimised multiplex RT-qPCR parameters and conditions on CFX96 PCR machine (BioRad) with 40 cycles of denaturation and annealing/extension. * Taqman Fast Virus (Thermo Fisher Scientific).*

Reaction mix

component	concentration	volume, µL
RNase free water	-	1.8
4x master mix*	1 ×	5.0
50 nM $MgSO_4$	2.5 nM	0.0
10 µM FMDV 3D forward	700 nM	1.4
10 µM FMDV 3D reverse	700 nM	1.4
10 µM FMDV 3D probe	300 nM	0.6
10 µM BVDV-1_2 forward	500 nM	1
10 µM BVDV-1_2 reverse	500 nM	1
10 µM BVDV-1 probe	100 nM	0.2
10 µM BVDV-2 probe	100 nM	0.2
10 µM QB forward	500 nM	1
10 µM QB reverse	500 nM	1
10 µM QB probe	200 nM	0.4
PCR template	-	5.0
Volume per reaction		20

Cycling conditions

Step	T, °C **	Time	cycle no.
RT***	50	5 min	1
Hold	95	20 s	1
Denaturation	95	3 s	40
Annealing / Extension	60	30 s	

** T: temperature
*** RT: reverse transcription

5.3.2. Implementation of mRT-qPCR on the T-COR

The standard curve generated in the initial testing of mRT-qPCR on T-COR using the conditions optimised on the CFX96 machine (Table 5-5) with three R3+ standards produced sub-optimal PCR *efficiencies* (data not shown) hence the primer and probe concentrations were re-optimised.

Details of the primer and probe optimisation run on the T-COR are shown in Table 5-6. Although the mean Cq values were similar, the primers (forward/reverse) and probe concentrations that produced the lowest Cq were selected (900/400 nM and 150 nM) for the FMDV assay. The BVDV-1 and BVDV-2 primers and probe concentrations of 250/250/50 nM were considered optimal for multiplexing. Optimisation of Qβ primers and probe also produced low Cq at 150/150/50 nM concentrations.

The optimised mRT-qPCR conditions for the T-COR are shown in Table 5-7. Cycling parameters were as follows: 50°C for 5 min reverse transcription (RT), 95°C for 20 s initial denaturation, and 45 cycles of 95°C for 10 s denaturation and 60°C for 30 s annealing and extension.

Side-by-side testing of the mRT-qPCR and each singleplex RT-qPCR using one R3+ standard (1.0×10^5/reaction) showed similar Cq values for each target (Figure 5-6). Acceptable assay repeatability was also achieved (Cq SD = ≤ 0.15, CV = ≤ 0.67 %) across all targets.

Table 5-6. Optimising the concentrations of four primer pairs and probes (foot and disease virus = FMDV, bovine viral diarrhoea virus type 1 = BVDV-1, BVDV type 2 = BVDV-2, and Qβ = Q Beta phage) using the T-COR portable device. The PCRs were performed as described in section 5.2.5.1. Two R3+ standard concentrations (1 × 10⁵ and 1 × 10⁴ copies/reaction) in triplicate were used in all experiments. Results are expressed as quantification cycle (Cq) values. F= forward, R=reverse, SD = standard deviation, nM = nanomolar.

FMDV	R+ standard, Cq mean (n=3) (SD)	
	1 × 10⁵ copies/reaction	1 × 10⁴ copies/reaction
Primers, F/R nM	Probe = 250 nM	
900/900	23.33 (0.115)	26.33 (0.058)
900/400	23.03 (0.058)	26.13 (0.058)
400/900	23.53 (0.058)	26.50 (0.100)
400/400	23.43 (0.153)	26.66 (0.153)
Probe, nM	Primers, F/R = 900/400 nM	
300	23.43 (0.058)	26.47 (0.058)
250	23.40 (0.100)	26.53 (0.058)
150	22.93 (0.058)	26.07 (0.115)

Qβ	R+ standard, Cq mean (n=3) (SD)	
	1 × 10⁵ copies/reaction	1 × 10⁴ copies/reaction
Primer (F/R) and Probe, nM		
250/250/100	22.80 (0.100)	26.0 (0.173)
150/150/50	22.27 (0.058)	25.53 (0.115)

BVDV-1	R+ standard, Cq mean (n=3) (SD)	
	1 × 10⁵ copies/reaction	1 × 10⁴ copies/reaction
Primers, F/R nM	Probe = 250 nM	
900/900	25.17 (0.153)	27.87 (0.153)
900/400	24.87 (0.058)	27.57 (0.058)
400/900	25.17 (0.058)	27.83 (0.153)
400/400	22.13 (0.058)	25.17 (0.058)
300/300	21.70 (0.000)	24.83 (0.058)
250/250	21.57 (0.058)	24.80 (0.100)
Probe, nM	Primers, F/R = 250/250 nM	
150	21.50 (0.000)	24.60 (0.100)
100	21.50 (0.100)	24.50 (0.173)
50	21.30 (0.100)	24.53 (0.058)

BVDV-2	R+ standard, Cq mean (n=3) (SD)	
	1 × 10⁵ copies/reaction	1 × 10⁴ copies/reaction
Probe, nM	Primers, F/R =250/250 nM	
150	22.27 (0.058)	25.60 (0.173)
100	22.37 (0.153)	25.40 (0.100)
50	21.43 (0.058)	24.73 (0.058)

*Table 5-7. Optimised multiplex RT-qPCR parameters and conditions on the T-COR™ 8 PCR machine (Tetracore) with 45 cycles of denaturation and annealing/extension. * Taqman Fast Virus Mix (Thermo Fisher Scientific).*

Reaction mix

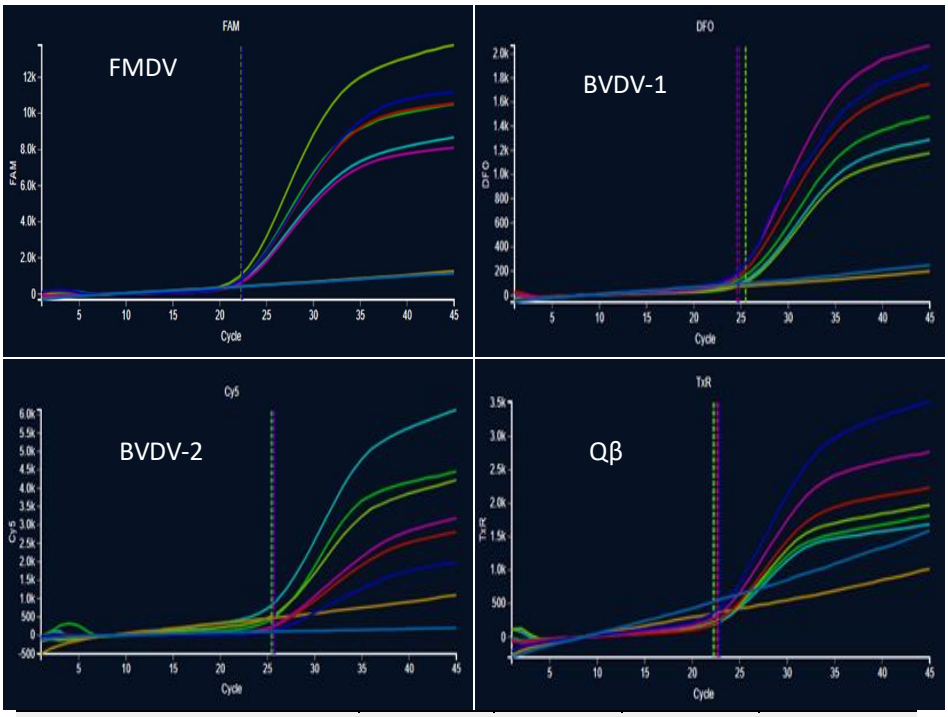
component	concentration	volume, µL
RNase free water	-	4.2
4x master mix*	1 ×	5.0
50 nM $MgSO_4$	2.5 nM	1.0
10 µM FMDV 3D forward	900 nM	1.8
10 µM FMDV 3D reverse	400 nM	0.8
10 µM FMDV 3D probe	150 nM	0.3
10 µM BVDV-1_2 forward	250 nM	0.5
10 µM BVDV-1_2 reverse	250 nM	0.5
10 µM BVDV-1 probe	50 nM	0.1
10 µM BVDV-2 probe	50 nM	0.1
10 µM QB forward	150 nM	0.3
10 µM QB reverse	150 nM	0.3
10 µM QB probe	50 nM	0.1
PCR template	-	5.0
Volume per reaction		20

Cycling conditions

step	T, ℃ **	Time	cycle no.
RT***	50	5 min	1
Hold	95	20 s	1
Denaturation	95	10 s	45
Annealing / Extension	60	30 s	

** T: temperature

*** RT: reverse transcription



RT-qPCR	FMDV	BVDV-1	BVDV-2	Qβ phage
singleplex				
Cq, mean of 3 replicates	22.30	25.50	25.50	22.27
SD	0.00	0.00	0.10	0.06
CV, %	0.00	0.00	0.39	0.27
multiplex				
Cq, mean of 3 replicates	22.40	24.70	25.70	22.73
SD	0.00	0.10	0.10	0.15
CV, %	0.00	0.40	0.39	0.67

Figure 5-6. Repeatability of the mRT-qPCR and singleplex RT-qPCR on the T-COR™ 8 device using an R3+ standard with 1.0×10⁵ copies/reaction (FMDV = foot and mouth disease virus, BVDV-1 = bovine viral diarrhoea virus type 1, BVDV-2, and Qβ). The PCR run conditions was specified in Table 5-7. The mean quantification cycle (Cq) value, standard deviation (SD) and % coefficient of variation (CV) for multiplex and singleplex RT-qPCRs are shown in the table below the amplification plots. Different wells are represented by different colours and the intersection of the perpendicular line and amplification plot represents the Cq value.

5.3.3. PCR efficiency

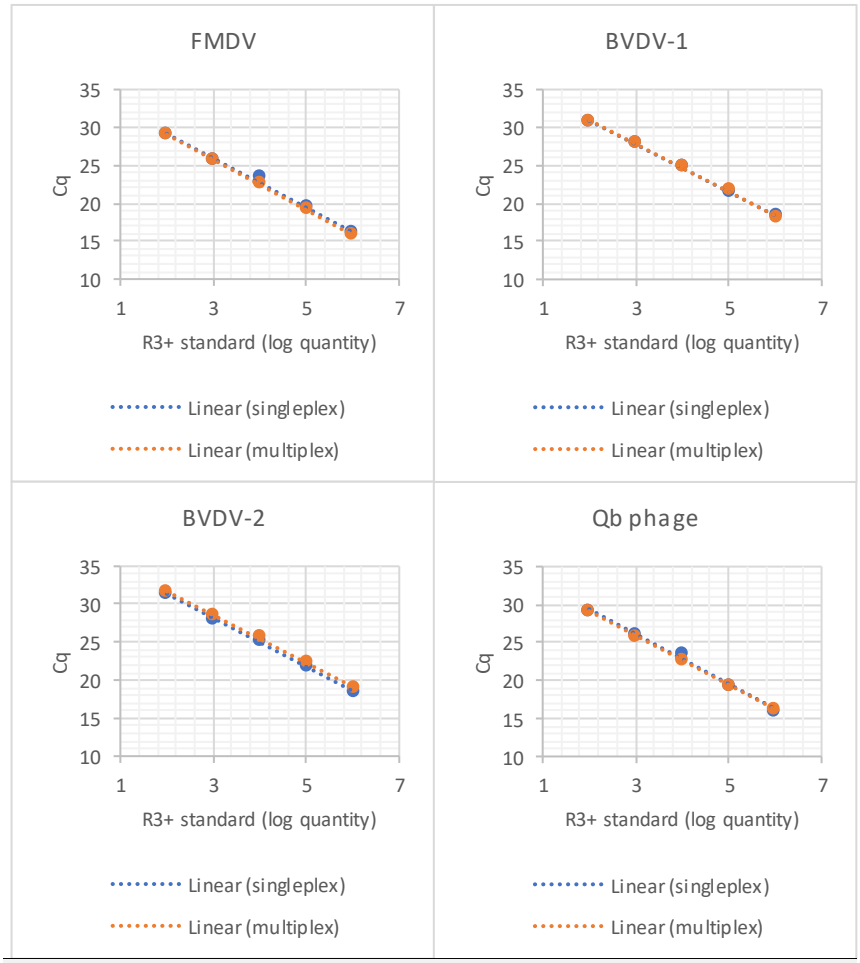
The mRT-qPCR amplification curve was linear for all four targets over a range of 1.0×10^3 to 1.0×10^7 copies/reaction R3+ RNA input. The R^2 of the singleplex assays ranged between 0.988 to 0.999 (Figure 5-7) and were similar to the R^2 of standard curves generated using the mRT-qPCR (FMDV: 0.999, BVDV-1: 0.999, BVDV-2: 0.998 and Qβ: 0.999). The mean efficiencies (±95% C.I.) obtained for both the singleplex (FMDV: 103±5.62, BVDV-1: 108±1.98, BVDV-2: 105.45±3.16 and Qβ: 101.78±3.15) and multiplex (FMDV: 101.35±5.62, BVDV-1: 107.71±6.91, BVDV-2: 108.2±3.05 and Qβ: 106.28±4.3) assays overlapped and were within the acceptable range of 90-110%. The linear dynamic range of the mRT-qPCR was from 1.0×10^3 to 1.0×10^7 copies per reaction.

5.3.4. Analytical sensitivity

The mRT-qPCR detection limits were 100 (FMDV), 100 (BVDV-1) and 1000 (BVDV-2 and Qβ) copies per reaction of the R3+ standard (Table 5-8). This was similar to the analytical sensitivity of singleplex assays with the same R3+ standards. Furthermore, 2/3 replicates of the R3+ template with 1 and 10 copies/reaction were positive for the FMDV 3D target, suggesting that the sensitivity of this target may extend beyond the conservative limit of 100 copies/reaction.

5.3.5. Analytical specificity

In the mRT-qPCR analytical specificity experiments (Table 5-9), all specific targets (seven FMDV serotypes, two isolates of BVDV-1, two isolates of BVDV-2, and Qβ phage) were correctly amplified. No cross-reaction was observed between the four targets. No false positives were observed with any samples containing non-target nucleic acids that may be present in field samples (Table 5-9).



RT-qPCR performance	FMDV	BVDV-1	BVDV-2	Qb phage
Singleplex				
slope, 95% CI	-3.34 ± 0.12	-3.13 ± 0.04	-3.20 ± 0.07	-3.28 ± 0.07
R^2, 95% CI	0.988 ± 0.019	0.999 ± 0.00	0.999 ± 0.00	0.994 ± 0.00
% Efficiency, 95% CI	103 ± 5.62	108.93 ± 1.98	105.45 ± 3.16	101.78 ± 3.15
Multiplex				
slope, 95% CI	-3.31 ± 0.12	3.14 ± 0.14	-3.14 ± 0.06	-3.24 ± 0.08
R^2, 95% CI	0.999 ± 0.019	0.999 ± 0.00	0.998 ± 0.00	0.999 ± 0.00
% Efficiency, 95% CI	101.35 ± 5.62	107.71 ± 6.91	108.2 ± 3.05	106.28 ± 4.3

Figure 5-7. Comparison between the standard curves generated in the singleplex and mRT-qPCR assays. Overlapping multiplex and counterpart singleplex plots of quantification cycle (Cq) values against a ten-fold (1.0×10^3 to 1.0×10^7) dilutions of the R3+ standard in water. The multiplex plot represents the mean values of three independent runs while the singleplex plot represents the mean values of four independent runs. Each point of the standard was tested in a single well of the T-COR™ 8 device. A comparison of PCR efficiencies and correlation coefficient (R^2) for each virus target are shown in the table. The assays were performed using conditions described in Table 5-7.

Table 5-8 Analytical sensitivity comparison of the multiplex and singleplex RT-qPCR assays based on three runs of the assay. In each run, seven serial dilutions of an R3+ artificial transcript in RNase-free water were used as standards. The symbol (-) means no quantification cycle value was produced.

R3+ copies/ reaction	FMDV 3D		BVDV-1		QB		BVDV-2	
	no. of pos/no. of runs		no. of pos/no. of runs		no. of pos/no. of runs		no. of pos/no. of runs	
	multiplex	singleplex	multiplex	singleplex	multiplex	singleplex	multiplex	singleplex
1.0×10^6	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
1.0×10^5	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
1.0×10^4	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
1.0×10^3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
1.0×10^2	3/3	3/3	3/3	-	-	2/3	1/3	1/3
1.0×10^1	2/3	3/3	-	-	-	2/3	-	1/3
1.0×10^0	2/3	-	-	-	-	-	-	-

5.3.6. Intra and inter-assay variation

To determine the repeatability of the mRT-qPCR assay on the T-COR, the intra-assay variation was calculated based on the Cq values produced from five replicates of each of the three R3+ concentrations (1.0×10^7, 1.0×10^5 and 1.0×10^3 copies/reaction). The mean SD and CV of the Cq values obtained for each dilution of each target are shown in Table 5-10. The observed intra-assay CV was no greater than 1.5% for any of the target R3+ concentrations tested, which indicated good repeatability of the PCR reactions.

The inter-assay variation (Table 5-11) was examined by performing two or three runs of the multiplex assay on three different days (for a total of 8 runs) by two operators using the R3+ standards (1.0×10^4 to 1.0×10^8 copies/reaction). For the FMDV target, the Cq mean CV for each template concentration ranged from 0.83 % to 4.59 %, with an overall mean of 1.95%. The Cq mean CV for BVDV-1 and BVDV-2 ranged from 1.58 % to 4.59 % and 1.76 % to 4.13 %. The overall mean CV of all copy numbers was 3.06 % for BVDV-1 and 2.71 % for BVDV-2. The Qβ target had a Cq mean CV range from 0.66 % to 1.35 % and an overall 1 % mean CV at all concentrations. Although the more concentrated R3+ template in all targets had a slightly higher mean CV range (1.22% to 4.49 %), the variation in Cq values improves to a range of 0.66 % to 1.76 % CV at 1.0×10^4 copies/reaction, testifying to the reproducibility of the reactions.

Table 5-9. The analytical specificity of the multiplex RT-qPCR is based on the results of testing a panel of nucleic acids from various organisms that may be present in a clinical sample.

Agent	Sample names	Origin	Sample type	multiplex RT-qPCR			
				FMDV 3D	BVDV-1	QB	BVDV-2
				Cq	Cq	Cq	Cq
FMDV serotype O*	O Manisa	Pirbright, UK	inactivated virus	19.2/19.2	-/-	-/-	-/-
FMDV serotype A	A5 Allier RNA	Pirbright, UK	inactivated virus	17.1/17.4	-/-	-/-	-/-
FMDV serotype Asia 1	Asia 1 Shamir	Pirbright, UK	inactivated virus	24.7/24.5	-/-	-/-	-/-
FMDV serotype C	C Noville	Pirbright, UK	inactivated virus	18.3/18.3	-/-	-/-	-/-
FMDV serotype SAT 1	SAT 1/BOT/ 1/68	Pirbright, UK	inactivated virus	19.2/19.3	-/-	-/-	-/-
FMDV serotype SAT 2	SAT 2/ ZIM 5/81	Pirbright, UK	inactivated virus	21.8/22.1	-/-	-/-	-/-
FMDV serotype SAT 3	SAT 3/ ZIM 4/81	Pirbright, UK	inactivated virus	20.1/20.1	-/-	-/-	-/-
MCFV (OvHV-2)	1256/1	New Zealand	DNA	-/-	-/-	-/-	-/-
MCFV (OvHV-2)	1256/17	New Zealand	DNA	-/-	-/-	-/-	-/-
VSV Indiana strain	Type Ind-1 Batch 1	Pirbright, UK	inactivated virus	-/-	-/-	-/-	-/-
VSV New Jersey strain	Type NJ Batch 1	Pirbright, UK	inactivated virus	-/-	-/-	-/-	-/-
IBRV	05-323/1	New Zealand	DNA	-/-	-/-	-/-	-/-
IBRV	06-2553	New Zealand	DNA	-/-	-/-	-/-	-/-
BVDV-1	C24v	New Zealand	RNA	-/-	13.5/13.5	-/-	-/-
BVDV-1*	Bovax	New Zealand	tissue cell supernatant	-/-	18.3/18.5	-/-	-/-
BVDV-2*	97/730	USA	tissue cell supernatant	-/-	-/-	-/-	23.1/22.9
BVDV-2	97/1273B	USA	tissue cell supernatant	-/-	-/-	-/-	28.9/28.8

Continuation Table 5 9

Orf virus	**15-363**	New Zealand	DNA	-/-	-/-	-/-	-/-
Theileria buffeli	906/12	New Zealand	DNA	-/-	-/-	-/-	-/-
Theileria chitose	906/12	New Zealand	DNA	-/-	-/-	-/-	-/-
Border disease virus	10-1118/2	New Zealand	RNA	-/-	-/-	-/-	-/-
Border disease virus	91-5809	New Zealand	RNA	-/-	-/-	-/-	-/-
Bovine papillomavirus	79-6129	New Zealand	DNA	-/-	-/-	-/-	-/-
Q beta bacteriophage*	Attostar 1	USA	virus suspension	-/-	-/-	17.5/17.6	-/-
Mycoplasma bovis	ATCC 255	USA	DNA	-/-	-/-	-/-	-/-
Serum	NA	New Zealand	serum from apparently healthy cattle	-/-	-/-	-/-	-/-
oral swab	NA	New Zealand	oral swab from apparently healthy cattle	-/-	-/-	-/-	-/-

* Reference viruses in this study; - /- means no Cq value generated

Table 5-10. Repeatability of the multiplex RT-qPCR assay using three concentrations of R3+ RNA standard on the T-COR. Cq= quantification cycle; SD= standard deviation; CV= coefficient of variance; CI=confidence interval.

R3+ standard	**FMDV 3D** n = 5				**BVDV-1** n = 5				**QB** n = 5				**BVDV-2** n = 5			
copy number/reaction	Cq mean	SD	CV (%)	CI (95%)	Cq mean	SD	CV (%)	CI (95%)	Cq mean	SD	CV (%)	CI (95%)	Cq mean	SD	CV (%)	CI (95%)
1.0×10^7	15.9	0.05	0.3	*15.82-15.94*	18.5	0.28	1.5	*18.15-18.85*	16.22	0.08	0.5	*16.12-16.32*	19.3	0.22	1.2	*19.02-19.58*
1.0×10^5	22.7	0.05	0.2	*22.62-22.74*	25	0.21	0.9	*24.74-25.26*	23.06	0.09	0.4	*22.95-23.17*	25.7	0.16	0.6	*25.50-25.90*
1.0×10^3	28.9	0.05	0.2	*28.86-28.98*	30.82	0.11	0.4	*30.68-30.96*	29.1	0.16	0.5	*28.90-29.30*	31.1	0.08	0.3	*30.98-31.18*

Table 5-11. Two operators' repeatability of four (FMDV, BVDV-1, BVDV-2, Qβ) target components of the multiplex RT-qPCR system on different times and days. Each dilution of the R3+ standard panel was assayed once per run on the T-COR machine. Runs 1 and 2 were run on day 1, runs 3, 4 and 5 on day 2 and runs 6, 7 and 8 on day 3. Results are presented as quantification cycle (Cq) values. CV= coefficient of variance; CI=confidence interval.

R3+ Standard, copy/reaction	Operator 1				Operator 2				Mean	CI (95 %)	SD	%, CV
	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8				
FMDV target												
1.0×10^8	15.8	15.7	15.9	15.9	15.9	17.1	16.9	16.7	16.24	*15.77-16.71*	0.56	3.45
1.0×10^7	19.0	18.9	19.3	19.2	19.2	20.2	20.1	19.8	19.46	*19.04-19.88*	0.5	2.57
1.0×10^6	22.5	22.4	22.7	22.6	22.8	23.4	23.3	23.4	22.89	*22.54-23.23*	0.42	1.83
1.0×10^5	25.7	25.6	25.9	25.7	25.6	26.6	26.6	26.6	26.04	*25.64-26.43*	0.47	1.8
1.0×10^4	29.1	29.0	29.0	29.0	28.8	29.5	29.4	29.3	29.14	*28.94-29.34*	0.24	0.82
BVDV-1 target												
1.0×10^8	18.6	18.4	17.5	17.4	17.3	19.3	19.2	19.1	18.35	*17.64-19.06*	0.843	4.59
1.0×10^7	22.2	22.1	20.8	20.7	20.8	22.5	22.3	22.3	21.71	*21.05-22.37*	0.792	3.65
1.0×10^6	25.3	25.1	24.2	24.2	24.3	25.9	25.9	25.9	25.1	*24.45-25.75*	0.776	3.09
1.0×10^5	28.2	28.1	27.1	26.9	27.2	28.4	28.5	28.4	27.85	*27.29-28.41*	0.665	2.39
1.0×10^4	31.0	31.2	30.3	30.0	30.0	30.9	31.0	30.9	30.66	*30.26-31.07*	0.484	1.58

Continuation of Table 5-11.

R3+ Standard, copy/reaction	Operator 1				Operator 2				Mean	CI (95 %)	SD	%, CV
	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8				
BVDV-2 target												
1.0×10^8	19.2	19.3	18.3	18.2	18.3	20	20	19.9	19.15	*18.49-19.81*	0.79	4.13
1.0×10^7	22.6	22.6	21.9	21.6	21.4	23.1	22.9	22.7	22.35	*21.82-22.88*	0.63	2.82
1.0×10^6	26.0	26.1	25.1	25.0	25.1	26.4	26.4	26.2	25.79	*25.28-26.30*	0.61	2.37
1.0×10^5	28.9	28.6	27.9	27.3	27.7	29.1	29.0	29.0	28.44	*27.85-29.02*	0.7	2.46
1.0×10^4	31.6	32.0	31.1	30.5	30.4	31.5	31.5	31.1	31.21	*30.75-31.67*	0.55	1.76
Qβ target												
1.0×10^8	16.1	16.0	16.3	16.4	16.3	16.6	16.5	16.4	16.33	*16.16-16.49*	0.2	1.22
1.0×10^7	19.4	19.3	19.7	19.5	19.5	19.8	19.8	19.6	19.58	*19.42-19.73*	0.18	0.92
1.0×10^6	22.8	22.6	23.1	23.0	23.2	22.9	23.1	23.1	22.98	*22.81-23.14*	0.2	0.87
1.0×10^5	25.8	25.9	25.8	25.9	25.1	26.3	26.0	26.1	25.86	*25.57-26.16*	0.35	1.35
1.0×10^4	29.1	29.2	29.1	29.0	28.7	28.7	28.9	28.8	28.94	*28.78-29.10*	0.19	0.66

5.4. Discussion

Pen-side testing can rapidly confirm a provisional diagnosis of a "fast-moving" disease such as FMD in affected animals and is a valuable tool for national diagnostic capability. The availability of pen-side testing could hasten sample-to-result turn-around time, enabling rapid implementation of disease control measures. This chapter describes the development of a multiplex qPCR assay that will be adopted into a deployable pen-side format to support the presumptive diagnosis of suspected FMD incursion in New Zealand cattle. Along with FMDV-specific reagents, the mRT-qPCR assay included primers and probes targeting endemic BVDV-1 and exotic BVDV-2 to allow the detection of two other viruses that may cause disease with clinical signs similar to those produced by FMDV infection. The developed multiplex PCR also contained a fourth Qβ phage target, which serves as an internal amplification control of the assay. The assay was optimised and evaluated for efficiency, repeatability, analytical specificity and analytical sensitivity on a mobile T-COR PCR device.

The newly developed mRT-qPCR in the T-COR had all the characteristics of an optimal PCR test with low detection limits for FMDV (10-100 copies/reaction), BVDV-1 (10-100 copies/reaction) and BVDV-2 (1000 copies/reaction). These findings agreed with the published limits of detection of the original singleplex pan serotypic FMDV 3D (Callahan et al., 2002); and BVDV-1 and 2 (Willoughby et al., 2006) assays. Wernike et al. (2015) described a multiplex PCR system for cattle diseases similar to mRT-qPCR but with the addition of three more viruses (bluetongue, epizootic haemorrhagic, and Rift Valley fever). In that study, the limit of detection for FMDV and BVDV RNA components (100 copies) was also comparable to those described in this chapter for mRT-qPCR.

PCR performance parameters, including amplification *efficiency* and linearity (R^2), are derived from the analysis of standard curves generated using a range of analyte concentrations (Bustin et al., 2009). This approach has been used for the determination of the optimal test conditions in other multiplex assay development studies (Haines et al., 2013; Wernike et al., 2015; Yue et al., 2009). The biggest challenge to this method using the T-COR was the small number of reaction wells (eight) available on the device, making it impossible to generate a standard curve using replicate standards. Therefore, the mean Cq values of three independent mRT-qPCR runs using a singleton of each standard were used to create the

standard curve. A disadvantage of this approach is that it reduces the precision in estimating PCR efficiency (Svec et al., 2015) compared to estimation using two to three replicates (Panina et al., 2019). Svec et al. (2015) demonstrated that a standard analysed without replicate has a higher imprecision effect of about 8.3% than testing in triplicate (2.3%) in efficiency estimates. Despite such restriction in testing capacity, the multiplex assay was repeatable, as shown by the low intra and inter-assay variability (Cq mean CV <1.5%), corroborating the ability of the mRT-qPCR assay to withstand variations in testing circumstances (e.g., minor changes in cycling temperatures, batches of master mixes, or variability associated with pipetting).

Thermocycling device is one factor that could affect PCR performance. Optimisation of primer and probe concentrations was initially carried out on the CFX96 due to higher plate capacity of that machine in comparison with T-COR. However, the CFX96 optimised PCR assay performed poorly (as judged by low efficiency and R^2) when the assay was directly transferred to the T-COR. The two PCR machines (T-COR and CFX96) may have differences in the heating and cooling rates, affecting the efficiency of the PCR assay. In addition, the two thermocyclers use different software algorithms to derive Cq values. The inability to directly transfer an assay from one machine to another was also reported by others. In one study, six different PCR machines produced significantly different ($p<0.0001$) qPCR efficiencies calculated from standard curves prepared using four replicates of six ten-fold dilutions of reversed transcribed murine 18S rRNA (Svec et al., 2015). Nevertheless, the CFX96 optimised assay provided a starting point to strategically re-optimise primer and probe concentrations on the T-COR. This was done using a stepwise approach using a limited number of different combinations of primer concentrations that were selected based on the results of the initial optimisation experiments from the CFX96 machine. Compared to the checkerboard method (Mikeska & Dobrovic, 2009), where a comprehensive range of primer concentrations were tested, the stepwise approach was more efficient in empirically determining primers and probes concentrations given limitations in the number of available wells per run. Overall, the T-COR-optimised mRT-qPCR assay showed efficiencies within the acceptable range (90-110%) for each target test and the reactions were linear over five magnitudes of the R3+ standard (R^2 = 0.988 to 0.999).

The mRT-qPCR assay has a high analytical specificity (100%) against the non-targeted pathogens tested in this study including those expected to be in New Zealand. Specificity encompasses the ability of an assay to detect the target analyte and not the other components present in the sample (Saah & Hoover, 1997). Such non-cross reactivity is an essential trait of mRT-qPCR assay to avoid the unwanted effect of a false positive test classification. A false positive FMDV classification could lead to high emotional and financial costs due to the seriousness of such a diagnosis for the New Zealand trade combined with the necessity to implement the necessary control measures while waiting for the confirmatory results.

The analytical sensitivity of the mRT-qPCR to detect BVDV-1 and BVDV-2 may not have reflected the sensitivity of the assay to detect both BVDV-1 and BVDV-2 in a sample that contains both viruses. The R3+ contained only one target fragment for both assays. Since both BVDV assays use the same primers, competition between the targets may occur if the diagnostic sample is positive for both BVDV-1 and BVDV-2. Such competition may alter the sensitivity of the detection of either virus. BVDV-2 is not present in New Zealand (Reichel et al., 2018). Hence, concomitant BVDV-1 and BVDV-2 infections in New Zealand cattle are unlikely.

The mRT-qPCR was optimised based on synthetic RNA template (R3+) standards, which do not represent a clinical sample containing the target pathogen in a complex biological matrix. The latter may have various PCR inhibitors, affecting the test's sensitivity (Thatcher, 2015). The analytical and diagnostic performance of the mRT-qPCR field assay using clinical samples is described in Chapter 6 (for FMDV component) and Chapter 7 (for BVDV component).

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Chapter 6. Estimating the diagnostic performance of mRT-qPCR assay for detecting foot and mouth disease virus in a field setting

6.1. Introduction

Laboratory confirmation of presumptive FMD diagnosis is usually carried out in a national animal health reference laboratory using a range of diagnostic tests. Since the rapid confirmation of FMD diagnosis is crucial for successful FMD control and elimination, previous researchers have highlighted the need for the development of pen-side testing to assist in outbreak investigation (Howson et al., 2015; Madi et al., 2012; Yamazaki et al., 2013). However, the broad uptake of these technologies has been hampered by the lack of data on diagnostic accuracy estimates within the population of interest and the capability to simultaneously detect endemic FMD-like diseases such as BVDV-1.

A field-deployable mRT-qPCR was developed (Chapter 5) for detecting FMDV, BVDV-1 and BVDV-2 using simplified sample preparation without nucleic acid extraction (Chapter 3). The mRT-qPCR was demonstrated to have a high analytical specificity against unrelated pathogens, including those endemic in New Zealand. The mRT-qPCR also had low detection limit for FMDV (10-100 copies/reaction), BVDV-1 (10-100 copies/reaction) and BVDV-2 (1000 copies/reaction). However, because the initial ASe and ASp data were determined using a synthetic RNA target (R3+), the same analytical characteristics should be tested using crude clinical samples.

To determine the utility of the test for the detection of specific pathogens within a population, diagnostic accuracy studies must be conducted to generate estimates of diagnostic specificity (DSp) and diagnostic sensitivity (DSe) (WOAH, 2022). *Diagnostic specificity* is the proportion of known negative (non-infected) animals that test negative in the assay. *Diagnostic sensitivity* is the proportion of known positive (infected) animals that test positive in the assay. These parameters can also be used to estimate the likelihood ratios and predictive values of a positive or negative test result. Factors such as the stage of

infection, the severity of disease, immune status, age, sex, or breed of an individual animal may affect the test results and, therefore, the reliability of using such a test at a population level (Greiner & Gardner, 2000). Inherent differences related to the sample type (i.e., saliva, blood, other tissue samples), collection, storage, and processing methods can also affect the diagnostic performance of the test through the presence of components that can react non-specifically in the test (reducing DSp) or have an inhibitory effect on the test (reducing DSe).

The objectives of this study, therefore, were to (1) estimate the analytical sensitivity (ASe) and analytical specificity (Asp) of the mRT-qPCR test in the T-COR for detection of FMDV RNA without nucleic acid extraction, (2) estimate diagnostic performance (DSe and DSp) of the test when applied to New Zealand cattle for detection of FMDV in the event of a suspected FMDV incursion against established laboratory-based PCRs, and (3) evaluate the impact of field conditions on the test performance.

Experiments for DSp were conducted at the AHL-MPI, New Zealand. However, because FMD is historically absent in New Zealand, the DSe evaluation of the mRT-qPCR test was done on a panel of FMD outbreak samples in two FMD-endemic countries to provide indicative measures of the test performance in the field. DSe trials were performed at the National Animal Health Laboratory, Vientiane, Lao People's Democratic Republic (PDR) and FMD BSL2 Diagnostic Laboratory, Nay Pyi Taw, Myanmar. Field testing was also done on New Zealand dairy farms in the South Island, at a slaughter plant in Vientiane, Lao PDR, and a dairy farm in Meiktila, Myanmar.

6.2. Materials and methods

6.2.1. Sources of samples

6.2.1.1. Viruses and samples used for laboratory proficiency testing

Two types of inactivated samples (by binary ethylenimine inactivation) supplied by The Pirbright Institute (United Kingdom) were utilised in the study:

a. A collection of inactivated cell culture lysates (n=17) comprising FMDV serotypes O (n=5: O/SKR/6/2014, O/SAU/1/2013, O/PAK/28/2010, O/LIB/48/2012,

O/MOR/1/2015), A (n=5: A/TUR/16/2014, A/TUR/28/2010,A/SAU/2/2015, A/PAK/12/2015), Asia 1 (n=3: ASIA1/TUR/2/2013, As1/TUR/49/2011, As1/TUR/12/2015), SAT 1 (n=1: SAT1/TAN11/2012), SAT 2 (n=2: SAT2/EGY/2012, SAT2/EGY/24/2014), and swine vesicular disease virus (SVDV) (n=1: 2013 p2 7). Extracted viral RNA from these isolates is routinely used for PCR controls at AHL-MPI.

b. Inactivated (n=20) FMDV-positive epithelial suspension (ES) samples (serotype O, A, Asia 1, SAT 1, and SAT 2) obtained for the purpose of proficiency testing.

6.2.1.2. Bovine viral diarrhoea virus

BVDV-1 (n=2: Bovax, C24v) and BVDV-2 (n=2: 97/730, 97/1273B) cell culture isolates used for virus isolation controls at AHL-MPI.

6.2.1.3. Clinical samples spiked with inactivated FMDV

Experiments involving inactivated FMDV positive samples were conducted in a level 3 physical containment environment (PC3+) at AHL-MPI.

The mock-infected samples were prepared by spiking clinical material (pooled serum and pooled oral swab suspension with approximate dilution of 1:10 in VTM (n=5) collected from New Zealand cattle with an inactivated FMDV type O isolate (O Manisa, TPI) of known viral RNA quantity. The mock-infected panel was serially diluted to contain between 1.0×10^{1} and 1.0×10^{6} copies of viral RNA/µL of the sample.

The concentration of the FMDV type O isolate for spiking was estimated by mRT-qPCR testing using RNA prepared from an aliquot of the stock virus. Briefly, 200 µL (n=2) of the inactivated virus diluted in PBS (1:5) was purified using Magjet Viral DNA and RNA Purification extraction kit (Thermo Fisher Scientific) according to the manufacturer's instructions on the KingFisher™ Flex (Thermo Fisher Scientific) machine. The final elution volume was 100 µL. Five replicates of the eluent were then tested in mRT-qPCR on the CFX96 PCR machine as described in Table 5-5, with the exception that the primers and probes for targets other than FMDV were replaced with RFW. The run included a standard curve

prepared using the R3+ standards in quadruplicate (1.0×10^8 to 1.0×10^0 RNA copies/reaction, section 5.2.2). The concentration of FMDV serotype O RNA in the supplied stock virus was extrapolated from the R3+ standard curve.

6.2.1.4. Clinical samples from New Zealand cattle

Serum (n=306) and oral swab (n=200) samples (Table 6-1) collected from dairy and beef cattle of various breeds and genders throughout New Zealand for unrelated purposes were utilised in the current study. The North Island (NI) sera (n=176) were randomly selected from archival samples collected for the 2018 arbovirus sero-surveillance program (Peacock et al., 2018). Cattle sera from 128 farms (2-6 sera/per farm) were used. The South Island (SI) sera (n=130) and all oral swabs were collected as part of the *Mycoplasma bovis* testing for the research programme in 2019.

Table 6-1. The source of clinical samples from New Zealand cattle that were used in the study. Except for the 67 cattle from the South Island where both serum and oral swab samples were taken from the same animals, the rest of the samples came from different individual animals.

Source	Serum	Oral swab
Auckland	44	
Bay of Plenty	31	
Manawatu Wanganui	4	21
Northland	42	
Waikato	55	38
North Island	*176*	*59*
Canterbury	122	67
Marlborough		22
Tasman		52
Otago	8	
South Island	*130*	*141*
Grand Total	306	200
No. of farms/properties	*157*	*53*

For the SI panel, blood was collected mid-stream during bleeding at slaughter using an uncapped vacutainer tube (10 mL) for coagulated blood and sent to AHL-MPI in chilled conditions for processing. Upon arrival at the laboratory, the blood samples were centrifuged (1500 × g for 3 minutes), and serum was then collected using a plastic transfer pipette and stored at -80°C prior to analysis. Oral swab samples were also collected conveniently from cattle at the slaughter plant. Fresh cattle heads were sampled immediately after being bled

out by rolling and rubbing a rayon swab (Copan Diagnostics) on the mucosal surface of the gums, lips, and tongue for at least 30 seconds, saturating it with oral fluids and saliva. The swab tip was then suspended in a tube containing 1 mL of VTM (1:10). The swab solution was shipped to AHL in chilled condition and frozen at -80°C for further testing.

6.2.1.5. Archival samples submitted for FMDV detection during outbreaks of FMD (Lao PDR and Myanmar)

Since New Zealand does not allow the importation of samples from FMD endemic regions, FMDV-positive samples were obtained through a collaborative project (reported by Buckle et al., 2021) in Lao PDR and Myanmar coordinated through the World Organisation for Animal Health (WOAH)-Southeast Asia China Foot and Mouth Disease (SEACFMD) Campaign. Archival diagnostic samples of known FMDV-status stored in their respective national laboratories (Lao PDR and Myanmar) were tested in-country. The FMDV-positive samples (Table 6-2) consisted of the epithelial tissue suspensions (ES) from the tongue (n=7) and hoof (n=4), as well as saliva (n=1).

6.2.1.6. Samples collected as part of FMDV surveillance project (Lao PDR and Myanmar)

Pharyngeal swab samples from cattle and buffalo (n=41) collected as part of a pilot slaughterhouse surveillance project (reported by Buckle et al., 2021) for FMD in Lao PDR and Myanmar were also used to establish DSe of mRT-qPCR. The animals were selected by convenience and had no known history of exposure to FMDV, except that they originated from FMD-endemic areas. After the animal's head was separated at slaughter, two rayon swabs (Copan Diagnostics) were inserted simultaneously through the cut end of the eosophagus towards the dorsal nasopharynx to collect the pharyngeal swabs. One pharyngeal swab was inserted in 1 mL VTM (approximate dilution of 1:10) and the other in 1 mL DNA/RNA Shield™ (Zymo Research) and kept at -80°C for further testing. The VTM swabs were tested for FMDV by 3D qPCR in the national laboratories of respected countries (Appendix 1). Based on these results, the samples were classified as FMDV-positive (n=31) or FMDV-negative (n=10).

6.2.2. RNA extraction

Ribonucleic acid extraction from New Zealand samples (section, 6.2.1.1 a & b, 6.2.1.2, 6.2.1.3 and 6.2.1.4) was done using the Magjet DNA and RNA Purification kit on the KingFisher™ Flex high throughput extraction machine according to the manufacturer's instructions. Inactivated FMD type O virus (O Manisa, TPI, UK) was added into one well of a 96-well plate during each extraction run as a positive extraction control.

Table 6-2. Archival samples from FMD outbreaks in Lao PDR and Myanmar used for establishment of diagnostic sensitivity of mRT-qPCR. Samples were collected from cattle with vesicular lesions. Except for the saliva/oral fluid sample, all samples were positive for FMDV in at least one of the diagnostic assays used by the relevant national laboratory.

Sample number	Sample ID	Country	Year of outbreak	Sample type	In-country FMD test results
1	NL1725352	Lao PDR	2017	tongue ES[#]	Ag ELISA* and RT-qPCR** positive
2	NL1725381	Lao PDR	2017	tongue ES	Ag ELISA and RT-qPCR positive
3	NL1620179	Lao PDR	2016	tongue ES	Ag ELISA and RT-qPCR positive
4	NL1840602	Lao PDR	2018	tongue ES	RT-qPCR positive
5	NL1840607	Lao PDR	2018	tongue ES	RT-qPCR positive
6	Mya19/11	Myanmar	2019	hoof ES	LFD**** positive, serotype O
7	Mya19/12	Myanmar	2019	hoof ES	LFD positive, serotype O
8	Mya19/13	Myanmar	2019	tongue ES	LFD positive, serotype O and convPCR*** positive
9	Mya19/15	Myanmar	2019	hoof ES	Ag ELISA positive, serotype O
10	Mya19/17	Myanmar	2019	hoof ES	Ag ELISA positive, serotype O
11	Mya19/18	Myanmar	2019	tongue ES	Ag ELISA positive, serotype O
12	Mya19/19	Myanmar	2019	saliva/oral fluid	negative on both Ag ELISA and convPCR

* Ag ELISA = antigen enzyme-linked immunosorbent assay

**RT-qPCR = reverse transcription quantitative polymerase chain reaction

***convPCR = conventional polymerase chain reaction

****LFD = lateral flow device for FMD pan detection and serotyping (O, A, Asia 1)

[#]ES = epithelial suspension in 10% phosphate-buffered saline (PBS)

The RNA from FMDV-positive samples (section 6.2.1.5) and slaughterhouse surveillance samples (section 6.2.1.6) was manually extracted using either QIAamp Viral RNA Mini kit (Qiagen) or RNAEasy Mini Kit (Qiagen) according to the manufacturer's instructions. The extractions were performed in the country of sample collection, e.g., Lao PDR or Myanmar. RFW was included in each batch of extractions as a negative extraction control. All steps of the extraction process were done inside a class 2 biosafety cabinet (BSCII)

except for the centrifugation step, where the outside of the tubes was wiped with 0.5% citric acid solution. At the Myanmar laboratory, one BSCII was used for the first three steps of RNA extraction (sample lysis, ethanol precipitation and nucleic acid binding onto the spin column membrane), and another BSCII was used for the two washings and elution steps. BSCIIs were cleaned, disinfected, and exposed to UV light for 20 minutes before processing a batch of samples. This was to avoid cross-contamination between batches of samples.

6.2.3. FMD assays

6.2.3.1. Multiplex RT-qPCR

The mRT-qPCR (Table 6-3) assay was set up and performed as described in Table 5-7. The workflow for mRT-qPCR testing is shown in Figure 6-1. The clinical samples were prepared as described in section 3.2.5.1. The templates for mRT-qPCR (Figure 6-1) were prepared by diluting unextracted sera (1:5) in RFW, with or without heating (Chapter 3). However, because oral swab was already diluted in VTM (approximately at 1:10 dilution), swab homogenate (5 µL) was tested neat (with or without heating) to reduce dilution effect on target concentration.

Each mRT-qPCR run included a PC (the R3+ standard containing 1×10^4 copies per reaction) and NTC (water). Assay controls for the FMD reference PCRs also included RNA from inactivated FMDV type O (O Manisa, TPI, UK).

Table 6-3. FMD test abbreviations.

Abbreviations	Test descriptions
mRT-qPCR	multiplex reverse transcription quantitative polymerase chain reaction (FMDV target)
C2T FMD PCR	commercially prepared collect to test RT-qPCR (Tetracore)
LFD	lateral flow device (VDRG FMDV 3Diff/PAN Ag rapid kit, Median Diagnostics Inc.)
AHL_FMD PCR	reference FMD RT-qPCR (AHL-MPI, Appendix 1) (Callahan et al., 2002)
TPI_FMD PCR	reference FMD RT-qPCR performed at The Pirbright Institute, United Kingdom (Callahan et al., 2002)

6.2.3.2. Reference FMDV RT-qPCR tests

The reference RT-qPCR assays (Table 6-3) made up a pan-serotype-specific assay recommended by WOAH based on primers and probe described by Callahan et al. (2002). The AHL_FMD PCR was performed using cycling conditions standardised by the AHL-MPI (New Zealand) for diagnostic use (Appendix 1), while TPI_FMD PCR was performed at The Pirbright Institute, United Kingdom. The TPI_FMD PCR was used as a reference test for the surveillance samples collected in Laos PDR and Myanmar (section 6.2.1.6).

Each AHL_FMD PCR run included two positive controls: the R3+ standard containing 1×10^4 copies per reaction and the RNA from inactivated FMDV type O (O Manisa, TPI, UK), as well as NTC (water).

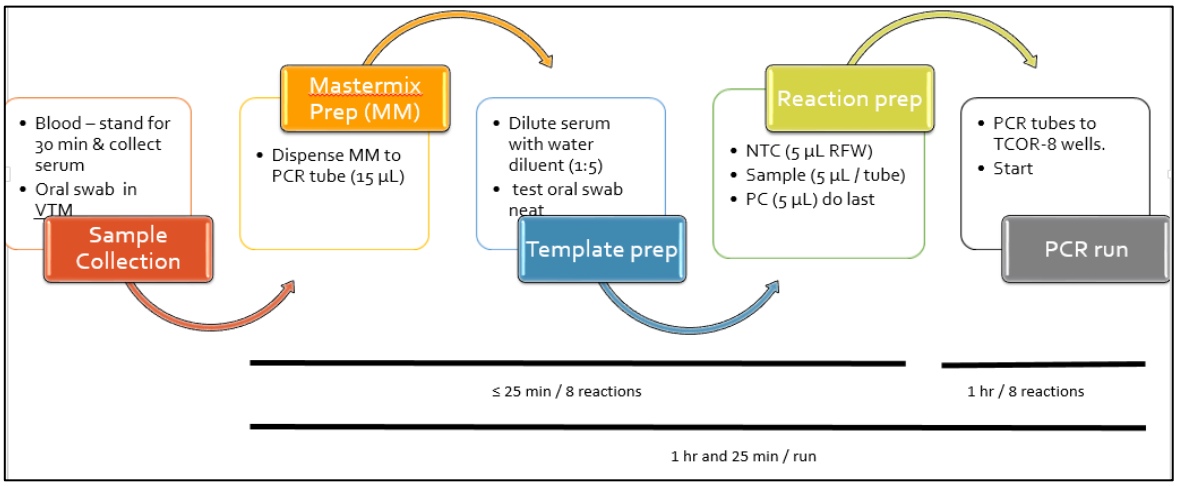

Figure 6-1. Pen-side testing workflow using the mRT-qPCR assay without nucleic acid extraction on the T-COR™ 8 machine (Tetracore). VTM-virus transport media, RFW-RNAse free water.

6.2.3.3. C2T FMD PCR

The C2T FMD PCR (Figure 6-2), is a commercial field-ready assay for the detection of FMDV designed specifically for the T-COR 8™ device. The test targets the FMDV 3D region and includes an internal amplification control (IC). The FMD C2T assay consists of a sample processing device (Figure 6-3A) containing sample diluent and a cartridge with a dried master mix inside (Figure 6-3B). The assay can be stored or transported at room temperature. Two

cartridge variants (mix 1 and mix 2) of the FMD C2T assay's proprietary reagents were kindly provided by Tetracore.

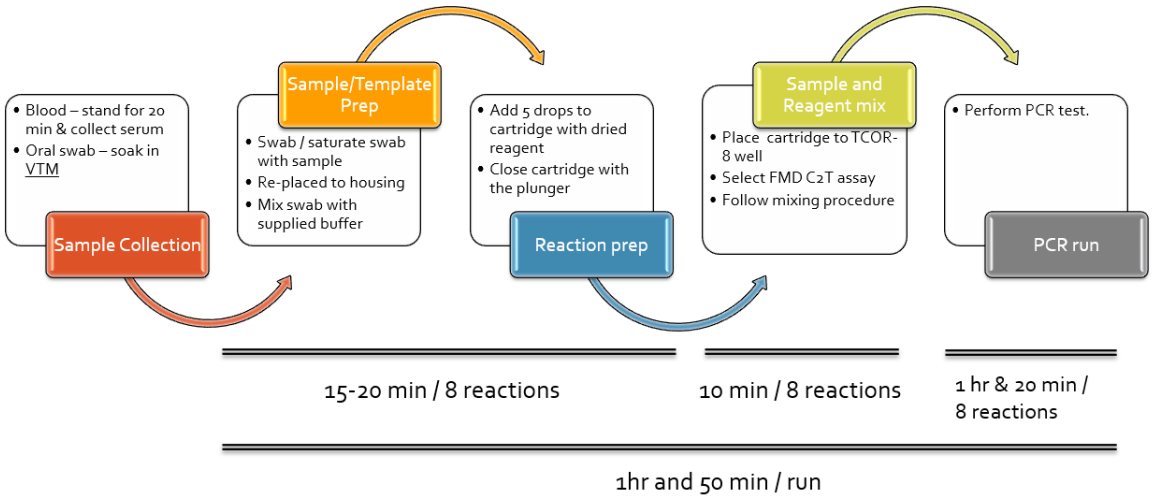

Figure 6-2. Test workflow of the C2T FMD PCR field assay (Tetracore) using the T-COR 8™ Real-time PCR platform.

The sample was processed according to the manufacturer's instructions (Figure 6-3). Briefly, the kit-supplied swab was saturated with the sample and then inserted into the sample diluent by breaking the bulb neck of the collection device (Figure 6-3A). Five drops of the diluted sample were added to the C2T cartridge well (Figure 6-3B) by unscrewing the white cap and squeezing the tube to dispense the liquid. The C2T cartridge was closed by pressing the plunger to rehydrate the dried reagent with the diluted sample and positioned into the reaction well of the T-COR 8™ device. The FMD C2T protocol was selected, and the instructions on the device's screen were followed to run the assay.

The results (Cq values) were displayed via the built-in software in tabular and graphical format. No positive/negative controls were used for the C2T assay, and the built-in internal control performance was used to evaluate each run's validity.

6.2.3.4. FMDV lateral flow device

The LFD (lateral flow device) for FMD (Table 6-3) is a rapid antigen test based on the immunochromatographic principle (Figure 1-4). The LFD (VDRG FMDV 3Diff/PAN Ag rapid kit, Median Diagnostics Inc.) test can detect all seven serotypes of FMDV but discriminates

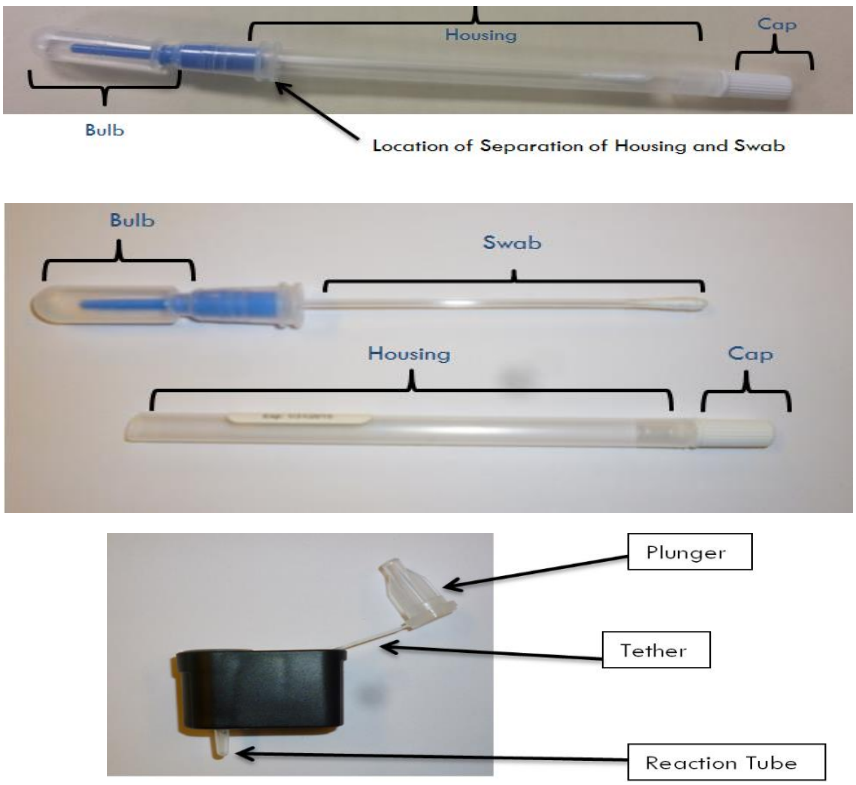

Figure 6-3. The C2T FMD PCR assay (Tetracore) composed of a sample collection device (A) with swab and a proprietary dilution buffer and a PCR cartridge containing dried master mix (B) designed for direct analysis using the T-COR PCR device (images courtesy of Tetracore).

only between the three common Southeast Asian serotypes (A, O, Asia 1). The LFD was performed by mixing the sample (120 µL) with the supplied sample dilution buffer (120 µL). Four drops or 100 µL of the diluted sample were added to each of the two device wells for serotyping and pan detection. After incubation (15 minutes), the sample was regarded as positive if two visible red lines (control and test line) were seen and negative if only a control line were present.

6.2.4. Analytical performance of mRT-qPCR (FMDV) using unextracted samples

After determining the analytical characteristics of mRT-qPCR using RNA templates (section 5.3.4 and 5.3.5), the ASe and ASp estimate of the pen-side format (section 6.2.3.1) was examined using diluted clinical samples (without RNA extraction) as templates. Table 6-4 summarised the various panels used to examine the test characteristics of mRT-qPCR.

Table 6-4. Test panels used to determine the analytical (ASe and ASp) and diagnostic characteristics (DSe and DSp) of the field deployable mRT-qPCR. ASe – analytical sensitivity, ASp – analytical specificity, DSe – diagnostic sensitivity, DSp – diagnostic specificity

Test under evaluation	**mRT-qPCR**					
Reference test	AHL_FMD PCR (Table 6-3)				TPI_FMD PCR (Table 6-3)	
Sample panel (section)	6.2.1.3*	6.2.1.1 (a)* 6.2.1.2*	6.2.1.5** 6.2.1.1 (b)	6.2.1.4	6.2.1.6	
Parameters	ASe	ASp	DSe	DSp	DSe	DSp
Data analysis (section)	6.2.7.1		6.2.7.2 & 6.2.7.3***	6.2.7.2 & 6.2.7.3***	6.2.7.2 & 6.2.7.3***	
*The C2T FMD PCR was also performed on these panels to examine the C2T's ASe and ASp.						
** C2T FMD PCR and LFD tests were also conducted on archived outbreak samples from Lao PDR and Myanmar.						
*** Bayesian latent class modelling was applied to outbreak panel (6.2.1.5), New Zealand cattle panel (6.2.7.2) and slaughterhouse surveillance samples (6.2.1.6).						

6.2.4.1. Analytical sensitivity

Mock FMDV-infected sera and oral swab panel (section 6.2.1.3) were tested to determine the analytical sensitivity (ASe) of mRT-qPCR. Each sample was diluted in RFW (1:5), and 5 µL was added immediately as a template into the reaction mix for analysis using mRT-qPCR on the T-COR machine. In addition, an aliquot (20 µL) of the diluted sample was heat-treated using the T-COR device for a total of 4 minutes (1 min at 80°C, 2 min at 95°C and 1 min at 42°C) before 5 µL was used as a template in the mRT-qPCR system (Chapter 3).

6.2.4.2. Analytical specificity

Analytical specificity (ASp) of the mRT-qPCR without RNA extraction was determined by testing inactivated virus isolates used as a source for PCR controls (n=17, section 6.2.1.1 a). In addition, BVDV-1 (n=2) and BVDV-2 (n=2) cell culture isolates were also included (section 6.2.1.2). The same set of samples was used for comparative purposes to determine the ASe and ASp of the C2T FMD PCR.

6.2.5. Diagnostic performance of mRT-qPCR

6.2.5.1. Diagnostic sensitivity

The aim was to test at least *n*=30 FMDV-positive samples based on a 98% DSe estimate, 5% error and 95% confidence (WOAH, 2022). The allowed error (5%) used in the estimate of DSe was slightly larger than the DSp (2%) because the purpose of field testing using mRT-qPCR was to support the clinical diagnosis of FMD as opposed to screening a population of healthy animals (Dohoo et al., 2014). Altogether, 32 FMDV-positive samples were used to determine the DSe of mRT-qPCR. These comprised ES suspensions (n = 11) and saliva (n = 1) collected/processed in Lao PDR and Myanmar; and mock-infected ES suspensions (n = 20) processed in New Zealand.

Processing of samples differed between the two countries. Since the volume of the positive ES suspension from Lao PDR was insufficient to allow complete immersion and soaking of the rayon swab (Copan Diagnostics), FMDV-positive ES (n=5, section 6.2.1.5) was diluted 1:5 or 1:10 in RFW. Alternatively, a rayon swab was immersed in each FMDV positive ES suspension from Myanmar (n=7, section 6.2.1.5) for 30 seconds and transferred to a vial with 1 mL VTM. Five microlitres of the processed sample were then tested with mRT-qPCR (section 6.2.3.1).

The inactivated FMDV-positive ES (section 6.2.1.1 b) to produce mock virus-infected samples (n=20) at AHL-MPI were processed similar to the Myanmar procedure.

The DSe of mRT-qPCR was assessed against results obtained using the reference AHL_FMD PCR (Appendix 1) with extracted RNA from the same panel (Table 6-4) used as a template (section 6.2.2). The testing was done on the CFX96 machine in Lao PDR and AHL-MPI. In Myanmar, however, because no real time PCR machine was available, extracted RNA was analysed on the T-COR device using the optimised singleplex protocol (Appendix 3) for the developed mRT-qPCR.

The mock FMDV-positive oral swabs (Table 6-4) from Lao PDR, Myanmar, and AHL were also tested with C2T (section 6.2.3.3) PCR and LFD (section 6.2.3.4) tests.

In addition to FMD-positive samples from proficiency testing, slaughterhouse pharyngeal swab samples in VTM from the FMD surveillance project (section 6.2.1.6) were tested neat (5 µL) without heating in mRT-qPCR. Duplicate swabs suspended in DNA/RNA Shield™ (Zymo Research) were sent frozen to The Pirbright Institute (UK) for comparative testing with TPI_FMD PCR (section 6.2.3.2).

6.2.5.2. Diagnostic specificity

Examination of DSp of the mRT-qPCR assay was conducted using serum and oral swab samples from New Zealand cattle (n=506, section 6.2.1.4). The number of each sample type for the negative panel was aimed to be *n*=279 (Greiner & Gardner, 2000) based on a conservative DSp estimate of 97%, 2% precision and 95% confidence (Dohoo et al., 2014; WOAH, 2022).

Five microlitres of diluted serum in RFW (1:5) or oral swab suspension in VTM (1:10) was analysed without heat treatment on the T-COR PCR machine with a pre-programmed cycling protocol (Table 5-7). The mRT-qPCR PCR master mix (Table 5-7) was prepared in batches of 200 reactions, aliquoted (255 µL per tube) and stored at -20°C. Prior to each run, one tube of the pre-mixed master mix was thawed, and 15 µL was dispensed to each of 8 T-COR specific PCR tubes. The QβIC (1 µL) was added to individual tubes. Each PCR run consisted of a maximum of 6 test samples and two controls (PC and NTC). The results were assessed against the results obtained using the reference PCR (AHL_FMD) with RNA (5 µL, section 6.2.3.2) extracted from the same samples.

6.2.6. Mock field deployment of mRT-qPCR

6.2.6.1. Stability testing of reagents

Reagents used for field testing were evaluated for stability at various storage conditions in the laboratory. The bulk master mix was prepared (Appendix 4) and aliquoted (500 µL) into three vials that were stored using three conditions. Vial 1 was stored at -20°C freezer. Vial 2 was stored in a chilled polystyrene box for 18 hours after preparation. Vial 2 was subsequently kept in a fridge at 2-8°C temperature to simulate excess reagents after a

day's work and for use the following day or approximately 42 hours post-preparation. The temperature of the chilled polystyrene box was monitored with a min-max thermometer. Vial 3 was kept at room temperature (initially inside a 21°C incubator and then on the bench until 42 hours post-preparation). The master mix from the three vials was then used at different time points (18, 24- and 42- hours post-preparation) to test serum spiked with 100 copies of FMDV RNA. Two samples were tested using mRT-qPCR as described in section 6.2.3.1 on the T-COR (in duplicate) and the CFX96 PCR machine (five replicates).

6.2.6.2. Field testing simulation

To simulate field testing conditions that may be encountered during an outbreak investigation in New Zealand, three farms in the South Island of New Zealand (Geraldine) were visited, and on-site molecular testing was carried out. All animals sampled (Animal Ethic approval: MUAEC Protocol 18/129) were considered healthy based on visual assessment during sampling. During travel, reagents (pre-mixed master mix and assay controls) were chilled in a polystyrene box with ice packs. The minimum and maximum temperature inside the ice box was between 1°C and 10°C. Upon arrival at a hotel, all reagents were transferred to a domestic freezer within the hotel room for overnight storage. The T-COR machine, protected by a hard-plastic carrier case, was checked in on the flight and carried at the back of a truck during land travel.

Upon arrival at the farm, a suitable location for sample processing and testing was determined before sample collection. Blood and oral swabs were then collected from 10 dairy cows restrained in a squeeze chute. Blood (5 mL) was taken from the caudal vein using a 10 mL vacutainer for coagulated blood. The blood was allowed to clot for at least 30 minutes before the serum was collected by aspiration into a microfuge tube. Oral swabs were collected by rolling the rayon swab tip (Copan Diagnostics) along the mucosal surface of the oral cavity and tongue. The swab tip was then submerged in 1 mL VTM. Both samples were processed as PCR templates on the dirt floor of a farm machine shed using a big sheet of clean paper as a working area (Figure 6-4), as described in section 6.2.3.1. The reaction mix was prepared (section 6.2.3.1) and analysed with the portable PCR machine positioned either in the farm shed or in the opened rear of a vehicle. Each run included wells for PC and NTC.

Figure 6-4. Field testing simulations in the South Island, New Zealand.

Field testing was also conducted at a slaughter plant in Vientiane, Lao PDR, and a dairy farm in Meiktila, Myanmar. Pre-mixed master mixes and controls were kept chilled inside a polystyrene box with ice packs for up to 6 hours. At the slaughter plant (Vientiane), oral swabs in VTM from freshly killed cattle (n=12) were tested on the T-COR machine. Sample preparation was performed in an open-air area at the back of a truck. At the same time, the PCR machine was situated inside the vehicle (Figure 6-5), parked immediately outside the slaughterhouse. Similarly, oral swabs were collected by in-country personnel from healthy cows (n=11) during a visit to a dairy farm in Meiktila (Myanmar). Farm testing was coincided with the scheduled surveillance sampling by in-country veterinary personnel. Cows were restrained with the aid of a nose grip while inside the milking pen. Sample processing and testing were done at the back of the vehicle parked in front of the farmhouse office, about 100 metres from the animal shed. The R3+ standard (1×10^4 copies/reaction) was used as a PC during all field tests, and water as NTC.

6.2.7. Data analysis

In all PCR tests, a Cq value of < 40 was used as a threshold for a positive result for the tested virus (Goris et al., 2009). Samples with a Cq value of ≥ 40 or no Cq value were considered negative.

Figure 6-5. Field testing conducted in Lao PDR (A, B) and Myanmar (C, D).

6.2.7.1. Analytical performance

Standard statistical methods to describe the mean, standard deviation (SD), coefficient of variance (CV) and 95% confidence interval (CI) of the Cq values were carried out using Microsoft Excel for Microsoft 365 (Version 2202) and Epitools (Sergeant, 2018).

6.2.7.2. Diagnostic performance

Binary results were cross classified into 2 × 2 table. The DSp (TN [true negative] / TN + FP [false positive]) and DSe (TP [true positive] / TP + FN [false negative]) of both the field tests and the reference PCRs were determined using Epitools with exact confidence limits (Sergeant, 2018). Kappa statistics were also conducted on slaughterhouse surveillance samples to compare mRT-qPCR and TPI_FMD reference PCR.

6.2.7.3. Latent class model assay comparison

A comparison of diagnostic accuracies of the FMD tests was also attempted using the Bayesian latent class model (BLCM) (Johnson et al., 2019) despite the very small size of the data. Bayesian latent class modelling is recommended when the true infection status of the tested animal is unknown and the reference or gold standard test being used for comparison does not have perfect accuracy (Johnson et al., 2019; Limmathurotsakul et al., 2012). The BLCM combines prior information about the diagnostic parameters being estimated with available data, and then through a likelihood function, the posterior distributions are generated for inferences (Branscum et al., 2005).

A three-test, two-population model where two correlated tests were conditionally independent to a third test (Branscum et al., 2005; Georgiadis et al., 2003) was applied to the FMDV-positive samples (section 6.2.1.5). Model 1 consisted of mRT-qPCR, AHL_FMD PCR and LFD while model 2 comprised C2T FMD PCR, AHL_FMD PCR and LFD. The assumption in models 1 and 2 was that test results for PCRs are conditionally dependent because both assays target viral nucleic acid in the sample. In contrast, the LFD test detects viral capsid protein. Therefore, Model 1 and Model 2 has 2 tests that were conditionally dependent (CD) and one with conditional independence (CI). The two populations consisted of Lao PDR and Myanmar cattle samples.

A third BLCM (model 3) was also fitted with two nucleic acid-dependent (CD) assays (mRT-qPCR and AHL_FMD PCR) using the tests results from two populations with dissimilar prevalence. Model 3 was applied to the combined FMD positive samples (section 6.2.1.5) from Laos PDR and Myanmar, and the New Zealand derived cattle samples (section 6.2.1.4). Since FMD is endemic in both Southeast Asian countries, the prior prevalence was assumed to be uniform (Table 6-5). In contrast, New Zealand is historically free from FMD, with zero prevalence among susceptible populations. Since beta distribution does not include zero, the New Zealand prevalence was assumed to be same to those described for Australia (also an FMD free country) when evaluating an FMD RT-LAMP assay (Bath et. al., 2020). In that study, Bath et al. (2020) assumed FMD prevalence for Australia was "95% sure to be <0.05 with mode = 0.01 (beta (1.88, 88.32)". Another variation of model 3 was no prior information for t1FMD was tested with a flat beta (1,1) distribution (model 3b).

For the cattle slaughterhouse surveillance panel (section 6.2.1.6), data were analysed using two tests (mRT-qPCR and TPI_FMD PCR) and two populations (Laos PDR and Myanmar) model with correlation (CD) (Johnson et al., 2019).

Informative prior information, summarised in Table 6-5, were obtained through literature searches and opinion from the field and laboratory veterinarians to support the identifiability of the model (i.e., enough information is available to estimate all test accuracy parameters). The unit of interest for the diagnostic accuracy study was an individual cow with apparent clinical signs compatible with FMD. It was assumed that 50% of samples collected from affected animals during an outbreak of FMD would test positive for FMDV in Lao PDR and Myanmar. It was presumed that the priors (DSe and DSp) of the FMD tests were the same when applied to animals in both countries, being in the same geographic region.

The prior distributions were produced by plugging the mode and the most likely value (95%) of the parameters into the BetaBuster[1] software program Chun-Lung Su's online version (https://betabuster.software.informer.com/). Using codes (Appendix 7) adopted from Geoff Jones (2019) of Massey Univeristy, BLCMs were done in OpenBUGS 3.2.3 (https://www.mrc-bsu.cam.ac.uk/software/bugs/openbugs/), discarding the initial 5000 iterations (burn-ins) and using the next 45,000 for deriving the median and 95% credible interval of the posterior distributions. Convergence of the models was assessed by checking the Brooks–Gelman– Rubin plots visually of at least three chains with dispersed initial values specified either manually or using the "gen inits" button of the software. The posterior distributions for specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) and prevalence were the interest here. PPV is the proportion of animals having the disease that test positive while NPV is the proportion of animals with a negative test that are not diseased (Dohoo et al., 2014). Sensitivity analyses were done to examine the effect of prior distributions on test accuracy estimates. The prior distribution of 25% instead of 50% prevalence was used to check DSe, followed by sets of optimistic and pessimistic priors for DSe (±10%) and DSp (<15% of mode).

[1] http://252s-epi.vet.unimelb.edu.au/~epi/epi_html/Courses/ISVEE_2018_Dx_Tests_Nov-2018/html/UC_Davis_Website/betabuster.html

Table 6-5. Informative priors used to conduct Bayesian latent class models for diagnostic accuracy studies of the field index PCR test and reference tests.

Three test two population model for detecting FMDV in Southeast Asia	
two populations	Lao PDR and Myanmar cattle samples
t1FMD	FMD test (mRT-qPCR) (section 6.2.3.1)
r1FMD	reference AHL_FMD RT-qPCR (section 6.2.3.2)
t2FMD	commercial FMD C2T field PCR (Tetracore) (section 6.2.3.3)
t3FMD	lateral flow device FMD antigen detection (section 6.2.3.4)
r2FMD	reference TPI_FMD RT-qPCR, The Pirbright Institute (6.2.3.2)
DSp_t1FMD	95% sure that DSp is greater than 90.0% and Mode at 99.0% – conservative estimate from small initial test data set by the author
DSe_t1FMD	95% sure that DSe is greater than 80% and Mode at 96.00% or 90.00% – conservative estimate from small initial test data set by the author and Howson et al., 2017
DSp_r1FMD and r2FMD	95% sure that DSp is greater than 97% and the Mode at 99.0% – *laboratory expert opinion and* Goris et al. (2009)
DSe_r1FMD and r2FMD	95% sure that DSe is greater than 95% and Mode at 98.00% - (King et al., 2006) and (Goris et al., 2009)
DSp_t2FMD	95% sure that DSp is greater than 90% and Mode at 99.0% - (Howson et al., 2017)
DSe_t2FMD	95% sure that DSe is greater than 80% and Mode at 96.00% or 90.00% - (Howson et al., 2017)
DSp_t3FMD	95% sure that DSp is greater than 90% and Mode at 99.00% - (Oem et al., 2009)
DSe_t3FMD	95% sure that DSe is greater than 60% and Mode at 87.00% (Oem et al., 2009)
Prev_pop1 and p02	95% sure that prevalence is greater than 20% and Mode at 50% - both for Lao PDR and Myanmar
Prev_NZ pop	95% sure that prevalence is less than 0.05 and Mode at 0.01 – (Bath et. al., 2020)

6.2.7.1. Evaluation of the field trial data

The performance of the mRT-qPCR assay on the T-COR machine under field conditions was assessed using the Cq values generated with the same PC standard at each test run. The mean Cq (95% CI) and standard deviation were calculated for each location. The inter-assay variability was expressed as the coefficient of variation (CV), calculated by dividing the mean Cq SD of PC from each location by the mean Cq value for PC obtained from all runs at a given location.

6.3. Results

6.3.1. Analytical specificity and sensitivity

6.3.1.1. mRT-qPCR

Extraction-free detection of FMDV RNA in spiked serum and oral swab matrices (by serial dilution), with or without heating, was compared (Table 6-6). Detection of FMDV RNA was successful without heating with an ASe between 10 and 100 viral RNA copies/reaction in both serum (2/3) and oral swab (3/3) samples. FMDV RNA was not amplified from heated samples.

High ASp was demonstrated (Table 6-7) with no cross-reactivity between FMDV-positive cell lysates (n=16), BVDV-1 positive sample (n=2), BVDV-2 positive lysate (n=2), and SVDV cell lysate (n=1). The mRT-qPCR detected all 5 serotypes (O, A, Asia 1, SAT 1, and SAT 2) in the panel.

Table 6-6. Analytical sensitivity of mRT-qPCR using spiked samples described in section 6.2.1.3. PCR templates, either pre-heated or not heated, were analysed directly without undergoing nucleic acid extraction in singleton. Results are expressed as quantification cycle (Cq), with Cq<40 considered positive. The limit of detection appeared to be lower when the sample preparation did not involve heating (10 R3+ copy/reaction) compared with heated samples. nd = not done, NTC = non template control, dash represents no Cq value.

FMDV serotype O RNA copies per reaction	Pre-heating						No pre-heating					
	Serum			oral swab			Serum			oral swab		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
1×10^6	28.4	-	-	-	29.6	-	21.5	23.6	22.0	23.0	22.6	22.7
1×10^5	31.2	-	-	31.6	31.8	-	27.2	27	24.6	26.1	25.6	25.6
1×10^4	-	-	-	-	-	-	29.3	28.9	25.6	27.6	26.3	27.2
1×10^3	-	-	-	-	-	-	29.6	30.9	27.5	28.9	29.2	28.5
1×10^2	-	-	-	-	-	-	30.9	30.7	-	30.7	30.4	29.4
1×10^1	-	-	-	-	-	-	31.6	31.4	-	31.1	31.6	30.9
neg serum or swab	-	-	-	-	-	-	-	-	-	-	-	-
NTC	-	-	-	-	-	-	-	-	-	-	-	-

Table 6-7. Analytical specificity (ASp) of the field mRT-qPCR) using virus panel in section 6.2.1.1 A and 6.2.1.2. Lysate were tested by simple dilution (1:5) without heat treatment. Results are expressed as quantification cycle (Cq), with Cq<40 considered positive. Negative control, R3+ positive control and Qβ internal control were included in each run, and all generated the expected results. (-) dash represents no Cq value.

Virus	sample type	Cq (tested single in TCOR-8)
FMDV O/SKR/6/2014	inactivated cell lysate	28.7
FMDV O/SAU/1/2013	inactivated cell lysate	23.1
FMDV O/PAK/28/2010	inactivated cell lysate	32.5
FMDV O/LIB/48/2012	inactivated cell lysate	31.2
FMDV O/MOR/1/2015	inactivated cell lysate	25.3
FMDV A/TUR/16/2014	inactivated cell lysate	26.5
FMDV A/TUR/28/2010	inactivated cell lysate	26.8
FMDV A/SAU/2/2015	inactivated cell lysate	25.6
FMDV A/SAU/2/2015	inactivated cell lysate	23.3
FMDV A/PAK/12/2015	inactivated cell lysate	25.5
FMDV Asia 1/TUR/2/2013	inactivated cell lysate	26.5
FMDV Asia 1/TUR/49/2011	inactivated cell lysate	23.3
FMDV Asia 1/TUR/12/2015	inactivated cell lysate	26.4
FMDV SAT1/TAN11/2012	inactivated cell lysate	16.5
FMDV SAT2/EGY/2012	inactivated cell lysate	26
FMDV SAT2/EGY/24/2014	inactivated cell lysate	22.6
Swine vesicular disease virus	inactivated cell lysate	-
BVDV-1 (Bovax)	cell lysate	-
BVDV-1 (C24V)	cell lysate	-
BVDV-2 (97/730)	cell lysate	-
BVDV-2 (97/1273B)	cell lysate	-
Negative serum	cattle serum	-
Negative oral swab	cattle oral swab	-

6.3.1.2. C2T FMD PCR

The ASe of the C2T FMD assay ranged between 1 to 10 copies of FMDV RNA for both infected serum and oral swab samples (Table 6-8). In all reactions with internal control signals, both the C2T mix 1 and mix 2 had acceptable ASp with no cross-reactions observed with BVDV-1 (n=1), BVDV-2 (n=1) and SVDV (n=1) positive lysates (Table 6-9).

Table 6-8. The analytical sensitivity of C2T FMD PCR (section 6.2.3.3) was determined using limited runs of two variants of the assay (mix 1 and mix 2). Mock-infected sera and oral swabs were used as templates (section 6.2.1.3). Results are expressed as quantification cycle (Cq). Samples with Cq<40 were considered positive for FMDV irrespective of the Cq value of the internal control (IC). Samples with Cq≥40 or no Cq value were considered negative for FMDV if IC produced a Cq value <40 and undetermined if IC did not amplify. The ASe of the assay ranged between 1 and 10 FMDV RNA copies per reaction for both variants of the assay. High variability was observed in the Cq of the IC in all three PCR runs (SD = 0.811 to 1.11.) possibly due to the effect of inhibitors present in the unextracted sample matrix. Neg= negative.

FMDV O RNA copies/reaction	**mix 1 – run 1**		**mix 2- run 1**		**mix 2 - run 2**	
	FMDV	IC	FMDV	IC	FMDV	IC
oral swab						
1×10^6	32.7	33.8	29.0	33.9	29.3	34.7
1×10^5	33.5	34.7	31.3	32.8	30.5	32.3
1×10^4	33.4	33.9	32.1	33.8	34.3	33.3
1×10^3	neg	neg	36.4	33.6	36.3	34.8
1×10^2	neg	32.9	34.9	32.3	35.3	34.6
1×10^1	neg	neg	neg	34.0	neg	33.7
1×10^0	neg	35.4	neg	37.6	35.7	33.4
Serum						
1×10^6	29.9	33.9	27.2	neg	28.2	33.2
1×10^5	30.1	33.7	27.1	30.4	29.6	34.7
1×10^4	31.0	34.3	28.3	34.1	29.3	33.6
1×10^3	32.9	34.0	29.2	34.4	32.7	34.7
1×10^2	34.6	35.8	33.3	neg	34.5	36.4
1×10^1	34.0	35.1	31.9	neg	35.0	36.0
1×10^0	35.5	34.1	neg	neg	35.2	34.7
mean Cq		34.3		33.69		34.29
SD		0.814		1.816		1.11
% CV		2.37		5.39		3.25

Table 6-9. Analytical specificity of C2T FMD field ready assay (mix 1 and 2) as determined by a panel of FMDV inactivated viruses (Pirbright Institute), bovine viral diarrhoea virus (BVDV) type 1 and type 2, and swine vesicular disease virus (SVDV) (section 6.2.1.1 A and 6.2.1.2). Results are expressed as quantification cycle (Cq), with Cq<40 considered positive. (-) dash represents no Cq value. The sample with Cq value, with or without Cq generated from the internal control (IC), was considered positive for FMDV. Test was not valid if no Cq is present in both targets.

Description	Sample type	mix 1, Cq		mix 2, Cq	
		FMDV	IC	FMDV	IC
FMDV O/SKR/6/2014	inactivated cell lysate	neg	neg	20.4	35.7
FMDV O/SAU/1/2013	inactivated cell lysate	22.8	34.5	20.9	33.2
FMDV O/PAK/28/2010	inactivated cell lysate	neg	neg	29.6	33.4
FMDV O/LIB/48/2012	inactivated cell lysate	29.7	33.7	28.0	33.0
FMDV O/MOR/1/2015	inactivated cell lysate	34.1	38.1	24.0	32.9
FMDV A/TUR/16/2014	inactivated cell lysate	22.0	neg	18.8	36.1
FMDV A/TUR/28/2010	inactivated cell lysate	26.1	33.2	25.6	33.2
FMDV A/SAU/2/2015	inactivated cell lysate	28.6	34.5	28.0	neg
FMDV A/PAK/12/2015	inactivated cell lysate	neg	neg	23.4	33.7
FMDV ASIA1/TUR/2/2013	inactivated cell lysate	neg	neg	29.8	30.0
FMDV As1/TUR/49/2011	inactivated cell lysate	23.8	32.9	23.0	33.4
FMDV As1/TUR/12/2015	inactivated cell lysate	neg	neg	28.5	35.1
FMDV SAT1/TAN11/2012	inactivated cell lysate	16.7	37.0	15.9	neg
FMDV SAT2/EGY/2012	inactivated cell lysate	32.8	neg	26.6	32.9
FMDV SAT2/EGY/24/2014	inactivated cell lysate	30.8	neg	22.9	neg
SVDV 2013 p2 7	inactivated cell lysate	neg	32.7	neg	33.6
BVDV-1 Bovax	cell lysate	neg	32.9	neg	33.5
BVDV-2 97/730	cell lysate	neg	32.7	neg	33.3
C2T sample diluent	buffer	neg	34.3	neg	32.9

6.3.2. Diagnostic specificity

Overall, 306 sera and 200 oral swabs from New Zealand cattle were analysed to estimate the DSp of the tests for the detection of FMDV (Table 6-10). DSp of the (mRT-qPCR for FMDV detection was high (100%) and similar to the reference AHL_FMD PCR using either sera (95% CI 98.8-100) or oral swabs (95% CI 98.12-100).

Table 6-10. Diagnostic specificity (DSp) of mRT-qPCR (FMDV detection) using samples from New Zealand cattle (section 6.2.1.4). Sample templates were not heat-treated and tested as described in section 6.2.3.1. The DSp of mRT-qPCR (FMD) was in agreement with the reference AHL_FMD PCR (section 6.2.3.2). Diagnostic specificities were calculated using Epitools with exact confidence limits for test evaluation (Sergeant, 2018). CI- confidence interval.

Sample type	N	mRT-qPCR (FMD)				reference AHL_FMD PCR			
		pos	neg	DSp, %	95% CI	pos	neg	DSp, %	95% CI
Serum	306	0	306	100	98.8-100	0	306	100	98.8-100
Oral swab	200	0	200	100	98.12-100	0	200	100	98.12-100

6.3.3. Diagnostic sensitivity

Mock-infected oral swabs (n=12) prepared from FMDV-positive clinical samples (section 6.2.1.5) were all positive (Table 6-11) in both the mRT-qPCR and AHL_FMD PCR. Nine of the ten FMDV positive samples (section 6.2.1.5) tested with the C2T (mix 2 assay) were positive for the FMDV RNA.

All oral swabs (n=20) spiked with inactivated FMDV-positive ES (section 6.2.1.1 b) at AHL (section 6.2.5.1) tested positive for FMDV in both mRT-qPCR and AHL_FMD PCR (100% DSe). The DSe for C2T FMD assay (17/17) was also high for the inactivated FMDV positive ES samples (section 6.2.1.1 b). Overall, the DSe estimate in both mRT-qPCR and AHL_FMD PCR in oral swabs was 100% (95% CI 89.29-100). A high DSe of 96.43% (95% CI 82.29-99.37) was also observed with C2T (27/28).

The DSe of LFD FMD pan-serotype detection was 80% (95% CI 49.02-94.33), all of which were serotyped as O (Table 6-11).

Table 6-11. Diagnostic sensitivity of mRT-qPCR, C2T FMD PCR, and LFD (refer to Table 6-3 for the test abbreviations) assessed using archival samples from past FMD outbreaks in Lao PDR and Myanmar (section 6.2.1.5) and inactivated FMDV epithelial suspension (ES) samples (section 6.2.1.1 b). The samples were used to prepare a simulated oral swab sample either by dilution (1:5 and 1:10) with RNAse free water (Lao PDR samples) or placing a Copan swab that had been saturated with ES into 1 mL of virus transport media (Myanmar and New Zealand or NZ samples). Simulated oral swabs were used directly as templates in mRT-qPCR or used for RNA extraction. The extracted RNA was used as a template in a reference AHL_FMD PCR (section 6.2.3.2) either on a CFX96 (BioRad) machine (Lao PDR and NZ) or the T-COR portable equipment (Myanmar samples). Lao PDR and Myanmar samples were also tested with LFD (section 6.2.3.4) with pan-detection and serotyping (A, O, Asia 1) capability. Diagnostic sensitivity was derived with Epitools using exact confidence limits for test evaluation (Sergeant, 2018). Results are expressed as quantification cycle (Cq), with Cq<40 considered positive. na = not applicable, nt = not tested.

Sample ID	Country	Sample type	FMD (mRT-qPCR)			C2T PCR, mix 2			Reference AHL_FMD RT-qPCR	LFD - Lateral flow device
			1:5, Cq	1:10, Cq	swab	1:5, Cq	1:10, Cq	swab	Cq	pan/serotype
NL1725352	Lao PDR	tongue ES[#]	24.20	24.90	na	25.90	25.70	na	20.33	positive / O
NL1725381	Lao PDR	tongue ES	24.00	23.90	na	25.70	25.30	na	17.28	positive / O
NL1620179	Lao PDR	tongue ES	29.70	28.20	na	30.40	29.10	na	22.74	positive / O
NL1840602	Lao PDR	tongue ES	35.30	34.90	na	35.50	30.90	na	22.25	negative
NL1840607	Lao PDR	tongue ES	34.00	31.80	na	no Cq	34.10	na	25.23	negative
Mya19/11	Myanmar	hoof ES	na	na	29.30	na	na	nt	17.30	nt
Mya19/12	Myanmar	hoof ES	na	na	25.00	na	na	31.90	16.80	positive / O
Mya19/13	Myanmar	tongue ES	na	na	28.20	na	na	23.90	19.70	positive / O
Mya19/15	Myanmar	hoof ES	na	na	25.70	na	na	20.90	16.50	positive / O
Mya19/17	Myanmar	hoof ES	na	na	25.80	na	na	19.90	15.60	positive / O
Mya19/18	Myanmar	tongue ES	na	na	27.20	na	na	29.10	20.00	positive / O
Mya19/19	Myanmar	saliva	na	na	33.70	na	na	neg	29.00	negative
W13_1437/1	NZ	ES	na	na	32.50	na	na	29.60	31.17	nt
W13_1437/2	NZ	ES	na	na	31.20	na	na	29.70	26.54	nt
W13_1437/5	NZ	ES	na	na	26.80	na	na	25.60	19.91	nt
W13_1437/6	NZ	ES	na	na	23.30	na	na	23.80	19.68	nt
W13_1437/3	NZ	ES	na	na	16.50	na	na	15.90	15.56	nt
W13_1437/4	NZ	ES	na	na	26.00	na	na	26.60	20.10	nt
W14_1170/1	NZ	ES	na	na	18.80	na	na	nt	15.03	nt
W14_1170/5	NZ	ES	na	na	29.60	na	na	nt	20.14	nt
W14_1170/7	NZ	ES	na	na	26.00	na	na	22.50	19.82	nt
W14_1170/8	NZ	ES	na	na	23.30	na	na	21.80	17.11	nt

Continuation of Table 6-11

Sample ID	Country	Sample type	FMD (mRT-qPCR)			C2T PCR, mix 2			Reference AHL_FMD RT-qPCR	LFD - Lateral flow device
			1:5, Cq	1:10, Cq	swab	1:5, Cq	1:10, Cq	swab	Cq	pan/serotype
W15_1361/1	NZ	ES	na	na	28.70	na	na	20.40	23.22	nt
W15_1361/2	NZ	ES	na	na	23.10	na	na	22.80	19.34	nt
W15_1361/5	NZ	ES	na	na	26.50	na	na	18.80	21.04	nt
W15_1361/7	NZ	ES	na	na	26.50	na	na	29.80	21.93	nt
W15_1361/4	NZ	ES	na	na	22.60	na	na	22.90	19.04	nt
W16_1281/2D	NZ	ES	na	na	25.30	na	na	24.00	21.99	nt
W16_1281/1Aa	NZ	ES	na	na	25.60	na	na	28.00	16.51	nt
W16_1281/1Ab	NZ	ES	na	na	23.30	na	na	nt	16.90	nt
W16_1281/2B	NZ	ES	na	na	25.50	na	na	23.40	19.92	nt
W16_1281/2C	NZ	ES	na	na	26.40	na	na	28.50	20.86	nt
no. of positive			5/5	5/5	27/27	4/5	5/5	22/23	32/32	8/10
DSe			100%	100%	100%	80%	100%	95.66	100%	80%
Combined DSe -FMD (mRT-qPCR) = 100% (95% CI 89.29-100)										
Combined DSe - C2T FMD = 96.43% (95% CI 82.29-99.37)										

6.3.3.1. FMD surveillance panel

Slaughterhouse surveillance swab samples (section 6.2.1.6) from cattle (n=37) and buffaloes (n=4) were tested both with mRT-qPCR (in Lao PDR and Myanmar) and TPI_FMD PCR (Appendix 5). Among the animals tested, 16 came from Lao PDR and 25 from Myanmar. Overall, a moderate test agreement (kappa=0.602) between the mRT-qPCR and TPI_FMD PCR was observed in this animal cohort (Table 6-12). Five samples were positive for FMDV RNA, and 31 were negative in both tests. The remaining five samples were positive in TPI_FMD PCR only. Three of those were buffaloes. Using TPI_FMD PCR as the gold standard test, the mRT-qPCR has a combined specificity of 100 % (95% CI 89-100) and a relatively low sensitivity of 50% (95% CI 19 – 81) for detecting sub-clinical FMDV infection.

Table 6-12. Agreement between results obtained using mRT-qPCR (FMD) and the TPI_FMD PCR (Table 6-3) to test pharyngeal swab samples from apparently healthy cattle and buffaloes from Lao PDR and Myanmar. The data were analysed using 2 x 2 table. Epitools with exact confidence limits for test evaluation (Sergeant, 2018) was used to analysed Kappa. CI- confidence interval.

	TPI_FMD+	TPI_FMD-	
mRT-qPCR +	5	0	5
mRT-qPCR -	5	31	36
	10	31	41

Kappa	0.601
SE for kappa = 0	0.143
Z(kappa)	4.200
Proportion positive agreement	0.667
Proportion negative agreement	0.925
Overall proportion agreement	0.878
McNemar's Chi sq	3.200
p (Chi sq)	0.074
Absolute diff. in proportions	-0.122
Relative diff. in proportions	-0.161
SE for non-zero kappa	0.153
Kappa lower 95% limit	0.302
Kappa upper 95% limit	0.902
Sensitivity	0.5, 0.19 - 0.81 95% CI
Specificity	1.0, 0.89-1.00 95% CI

6.3.4. Latent class model tests comparison

Since low numbers of FMDV-positive samples were available and no assumption was made regarding the samples' true disease status, BLCM was used to provide estimates for DSe and DSp, including disease prevalence. The cross-classifications of the FMD test results used for BLCM are shown in Appendix 6, and the posterior medians of the four models are detailed in Table 6-13. The point estimate for AHL_FMD PCR DSe (98%, 95-99% credible interval) and DSp (98%, 96-100% to 99%, 96-100% credible interval) was consistently high in all models. The DSe estimate of mRT-qPCR (model 1) was 95% (84-99 % credible interval). Similar DSe result was also obtained for C2T FMD PCR (model 2) with 93% (80-99% credible interval). The wide credible interval (mRT-qPCR and C2T FMD PCR) observed, although not unexpected, was due to the small sample size involved. DSe of both tests were influenced easily by the changes in the DSe prior estimate (96% to 90%) by about 4-5%. Among the four FMD tests in models 1 and 2, the LFD test, although with comparable DSp (97%, 88-100%

credible interval) to the other three FMD tests, had the lowest DSe of 79-% (61-91% credible interval) and 84% (66-95% credible interval).

Comparatively, the credible interval (also known as the posterior probabilities) in model 3 for mRT-qPCR DSe was the same with model 1 (Table 6-13). Model 3 was fitted with two CD tests and two animal populations with extreme disease prevalence. However, the DSp of mRT-qPCR in model 3 (a and b) was slightly higher than the first two models. In model 3b, where no priori assumptions were made for the new assay (mRT-qPCR), aside from a wider DSe estimate (73-99 credible interval), the posterior probabilities of other parameters were similar to the other models.

Table 6-13. Posterior estimates (95% credible interval) of diagnostic sensitivity (DSe) and specificity (DSp) using Bayesian latent class model. Model 1 and 2 are three test, two population models applied to archived outbreak samples (section 6.2.1.5) from Lao PDR and Myanmar. Model 3 (a and b) is a two test, two population model with dissimilar prevalence (Lao PDR + Myanmar and New Zealand). In Model 3b, no prior information was assumed for the new test (t1FMD) being examined. t1FMD is mRT qPCR (section 6.2.3.1), t2FMD is C2T FMD PCR (section 6.2.3.3) and t3FMD is LFD test (section 6.2.3.4). The r1FMD is the reference AHL_FMD PCR (section 6.2.3.2). pre = prevalence, PPV=positive predictive value, NPV=negative predictive value.

Parameters	Model 1	Model 2	Model 3a	Model 3b	Using a reference gold standard negative and positive panel
DSe_t1FMD	0.95 (0.84-0.99)		0.95(0.85-0.99)	0.94 (0.73-0.99)	1.0
DSe_t2FMD		0.93 (0.80-0.99)			1.0
DSe_r1FMD	0.98 (0.95-0.99)	0.98 (0.95-0.99)	0.98 (0.94-0.99)	0.98 (0.95-0.99)	1.0
DSe_t3FMD	0.79 (0.61-0.91)	0.84 (0.66-0.95)			0.80
DSp_t1FMD	0.97 (0.88-1.0)		0.99 (0.99-1.00)	1.00 (0.99-1.00)	1.0
DSp_t2FMD		0.97 (0.88-1.0)			
DSp_r1FMD	0.99 (0.97-1.0)	0.99 (0.96-1.0)	0.99 (0.97--1.00)	0.99 (0.97-1.0)	1.0
DSp_t3FMD	0.97 (0.88-1.0)	0.97 (0.88-1.0)			
prev_Lao	0.73 (0.44-0.92)	0.7 (0.40-0.92)			
prev_Myanmar	0.75 (0.48-0.93)	0.72 (0.42-0.92)			
prev_Lao+Myanmar			0.83 (0.61-0.95)	0.83 (0.61-0.95)	
prev_New Zealand			0.002 (0.00 - 0.002	0.002 (0.00 - 0.008)	
PPV_Lao+Myanmar cattle population			1.00 (0.99-1.00)		
NPV_Lao+Myanmar cattle population			0.82 (0.41-0.98)		
PPV_New Zealand cattle population			0.40 (0.10-0.80)		
NPV_New Zealand cattle population			1.00 (0.99-1.00)		

Model 1 - two conditionally dependent tests (t1FMD and r1FMD PCR) but conditionally independent to a third test (t3FMD lateral flow device) using two populations (Lao PDR and Myanmar).
Model 2 - two conditionally dependent tests (t2FMD and r1FMD PCR) but conditionally independent to a third test (t3FMD lateral flow device) using two populations (Lao PDR and Myanmar).

Model 3a - two conditionally dependent tests (t1FMD and r1FMD) using two cattle populations (Lao PDR +Myanmar and New Zealand) with different disease prevalence. DSe prior estimate for t1FMD was 96% and greater than 80% most of the time (95%).
Model 3b - two conditionally dependent tests (t1FMD and r1FMD) using two cattle populations (Lao PDR +Myanmar and New Zealand) with different disease prevalence. No prior estimates were given for t1FMD.

The PPV (100%, 99-100% credible interval) of mRT-qPCR was extremely high in FMD endemic countries (Lao PDR and Myanmar) while expected low (40%, 10-80% credible interval) in FMD-free New Zealand. However, the NPV of mRT-qPCR when applied to New Zealand cattle was high (100%, 99-100% credible interval), meaning a negative result in the pen-side test most likely predict that the suspect animal does not have FMD.

Not surprisingly, the DSe posterior estimate of mRT-qPCR when detecting FMDV in pharyngeal swabs from apparently healthy cattle but presumed to have sub-clinical infection (Table 6-14) was lower (83%, 65-96% credible interval) compared to DSe of TPI_FMD PCR (98%, 93-99% credible interval). The latent class model, however, had a considerably higher DSe estimate for mRT-qPCR than a 2 × 2 table calculation using TPI_FMD PCR as the reference standard (sensitivity = 50%, 19-81% 95% CI) as stated in section 6.3.3.1.

Table 6-14. Posterior estimates of two dependent FMD test (t1FMD=mRT-qPCR and r2FMD=TPI_FMD PCR), two population (Lao PDR and Myanmar) Bayesian latent class model of sensitivities and specificities using slaughterhouse surveillance samples (section 6.2.1.6). The t1FMD is the field multiplex PCR test (section 6.2.3.1) and r2FMD (section 6.2.3.2) is the PCR results from test conducted at The Pirbright Institute, UK. The median was also compared when r2FMD was treated as the gold standard test. CI=credible interval.

Parameters	two tests, two populations model with correlation median, 95% C.I.	r2FMD as reference gold standard, 95% CI
DSe_t1FMD	0.83 (0.65-0.96)	0.5 (0.19 - 0.81)
DSe_r2FMD	0.98 (0.93-0.99)	1.0 (0.89-1.0)
DSp_t1FMD	0.98 (0.95-0.99)	
DSp_r2FMD	0.99 (0.96-0.99)	
prevalence_Lao PDR	0.26 (0.10-0.49)	
prevalence_Myanmar	0.24 (0.10-0.42)	

6.3.5. Stability testing of reagents

Ready-to-use master mixes (section 6.2.6.1) were used for mRT-qPCR after 18-, 24- and 42-hours storage at various temperatures (Table 6-15 and Table 6-16). Master mix in vial

1 that has been kept at chilled conditions appeared stable for up to 24 hours since both FMDV RNA and QB RNA were amplified at all time points. The FMDV RNA and Qβ RNA were amplified using vial 2 master mix stored at -20°C for the duration of the experiment (up to 42 hours). FMDV RNA was not amplified with vial 3 master mix that was stored at room temperature for any of the three time periods. Further experiments are needed to complete the stability measurements by testing at least three batches of reagents for the three temperature conditions.

Table 6-15. Stability of mRT-qPCR master mix (section 6.2.6.1) FMDV-spiked serum sample (100 copies/reaction) was tested using mRT-qPCR on the T-COR and CFX96 machine using master mix that has been stored for an indicated time at three different temperatures. The storage conditions of vial 1 was mimicked conditions expected to be present during field deployment of mRT-qPCR assay. Results are expressed as quantification cycle (Cq), with Cq<40 considered positive.

Master mix	Storage condition	Temperature, °C	Storage time					
			18 hrs, Cq mean		24 hrs, Cq mean		42 hrs, Cq mean	
			TCOR-8 n = 2	CFX96 n = 5	TCOR-8 n = 2	CFX96 n = 5	TCOR-8 n = 2	CFX96 n = 5
Vial 1	Polystyrene box with chilly packs	-14-4.1°C (1st 18 hrs) 0.8-7.7°C (19-24hrs)	33.45 (2/2)	36.86 (5/5)	33.15 (2/2)	35.09 (5/5)	-/-	-/-
Vial 2	Freezer	-20°C	34.6 (2/2)	35.29 (4/5)	33.65 (2/2)	35.22 (5/5)	33.45 (2/2)	35.63 (5/5)
Vial 3	Incubator and on the bench	19-22°C	-/-	-/-	-/-	-/-	-/-	-/-

T-COR™ 8 = 2 replicates; CFX96 = 5 replicates

Table 6-16. The performance of mRT-qPCR master mix kept at different storage condition. Detection of Qβ internal control target that has been spiked into the serum sample using mRT-qPCR master mix that has been stored for an indicated time at three different temperatures on the T-COR and CFX96 machine. Qβ target was readily detected in Vial 1 experiments stored between 24 to 42 hours. Results are expressed as quantification cycle (Cq), with Cq<40 considered positive. hrs = hours

Master mix	Storage condition	Storage time						
			18 hrs, Cq mean		24 hrs, Cq mean		42 hrs, Cq mean	
		Temperature, °C	TCOR-8 n=2	CFX96 n=5	TCOR-8 n=2	CFX96 n=5	TCOR-8 n=2	CFX96 n=5
Vial 1	Polystyrene box with chilly packs	-14-4.1°C (1st 18 hrs) 0.8-7.7°C (19-24hrs)	*	*	26.1 (2/2)	26.84 (5/5)	24.7 (2/2)	-/-
Vial 2	Freezer	-20°C	*	*	26.2 (2/2)	26.92 (5/5)	25 (2/2)	28.15 (5/5)
Vial 3	Incubator and on the bench	19-22°C	*	*	-/-	-/-	-/-	-/-

*** Experimental run failed for an unknown reason.**

6.3.6. Field PCR control performance

While the testing conditions in the laboratory are stable and standardised, the testing conditions in the field can vary. For example, the recorded outside temperature during field testing in New Zealand was around 3°C, compared to 29°C in Vientiane and 35°C in Myanmar. To facilitate the development of protocols for field testing using mRT-qPCR on the T-COR, the entire process of deployment from AHL-MPI based in Wallaceville (North Island) to a farm with a suspect positive case was simulated. Three farms in the South Island of New Zealand were selected to mimic various conditions that may be encountered during testing. The T-COR PCR machine was also subjected to airplane and land travel conditions, with the pre-mixed master mix kept chilled (about 1-10°C) during travel. Across the three locations, the Cq values generated with the R3+ standard (Appendix 10) was consistent among four targets with a CV of no more than 0.059 (Table 6-17), even if the testing was performed 36-72 hours after preparation of the master mix. The overall % CV across the three testing locations was low and ranged between 2.83 and 4.10 for all targets.

Table 6-17. Performance variability of the mRT-qPCR during actual field testing in three countries with different prevailing temperatures (Lao PDR= 29°C, Myanmar=35°C and New Zealand=3°C) as indicated by the Cq values generated with the R3+ standard (1 × 10⁴ copies/reaction) in mRT-qPCR. Individual data points are shown in Appendix 10. CV = coefficient of variation, CI = confidence interval, n=number of test run, Cq=quantification cycle

		mRT-qPCR		
	FMD	BVDV-1	QβIC	BVDV-2
New Zealand	n=12			
mean, Cq	29.17	30.87	28.66	31.16
SD	1.079	0.973	1.691	0.949
CV, %	3.70	3.15	5.90	3.05
95% CI	28.48-29.86	30.25-31.49	27.59-29.73	30.56-31.76
Lao PDR	n=12			
mean, Cq	29.61	30.55	29.26	30.84
SD	1.001	1.037	0.873	0.916
CV, %	3.38	3.39	2.98	2.97
95% CI	28.97-30.25	29.89-31.21	28.71-29.81	30.26-31.42
Myanmar	n=11			
mean, Cq	29.01	30.85	28.90	31.14
SD	0.712	0.781	0.729	0.802
CV, %	2.45	2.53	2.52	2.58
95% CI	28.53-29.49	30.33-31.37	28.41-29.39	30-60-31.68
Overall mean Cq	29.27	30.75	28.93	31.04
Overall SD	0.957	0.925	1.187	0.880
Overall % CV	3.27	3.01	4.10	2.83
95% CI	28.94-29.60	30.43-31.07	28.52-29.34	30.74-31.34

6.4. Discussion

Pen-side testing can hasten presumptive diagnosis when dealing with an index case with clinical signs consistent with FMD. In this study, the performance of a newly developed mRT-qPCR was evaluated for the detection of FMDV in samples from cattle with vesicular lesions. With New Zealand cattle herds as the target population, the field mRT-qPCR was compared to reference tests using estimates of DSp and DSe as performance indicators. A panel of serum and oral swab samples from New Zealand cattle was tested to estimate the DSp of mRT-qPCR. Cattle herds from New Zealand are considered a good source of gold standard reference samples for DSp estimation because FMDV has never been detected in the country (Dohoo et al., 2014). Since FMD is exotic to New Zealand, mock-infected swabs with archival samples from FMD outbreaks in Lao PDR and Myanmar and inactivated FMDV epithelial suspension samples stored at AHL were tested for FMDV to establish DSe of mRT-qPCR. Overall, the DSp of mRT-qPCR was very high and equivalent to the DSp of the established AHL_FMD PCR assay (DSp= 100%, 95% CI 98.2-100). The DSe of mRT-qPCR for detecting the virus in samples from clinically affected animals was high (DSe 100%, 95% CI 89.29-100), comparable to the DSe of FMD PCR methods employed by most reference laboratories (King et al., 2006).

The high DSe of mRT-qPCR for FMD detection is most likely overly optimistic. The panel used for DSe comprised swab samples spiked with archival clinical samples from past FMD outbreaks and inactivated viruses as a proxy for infected oral swabs. Hence, the infected samples did not represent the full range of FMDV infection stages. Viral loads are often very high in oral fluids and serum in cattle during the acute phase of FMD (Alexandersen et al., 2003). In one experimental infection study, FMDV was shed in oral swabs at the level of between 6 to 8.01 $\log_{10}$ genomic copy numbers/mL within five days after the appearance of clinical lesions (Stenfeldt et al., 2016). Such levels of virus are above the limit of detection of mRT-qPCR. However, some animals with visible lesions but in the later stages of disease could have low viral load that is beyond the test's detection limit (Howson et al., 2017). In a field-testing study conducted in East Africa using an earlier version of the C2T FMD assay, FMDV RNA was more likely to be detected in samples from 1–3-day old lesions (5/5 epithelium, 1/1 swab and 3/3 sera) than from 4-7-day old lesions (13/14 epithelium, 15/19 swabs,

3/4 oropharyngeal fluids and 3/29 sera) (Howson et al., 2017). Hence, samples from early clinical cases and animals that have been in contact with those should be preferentially tested during an outbreak investigation. The preliminary data of Howson et al. (2017) was used for the BLCM analysis, which revealed perhaps a more realistic DSe estimate of 95% (84-99% credible interval) for mRT-qPCR.

The mRT-qPCR had low sensitivity in detecting FMDV in pharyngeal swab samples from animals at slaughter in this study. Although the pharyngeal area is also the primary site of viral replication after exposure in cattle (Stenfeldt et al., 2018), pharyngeal swabs tested most likely came from FMDV carrier animals. No clinical lesions were observed during the slaughterhouse sampling, suggesting that there was no current outbreak. FMDV carrier animals invariably have a low viral load in probang samples (Stenfeldt et al., 2016), which may have been outside the analytical sensitivity of mRT-qPCR. The pharyngeal swabs in this study had considerably high Cqs (Appendix 5), indicating the low viral load in the samples. Acutely infected cattle normally shed high levels of FMDV in samples such serum and oral swabs (Pacheco et al., 2016). Regardless of the unknown infection status of the animals, latent class modelling of the two dependent tests (mRT-qPCR and TPI_FMD PCR) in two populations of surveillance samples (Table 6-14) demonstrated possibly a more representative DSe estimate for mRT-qPCR (83%, 65-96% credible interval).

In the clinical diagnosis of a suspected vesicular disease in cattle, an FMD-negative but BVDV-1-positive test in the pen-side mRT-qPCR would indicate that the clinical signs observed in the affected animal were due to BVDV-1 infection. The afflicted animal could be either transiently infected with BVDV-1 or be a PI animal with mucosal disease. Because PI animals have high levels of BVDV-1 in both blood and respiratory secretions, they would be easily detected by the mRT-qPCR test ((Bhudevi & Weinstock, 2003; Lanyon et al., 2014; Zoccola et al., 2017). The main concern is such a situation would be the ability of mRT-qPCR to detect FMDV in samples from animals that are concurrently infected with BVDV-1 and FMDV. The mRT-qPCR assay is capable of simultaneously detecting the three viral targets (FMDV, BVDV-1 and BVDV-2) in a single reaction, as judged by the analytical sensitivity data (Table 5-8) using a universal RNA standard (R3+). The R3+ standard contained all three virus targets, including a Qβ phage IC fragment, in one long RNA construct. The pen-side PCR test was able to amplify all four targets between 1.0×10^6 and 1.0×10^0 copies simultaneously,

indicating that the assay can detect all targets present at various concentrations of the R3+ standard. However, such premise was based on an artificial template, not actual samples. The main limitation of this approach is that all targets were present in the sample in equimolar concentrations. In the future, this ability of the pen-side test should be validated using clinical samples co-infected with both FMDV and BVDV-1 or at least mock samples spiked with varying levels of FMDV and a competing virus. Nevertheless, any positive FMD, regardless of the BVDV-1 or BVDV-2 test outcome, should be given the most urgent attention to minimise viral spread.

During field testing in Lao PDR, Myanmar, and New Zealand to determine if the test can withstand different environmental conditions, the mRT-qPCR assay consistently amplified the R3+ standard (10^4 RNA copies). The mean Cq values of FMDV, BVDV-1, BVDV-2 and Qβ targets had very narrow CVs (≤ 0.59) between the three testing sites (Table 6-17). This result suggests the negligible effect of various field conditions (such as variations in the outside temperature (3°C to 35°C), the time the reagents are kept at chilled condition during transit, or the physical stress that the PCR machine was subjected to during plane and land travel) on the results obtained. The robustness of the mRT-qPCR system was supported by a preliminary experimental data where FMDV RNA was detected in virus-spiked serum (100 RNA copies/reaction) using a master mix kept in a chilled polystyrene box (Table 6-16) for up to 24 hours. Although, using dried reagents for molecular testing could be synonymous with being "robustly stable" for field deployment (Hobbs et al., 2021; Howson et al., 2015). However, it is expected that during the investigation of suspected FMD index cases in New Zealand, field testing will be carried out within 24 hours. This timeframe is within the observed performance of the premixed mRT-qPCR reagents, which therefore, make the mRT-qPCR fit-for-purpose in detecting FMDV during suspected incursion in New Zealand. Nevertheless, stability data of the pen-side PCR reagents needs to be expanded by further testing at least three different batches of reagents for the three temperature conditions.

In conclusion, the mRT-qPCR detection system for suspected FMDV incursion in New Zealand cattle had high DSp. The mRT-qPCR also had comparable DSe to the established laboratory PCR. Unfortunately, the absence of an actual FMD outbreak while visiting Lao PDR and Myanmar prevented the opportunity to deploy the mRT-qPCR in an actual field

diagnostic scenario. Validation using additional FMDV-positive samples from actual outbreaks and negative clinical samples should be attempted in the future.

6.5. References

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Chapter 7. Estimating the diagnostic performance of mRT-qPCR assay for detecting bovine viral diarrhoea virus type 1 and type 2

7.1. Introduction

Pathogens that can cause vesicular disease can obscure clinical recognition of FMD (Holliman, 2005). One example is the mucosal disease form of BVDV-1 infection, which is endemic to New Zealand (Reichel et al., 2018). The proposed pen-side multiplex PCR assay (mRT-qPCR, described in Chapter 5) allows the simultaneous detection of FMDV, BVDV-1 and BVDV-2. The mRT-qPCR assay was developed primarily to support the clinical diagnosis of FMD in a field setting and reduce the time delay when sending samples to the laboratory for testing.

Chapter 6 describes the analytical and diagnostic performance of mRT-qPCR in detecting the FMDV target. When applied to a target population, it is essential to understand the characteristic of the multiplex assay in detecting all targets (WOAH, 2022). Therefore, the primary aim of this work was to estimate the analytical (ASe and ASp) and diagnostic characteristics (DSp and DSe) of the mRT-qPCR when applied to New Zealand cattle for the detection of BVDV-1 and BVDV-2. The crude unextracted clinical sample was examined in the mRT-qPCR assay, either without or with heating applied into the template.

7.2. Materials and methods

7.2.1. Sources of samples

7.2.1.1. Archival cattle sera positive for BVDV-1

The positive panel was composed of archival cattle sera (n=30) positive for BVDV-1 (either by antigen ELISA or virus isolation) from AHL-MPI. The history and clinical status of the source animals were unknown.

7.2.1.2. Other viruses

A panel of virus isolates composed of inactivated FMDV (serotypes O = 5, A = 5, Asia 1 = 3, SAT1 = 1, and SAT 2 = 2), swine vesicular disease virus (n=1), BVDV-1 (n=2), and BVDV-2 (n=2) was used in this study. This panel was also utilised to determine the ASp of the mRT-qPCR assay for detecting FMDV (Table 6-7).

7.2.1.3. Clinical samples spiked with BVDV-1 and BVDV-2

Panels of mock-infected sera and oral swabs were prepared by spiking clinical samples (pooled cattle serum (n=5) and oral swab (n=5)) with either BVDV-1 (Bovax, AHL) or BVDV-2 (97/730, AHL) with a known quantity of viral RNA. The spiked sample was then serially diluted to produce a panel of mock-infected samples containing between 1.0×10^1 and 1.0×10^6 copies of viral RNA/µL of sample.

Viral RNA concentration of the stock virus was estimated by testing BVDV-1 and BVDV-2 stock lysate in mRT-qPCR (Table 5-5). Following the kit's instructions, RNA from BVDV-1 (140 µL, n=2) was extracted manually with the QIAamp Viral RNA kit (Qiagen). Final elution volume was 60 µL. Similarly, RNA from BVDV-2 (200 µL, n = 2) was extracted with Magjet Viral DNA and RNA Purification extraction kit according to the manufacturer's instructions on the KingFisher™ Flex (Thermo Fisher Scientific) machine. Eluted volume for BVDV-2 RNA was 100 µL. Five replicates of both eluents were then tested with mRT-qPCR on the CFX96 PCR machine, and viral copies were estimated as described in section 6.2.1.3.

7.2.1.4. Clinical samples from New Zealand cattle

Sera (n=306) and oral swabs (n=200) (Table 6-1) were collected from various dairy and beef cattle throughout New Zealand for disease surveillance and research purposes (section 6.2.1.4).

7.2.2. RNA extraction

Ribonucleic acid was extracted from clinical samples with a KingFisher™ Flex high throughput extraction machine using Magjet DNA and RNA Purification kit (Thermo Fisher Scientific) following the manufacturer's instructions. BVDV-1 (Bovax, AHL) and BVDV-2 (97/730, AHL) isolates were included in each extraction plate (96-well) as positive extraction control. RNase-free water was used as a negative extraction control.

7.2.3. BVDV PCR assay

7.2.3.1. Multiplex RT-qPCR

Table 7-1 shows the abbreviations of the BVDV assays included in the mRT-qPCR. The mRT-qPCR test was performed using clinical samples diluted in water (with or without heating) as templates (section 6.2.3.1). An R3+ positive control (1×10^4 copies per reaction, described in Chapter 3) and NTC (water) were incorporated into all BVDV PCR experiments.

7.2.3.2. Reference PCR

The BVDV-1 (rBVDV1) and BVDV-2 (rBVDV-2) reference PCRs (Table 7-1) were based on published primers and probes (Willoughby et al., 2006). The rBVDV1 and rBVDV-2 were performed according to the cycling conditions optimised for diagnostic testing at AHL-MPI (Appendix 2). Each reference BVDV PCR run included positive controls, which included RNA from BVDV-1 (Bovax, AHL) and BVDV-2 (97/730, AHL) isolates.

Table 7-1 BVDV test abbreviations.

Abbreviations	Test descriptions
tBVDV-1a	mRT-qPCR (BVDV-1 target) - un-heated template
tBVDV-1b	mRT-qPCR (BVDV-1 target) - heated template
tBVDV-2a	mRT-qPCR (BVDV-2 target) - un-heated template
tBVDV-2b	mRT-qPCR (BVDV-2 target) - heated template
rBVDV-1	reference BVDV-1 RT-qPCR
rBVDV-2	reference BVDV-2 RT-qPCR

7.2.4. Analytical performance of mRT-qPCR (BVDV-1 and BVDV-2)

A summary of the study design including the sample panels used for the different parameters is shown in Table 7-2.

Table 7-2. Test panels used to determine the analytical (ASe and ASp) and diagnostic (DSe and DSp) characteristics of the mRT-qPCR in detecting BVDV-1 and BVDV-2 using unheated and heated unextracted template (Table 7-1). The reference PCR tests (rBVDV-1 and rBVDV-2) were described in section 7.2.3.2. ASe – analytical sensitivity, ASp – analytical specificity, DSe – diagnostic sensitivity, DSp – diagnostic specificity.

Test under evaluation - mRT-qPCR	**tBVDV-1a (unheated) & tBVDV-1b (heated)**				**tBVDV-2a (unheated) & tBVDV-2b (heated)**			
Reference test	rBVDV-1 (Table 7-1)				rBVDV-2 (Table 7-1)			
Sample panel (section)	7.2.1.3	7.2.1.2	7.2.1.1	7.2.1.4	7.2.1.3	7.2.1.2	none	7.2.1.4
Parameters	ASe	ASp	DSe	DSp	ASe	ASp	DSe	DSp
Data analysis (section)	7.2.7	7.2.7 & 7.2.7.1	7.2.7	7.2.7*	7.2.7	7.2.7	none	7.2.7

* Bayesian latent class modelling was applied to clinical samples from New Zealand cattle (7.2.1.4).

Mock BVDV-1 and BVDV-2 infected sera and oral swabs (section 7.2.1.3) were tested to ascertain the analytical sensitivity (ASe) of mRT-qPCR for detection of either virus in crude clinical samples. The unheated (tBVDV-1a, tBVDV-2a) and heated (tBVDV-1b and tBVDV-2b) templates were prepared and tested according to section 6.2.4.

The analytical specificity (ASp) of mRT-qPCR for the detection of BVDV-1 and BVDV-2 was examined using virus isolates (section 7.2.1.2) without heat treatment (tBVDV-1a, tBVDV-2a) (section 6.2.4). A BVDV-negative serum and oral swab were also included in the mRT-qPCR run.

7.2.5. Diagnostic sensitivity of mRT-qPCR (BVDV-1 and BVDV-2)

A total of 30 BVDV-1 positive cattle sera (section 7.2.1.1) were examined to determine the DSe of mRT-qPCR (tBVDV-1a and tBVDV-1b). The conservative target sample size for

BVDV-1 DSe determination was *n*=30 (prior value of 98% DSe, 5% precision and 95% confidence) (WOAH, 2022). The template was tested with or without heat treatment, as detailed in section 7.2.3.1. The mRT-qPCR results were evaluated against rBVDV-1 (section 7.2.3.2) results using 5 µL extracted RNA from the positive panel (section 7.2.1.1).

No positive samples were available to establish DSe of BVDV-2 because the virus was not present in New Zealand and importing such positive material into the country was difficult.

7.2.6. Diagnostic specificity of mRT-qPCR

A total of 506(section 7.2.1.4) clinical samples (serum and oral swabs) were analysed to assess DSp of mRT-qPCR BVDV component of the mRT-qPCR assays (tBVDV-1a and tBVDV-2a) without heating. The sample size to determine DSp was based on a 97% DSe prior, 2% precision and 95% confidence (Dohoo et al., 2014; WOAH, 2022). The testing approach was detailed in section 7.2.3.1. The outcomes of the DSp experiments were compared to the results of the two reference RT-qPCRs (r1BVDV-1 and r1BVDV-2) using extracted RNA (5 µL) from the same samples (section 7.2.3.2).

7.2.7. Data Analysis

The threshold for a positive result was < 40 Cq in all PCR runs. A Cq value of ≥ 40 or none was deemed negative. The mean, standard deviation (SD), coefficient of variance (CV) and 95% confidence interval (CI) of the Cq data were calculated using Microsoft Excel for Microsoft 365 (Version 2202) and Epitools (Sergeant, 2018).

A 2 × 2 contingency table was constructed using the binary results of the mRT-qPCR (BVDV-1 and BVDV-2) and the reference RT-qPCRs. The DSp (TN or true negative / TN + FP or false positive) and DSe (TP or true positive / TP + FN or false negative) were then calculated using Epitools with exact confidence limits (Sergeant, 2018).

7.2.7.1. Latent class analysis

DSp and DSe of t1BVDV-1a and rBVDV-1 were also estimated using test results from the cattle samples (section 7.2.1.4) with BLCM. WOAH (2022) recommends the use of BLCM analysis when the true infection status of the animals from which the samples were derived is unknown (Enøe et al., 2000) or the reference standard test being used for comparison have an imperfect accuracy (Johnson et al., 2019; Limmathurotsakul et al., 2012).

A latent class model (Branscum et al., 2005) was applied to two conditionally dependent assays (tBVDV-1a and rBVDV-1), which both target the 5'UTR region, using data for two populations (North Island - NI cattle and South Island - SI cattle). The two tests, however, lacked identifiability because of conditional dependency (Johnson et al., 2019). Prior information (Table 6-5) was needed to support the identifiability of the model. Since the clinical samples were of unknown history, it was presumed that samples from viraemic PI or transiently infected cattle may have been present in the panel. Priors for a herd-level prevalence of viraemic animals were extrapolated from limited data. BVDV-1 infected herd was assumed to contain at least 1-2 % of PI animals (Han et al., 2018). The prevalence of transiently infected animals based on seroconversion of 3793 cows from 10 herds using individually tested bulk milk samples was 5.1% (Weir, 2016). That study involved a tiny fraction of the cattle population in New Zealand. However, the observed animal level prevalence was within the estimated weighted mean (4.37, 95% CI = 3.10-5.82) in a meta-analysis study involving 123 prevalence inputs from a sample size of 1,169,120 animals around the world (Scharnböck et al., 2018). For the current exercise, the NI animal level prevalence was assumed to be 6% (PI=1% and transient infection=5 %). The SI prevalence was assumed to be at 7% based on a higher PI prevalence of 2%.

The prior distributions were produced using the BetaBuster[2] software program Chun-Lung Su's online version (https://betabuster.software.informer.com/). BLCMs were conducted in OpenBUGS 3.2.3 (https://www.mrc-bsu.cam.ac.uk/software/bugs/openbugs/) using codes (provided by Geoff Jones of Massey University) shown in Appendix 9 and were performed as described in section 6.2.7.3. Posterior distributions (median with 95% credible

[2] http://252s-epi.vet.unimelb.edu.au/~epi/epi_html/Courses/ISVEE_2018_Dx_Tests_Nov-2018/html/UC_Davis_Website/betabuster.html

interval) were used to present the specificity, sensitivity, and prevalence estimates in the two populations.

Table 7-3. Informative priors used to conduct Bayesian latent class models for diagnostic accuracy studies of the BVDV-1 target (mRT-qPCR test) using un-heated template method (tBVDV-1a) and a reference RT-qPCR test (rBVDV-1).

Two test two population model for detecting BVDV-1 in New Zealand	
two populations	North Island (NI) and South Island (SI) (section 7.2.1.4)
t1BVDV-1a	BVDV-1 target, un-heated sample template (section 7.2.3.1)
rBVDV-1	reference RT-qPCR (section 7.2.3.2)
DSp_t1BVDV-1a	95% sure that DSp is greater than 90.0% and Mode at 99.0% - *conservative estimate from small initial test data set by the author*
DSe_t1BVDV-1a	95% sure that DSe is greater than 40% and Mode at 80.00% - *conservative estimate from small initial data set by the author*
DSp_rBVDV-1	95% sure that DSp is greater than 90.0% and Mode at 99.0% – *based on AHL unpublished validation data*
DSe_rBVDV-1	95% sure that DSe is greater than 90% and Mode at 98.00% – *based on AHL unpublished validation data*
Prevalence_NI	95% sure that prevalence is less than 10% and Mode at 6 % *(section 7.2.7.1)*
Prevalence_SI	95% sure that prevalence is less than 10% and Mode at 7 % *(section 7.2.7.1)*

7.3. Results

7.3.1. Analytical sensitivity and specificity

The limit of detection of the BVDV-1 target using an unheated template (t1aBVDV-1a) was between 1×10^3 and 1×10^5 viral RNA copies/reaction for both serum and oral swab samples (Table 7-4). Similarly, the limit of detection for the BVDV-2 target without heating the template (tBVDV-2a) was at least 1×10^4 viral RNA copies/reaction.

The limits of detection for BVDV-1 and BVDV-2 targets were lower (between 1×10^2 and 1×10^3 viral RNA copies/reaction) in serum and oral swab when the un-extracted samples were heat treated (tBVDV-1b and tBVDV-2b) prior to direct analysis on the portable T-COR 8™ device than without heating (Table 7-4).

Table 7-4. Analytical sensitivity of BVDV-1 and BVDV-2 detection by the RT-qPCR using spiked samples by serial dilution described in section 7.2.1.3. PCR templates, either pre-heated or not heated, were analysed directly without undergoing nucleic acid extraction. Results are expressed as quantification cycle (Cq), with Cq<40 considered positive. The assays that used a heated template (tBVDV-1b and tBVDV-2b) had better limit of detection (100-1000 viral RNA copies/reaction) for sera than assays that used the unheated template (tBVDV-1a and tBVDV-2a). NTC = non template control, (-) dash represents no Cq value.

viral RNA copies per reaction	**With heat pre-treatment**						**No heat pre-treatment**					
	serum			oral swab			serum			oral swab		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
	tBVDV-1a						tBVDV-1b					
1×10^5	28.0	28.2	28.0	27.7	27.4	29.0	-	31.1	32.4	28.7	28.8	28.8
1×10^4	30.8	31.0	30.8	30.6	30.5	30.7	-	-	-	30.5	30.8	31.0
1×10^3	33.6	33.1	32.4	33.0	32.8	29.0	-	-	-	32.8	32.0	32.8
1×10^2	-	-	33.8	-	-	28.4	-	-	-	-	-	-
1×10^1	-	-	-	-	-	-	-	-	-	-	-	-
neg serum or swab	-	-	-	-	-	-	-	-	-	-	-	-
NTC	-	-	-	-	-	-	-	-	-	-	-	-
	tBVDV-2a						tBVDV-2b					
1×10^5	26.7	27.6	27.5	24.2	24.2	24.4	28.8	27.9	29.1	28.3	28.2	28.4
1×10^4	29.3	29.3	29.4	28.6	28.6	28.7	32.4	32.5	31.7	30.6	30.1	30.9
1×10^3	31.9	31.6	31.2	31.3	31.3	30.4	-	-	-	-	-	32.7
1×10^2	33.4	-	-	32.8	32.8	32.4	-	-	-	-	-	-
1×10^1	-	-	-	-	-	-	-	-	-	-	-	-
neg serum or swab	-	-	-	-	-	-	-	-	-	-	-	-
NTC	-	-	-	-	-	-	-	-	-	-	-	-

Analytical specificity was high (100%) for both BVDV-1 and BVDV-2 targets (Table 7-5) with non-heated samples (tBVDV-1a and tBVDV-2a). ASp was not assessed with heated samples for either BVDV target due to time limitations. On all occasions, no cross-reaction was observed between BVDV-1 (C24v and Bovax) and BVDV-2 (97/730 and 97/1273B), FMDV-positive lysate (n=16) and swine vesicular disease virus (n=1). There was also no non-specific amplification in an unextracted non-heated negative serum or oral swab samples.

Table 7-5. Analytical specificity of mRT-qPCR (BVDV-1 and BVDV-2 target) using unheated viral isolates (section 7.2.1.2) as templates (tBVDV-1a and tBVDV-2a). PI, UK – The Pirbright Institute, United Kingdom; AHL, NZ – Animal Health Laboratory, New Zealand. (-) dash represents no Cq value.

Virus isolates	Source	sample type	Cq (tested in single)
FMDV type O/SKR/6/2014	PI, UK	inactivated cell lysate	-
FMDV type O/SAU/1/2013	PI, UK	inactivated cell lysate	-
FMDV type O/PAK/28/2010	PI, UK	inactivated cell lysate	-
FMDV type O/LIB/48/2012	PI, UK	inactivated cell lysate	-
FMDV type O/MOR/1/2015	PI, UK	inactivated cell lysate	-
FMDV type A/TUR/16/2014	PI, UK	inactivated cell lysate	-
FMDV type A/TUR/28/2010	PI, UK	inactivated cell lysate	-
FMDV type A/SAU/2/2015	PI, UK	inactivated cell lysate	-
FMDV type A/SAU/2/2015	PI, UK	inactivated cell lysate	-
FMDV type A/PAK/12/2015	PI, UK	inactivated cell lysate	-
FMDV type ASIA1/TUR/2/2013	PI, UK	inactivated cell lysate	-
FMDV type As1/TUR/49/2011	PI, UK	inactivated cell lysate	-
FMDV type As1/TUR/12/2015	PI, UK	inactivated cell lysate	-
FMDV type SAT1/TAN11/2012	PI, UK	inactivated cell lysate	-
FMDV type SAT2/EGY/2012	PI, UK	inactivated cell lysate	-
FMDV type SAT2/EGY/24/2014	PI, UK	inactivated cell lysate	-
Swine vesicular disease virus, 2013 P2	PI, UK	inactivated cell lysate	-
BVDV-1 (Bovax)	AHL, NZ	cell lysate	18.4
BVDV-1 (C24v)	AHL, NZ	cell lysate	13.5
BVDV-2 (97/730)	AHL, NZ	cell lysate	23.0
BVDV-2 (97/1273B)	AHL, NZ	cell lysate	28.8
Negative serum	NZ cattle	serum	-
Negative oral swab	NZ cattle	oral swab	-

7.3.1. Diagnostic sensitivity

Thirty BVDV-1 positive sera (section 7.2.1.1) were examined to determine DSe (Table 7-6) of mRT-qPCR to detect BVDV-1. Both tBVDV-1b (heated template) and rBVDV-1 (extracted RNA) detected viral RNA in all 30 BVDV-1 positive samples (DSe = 100%, 95% CI 88.65-100). However, the unheated template protocol (tBVDV-1a) had lower DSe (40%, 95% CI 24.59-57.86) with only 12/30 positives. None of the BVDV-1 positive samples produced non-specific amplification with FMDV or BVDV-2 primers.

The mean Cq values obtained for tBVDV-1b (heated) were comparable to those obtained with rBVDV-1. They were significantly lower than the mean Cq obtained with the

unheated samples (tBVDV-1a) (Table 7-6) with a P-value = 0.003 and 0.019, respectively. Sixteen of the 30 positive sera had a Cq > 30 in the reference r1BVDV-1 PCR.

7.3.2. Diagnostic specificity

A total of 506 cattle samples (306 sera and 200 oral swabs, section 7.2.1.4) were analysed to estimate the DSp of the mRT-qPCR for the BVDV-1 and BVDV-2 targets (un-heated) (Table 7-7). DSp for the tBVDV-2a component was high (100%) for detection of the virus in both sera (95% CI 98.8-100) and oral swabs (95% CI 98.2-100). The observed DSp was confirmed with the laboratory-based rBVDV-2 PCRs using RNA extracted from the same clinical samples (section 7.2.1.4).

One serum (1/306) was positive for BVDV-1 in mRT-qPCR using the unheated samples (tBVDV-1a), resulting in a slightly reduced DSp of 99.67% (95% CI 98.2-100). The same serum, and an additional two sera (for a total of 3/306 sera) tested positive in the rBVDV-1 PCR, resulting in a slightly decreased DSp of 99.2% (95% CI 97.2-99.6) of the reference assay. A follow-up testing of all BVDV-1 RNA PCR reactors (n=3) with the heat treatment protocol (tBVDV-1b) also gave the same positive results. This suggests that those sera were truly positive for BVDV-1, supporting a DSp of 100% for BVDV-1 detection in serum using both tBVDV-1a and rBVDV-1.

An oral swab from the animal with BVDV-1 positive serum also tested positive for BVDV RNA in both assays (tBVDV-1a and rBVDV-1), giving a DSp of 99.5 % (95%, CI 97.2-99.9). The same oral swab was also re-tested positive with tBVDV-1b. Considering that both serum and oral swab from this one animal were consistently positive for BVDV-1 RNA, this animal was most likely infected with BVDV-1 at the time of sampling. Hence, the DSp of tBVDV-1a and rBVDV-1 for detection of BVDV-1 in oral swabs can be adjusted to 100%.

Table 7-6. Diagnostic sensitivity (DSe) for detection of the BVDV-1 target in mRT-qPCR and BVDV-1 reference qPCR (rBVDV-1) using archived BVDV-1 positive sera (section 7.2.1.1). The unheated (tBVDV-1a) and heated (tBVDV-1b) samples were prepared as described in section 7.2.3.1. The reference rBVDV-1 was performed according to section 7.2.3.2. DSe was calculated using Epitools with exact confidence limits for test evaluation (Sergeant, 2018). SD=standard deviation, CI=confidence interval. The mean Cq of tBVDV-1b (P-value = 0.003) and rBVDV-1 (P-value = 0.019) were significantly lower than the Cq mean of tBVDV-1a test.

Sample ID	**tBVDV-1a (unheated)**		**tBVDV-1b (heated)**		**rBVDV-1**	
	Cq	Results	Cq	Results	Cq	Results
1	33.4	+	30.5	+	31.67	+
2	33.5	+	28.9	+	29.7	+
3	0	-	31.2	+	35.59	+
4	34.5	+	32.1	+	33.56	+
5	0	-	32.5	+	36.14	+
6	33.4	+	28.6	+	33.55	+
7	0	-	32.0	+	34.83	+
8	35.9	+	33.2	+	34.93	+
9	36.2	+	31.9	+	34.12	+
10	0	-	30.4	+	29.87	+
11	0	-	30.0	+	29.36	+
12	0	-	29.7	+	29.39	+
13	0	-	29.1	+	28.08	+
14	0	-	29.8	+	28.54	+
15	0	-	32.1	+	29.56	+
16	0	-	32.4	+	31.84	+
17	31.4	+	26.0	+	25.13	+
18	31.2	+	26.0	+	23.88	+
19	30.8	+	26.4	+	24.5	+
20	0	-	32.5	+	30.65	+
21	30.1	+	27.6	+	25.46	+
22	0	-	31.9	+	30.36	+
23	0	-	31.8	+	29.41	+
24	30.5	+	27.1	+	26.01	+
25	0	-	32.1	+	30.54	+
26	0	-	32.3	+	31.04	+
27	0	-	32.5	+	29.99	+
28	0	-	32.0	+	31.22	+
29	32.9	+	31.3	+	30.36	+
30	0	-	32.4	+	31.24	+
Mean	32.81		30.54 (P-value =0.003)		30.35 (P-value = 0.019)	
SD	2.05		2.16		3.22	
DSe, 95% CI	40% (24.59-57.86%)		100% (88.65-100%)		100% (88.65-100%)	

Table 7-7. Diagnostic specificities (DSp) of mRT-qPCR (tBVDV-1a tBVDV-2a) and reference BVDV-1 PCRs (rBVDV-1 and rBVDV-2) (section7.2.3.2) using clinical samples from New Zealand cattle (section 7.2.1.4). Crude samples without heating (section 7.2.3.1) were used as templates in both tBVDV-1a and tBVDV-2a. DSp was calculated using Epitools with exact confidence limits for test evaluation (Sergeant, 2018). CI – confidence interval.

n	PCR	pos	neg	DSp, %	95% CI
serum=306	tBVDV-1a	1	305	99.67	98.2-100
	tBVDV-2a	0	306	100	98.8-100
	rBVDV-1	3	303	99.2	97.2-99.6
	rBVDV-2	0	306	100	98.8-100
Oral swab=200	tBVDV-1a	1	199	99.5	97.2-99.9
	tBVDV-2a	0	200	100	98.2-100
	rBVDV-1	1	199	99.5	97.2-99.9
	rBVDV-2	0	200	100	98.8-100

7.3.3. Latent class model tests comparison

Bayesian latent class analysis was applied to tBVDV-1a (un-heated) and rBVDV-1 due to the assumption that the disease status of the clinical samples from New Zealand cattle (section 7.2.1.4) was unknown, and the reference PCR had no perfect accuracy. Using two conditionally dependent tests and two population (NI and SI cattle) models, the DSp posterior estimates of tBVDV-1a and rBVDV-1 were consistently high (97 %) with a 95% credible interval of 88-100%, given that the two models had slightly different DSe prior distribution (Table 7-8). The BLCM DSp findings generally agreed with the estimates presented in Table 7-7 using calculations described in section 7.2.7.

Most likely, model 1 provided a better DSe posterior distribution (73%, 33–94 % credible interval) for the un-heated template (tBVDV-1a) because the prior was based on the previous estimate in Table 7-4 using the BVDV-1 positive panel (section 7.2.1.1).

Table 7-8. Posterior estimates of two tests (tBVDV-1a and reference rBVDV-1 RT-qPCR) and two populations (North Island -NI and South Island-SI) using Bayesian latent class modelling for diagnostic sensitivities and specificities. tBVDV-1a mRT-qPCR used un-extracted template without heat treatment (section 7.2.3.1). In model 1, the DSe prior distribution was based on a mode of 80%, greater than 40% most of the time (95%) while model 2 had a mode of 90% and greater than 70% most of the time (95%).

Parameters	Model 1	Model 2
	median, 95% credible interval	median, 95% credible interval
DSe_tBVDV-1a	0.73 (0.33-0.94)	0.87 (0.66-0.97)
DSe_rBVDV-1	0.96 (0.90-0.99)	0.96 (0.89-0.99)
DSp_tBVDV-1a	0.97 (0.88-1.0)	0.97 (0.882-1.0)
DSp_rBVDV-1	0.97 (0.88-1.0)	0.97 (0.88-1.0)
Prevalence NI	0.06 (0.03-0.1)	0.06 (0.03-0.10)
Prevalence SI	0.07 (0.05-0.1)	0.013 (0.03-0.10)

7.4. Discussion

Bovine viral diarrhoea virus type 1 causes mucosal disease in persistently infected (PI) cattle that can be difficult to distinguish from FMD based on clinical presentation alone. To facilitate rapid differential detection of both viruses in the event of a suspected FMDV incursion, a pen-side multiplex PCR (mRT-qPCR) including the detection of BVDV-1 and BVDV-2 targets, was developed (Chapter 5). The performance of the DSe and DSp estimates of the assay, specifically for BVDV-1 and BVDV-2, including ASe and ASp in mock virus-infected samples was examined.

A panel of BVDV-1 positive sera was analysed to determine the DSe of mRT-qPCR in detecting the BVDV-1 target. Clinical samples (sera and oral swabs) collected from New Zealand cattle were also tested to determine the DSp of mRT-qPCR in detecting both BVDV types. The mRT-qPCR was applied to unextracted samples with or without heat treatment, and reference BVDV PCR tests were used to analyse purified RNA from the same sample set. Overall, the DSp of mRT-qPCR for BVDV-1 and BVDV-2 targets using unheated samples (n=506) was close to 100% (95% CI= 98.2-100). However, the DSe for detection of BVDV-1 with unheated samples was low (40%, 95% CI 24.59-57.86), although the estimate dramatically improved when heat was applied to the samples before testing (DSe=100%, 95% CI 88.65-100). Heat treatment improves the detection of BVDV-1 and BVDV-2 in samples with low viral copies. This observation agreed with the initial findings in Chapter 3, where heating

the prepared PCR template before analysis enhanced the detection rate of BVDV targets. However, this approach compromises the DSe of the test for FMDV detection. A higher DSe for FMDV detection was achieved with unheated samples (Chapter 6).

Judging by the spread of Cq values acquired, it is likely that the samples used to estimate DSe (BVDV-1) included those collected from both transiently infected and PI cattle. Transient infection is synonymous with acute BVDV-1 infection in cattle with mild or subclinical disease (Evans et al., 2019). The duration of viremia is short and may last up to three weeks post-infection when the virus is cleared by the host's immune response including production of virus-specific antibodies (Liebler-Tenorio et al., 2004; Meyling et al., 1990). More than half of the positive sera (Table 7-6) had a Cq value of >30 when heat-treated samples were tested (tBVDV-1b). Samples from transiently infected cattle were reported to have a low viral load (Lanyon et al., 2014) and hence would be expected to produce high Cq values in qPCR. Peddireddi et al. (2018) supported this argument when samples (ear notch, buffy coat, serum, and nasal swab) from acutely infected cattle produced Cq values ranging from 34 to 39 (mean Cq 37) after RT-qPCR testing. A high Cq value in qPCR is correlated to a low concentration of the target genomic fragment in the sample (Wong & Medrano, 2005).

In contrast, PI cattle shed massive quantities of BVDV and are the primary source of viral infections in the herd (Lanyon et al., 2014). Zoccola et al. (2017) showed evidence of high viral copies per reaction (1×10^4 - 1×10^7) in samples (hair bulbs, ear notch, plasma, and peripheral blood mononuclear cells) from PI animals. The low Cq values (19-30) obtained after PCR analysis of formalin-fixed tissues also supported this premise of high BVDV-1 load in PI cattle (Bhudevi & Weinstock, 2003).

The DSe for tBVDV-1a (without heating) would have probably been higher than that calculated in the current study if most of the BVDV-1 positive sera were from PI animals. The limit of detection range (1×10^4 to 1×10^5 viral copies) for BVDV-1 (mRT-qPCR) with unheated samples encompasses the expected viral load in clinical samples from PI animals. Serum from PI cattle was approximated to contain between 1×10^5 to 7×10^5 viral copies/microlitre (Fichtelová & Kovařčík, 2020), which is within the limit of detection of the established mRT-qPCR. In a study by Zoccola et al. (2017), 1×10^4 viral copies per reaction were also estimated to be present in hair bulb suspension from PI cattle based on detection by RT-qPCR using

crude samples without nucleic acid extraction. Nevertheless, further testing of additional samples from PI and transiently infected cattle is needed to fully establish the DSe of mRT-qPCR for BVDV-1 detection in serum samples without heating in this group of animals.

Since the primary purpose of the pen-side mRT-qPCR is to help rule in/out the suspected FMD index case, the method with greater sensitivity for detecting FMDV (i.e., no heating of template) should be prioritised. Most likely, if mucosal disease was involved, given the expected high BVDV load in PI cattle, the odds of detecting BVDV are high, even when using unheated samples. However, following a negative result for all targets, heat-treated samples can be tested to increase the probability of detecting BVDV that may be present at low concentrations. It is also possible to run simultaneous PCR reactions with the unheated and heated samples on the T-COR 8 platform.

In the clinical diagnosis of a suspected vesicular disease in cattle, an FMD negative but BVDV-1 positive result in the pen-side mRT-qPCR would indicate that the disease was most likely due to BVDV-1 infection. The affected animal could be either in the acute stage of transient BVDV infection or be a PI animal that developed mucosal disease. Acute BVDV-2 infection can induce severe disease clinically indistinguishable from mucosal disease but is currently absent from New Zealand and most BVDV-1 infections in immunologically competent animals present as mild disease (Reichel et al., 2018). Hence, mucosal disease is more likely to be clinically resemble FMDV than acute BVDV-1 infection. The PI animals would be easily detected by the mRT-qPCR test as they have high levels of BVDV-1 in both blood and respiratory secretions (Bhudevi & Weinstock, 2003; Lanyon et al., 2014; Zoccola et al., 2017). The high specificity of the mRT-qPCR for detection of BVDV-1 makes it unlikely that a positive animal is not infected with BVDV-1. However, further testing is needed to confirm the PI status of the affected animal. The animal could be re-sampled for detection of viral RNA (either by pen-side mRT-qPCR test or another lab-based test). A negative PCR result in the second test will confirm the infection was transient because the humoral immune response clears the virus within three weeks post-infection (Liebler-Tenorio et al., 2004; Meyling et al., 1990). A positive PCR result in the blood would suggest that the animal was persistently infected because BVDV remains in the PI animal throughout its lifetime (Houe, 1999).

Alternatively, the serum of the BVDV-1 infected animal can be tested for antibody against BVDV-1 approximately three weeks after the development of clinical disease. While a transiently infected animal will mount BVDV-1-specific antibodies within three weeks post-infection, a PI animal is antibody-negative, unless it is young enough to have some levels of BVDV-1 maternal antibodies. Immuno-tolerance of the PI animals is believed to develop in the foetus where an infecting BVDV-1 prevented the interaction of type I interferon and dendritic cells, vital in the induction of an efficient adaptive immunity (Fitzgerald-Bocarsly & Feng, 2007). Culling a PI animal is always recommended to prevent the persistence of BVDV-1 in cattle herds (Houe, 1999). However, regardless of whether the animal is positive or negative with BVDV-1 on the mRT-qPCR, a positive FMDV should take precedence over BVDV-1 and should be dealt with promptly by further confirmatory testing, simultaneously with rapid disease emergency response measures.

As already discussed in Chapter 6, it is possible for an animal to be simultaneously infected with BVDV-1 and FMDV. In such situation, both PCR targets would be expected to be amplified. One limitation of the work described in this chapter is the lack of testing results with samples containing various concentrations of both FMDV and BVDV-1, which could affect the sensitivity of between assays.

In conclusion, the mRT-qPCR had a relatively good diagnostic performance (DSe and DSp) for the detection of BVDV-1 and BVDV-2. The DSp of both BVDV targets was high. The mRT-qPCR (BVDV-1) also had high DSe for detecting viral RNA levels expected to be present in either PI or transiently infected animals, particularly when heating was applied to samples. Reformatting the mRT-qPCR only to include BVDV targets may be done if used solely for BVDV-1 detection. Theoretically, the assay can help remove PI cattle in the herd or screen for BVDV-1 infection in unvaccinated ten-month-old cattle and replacements heifer calves being introduced to the farm. However, considering the low throughput of the T-COR, this is probably unrealistic, unless T-COR instruments became a part of portable equipment owned and used by veterinary practitioners throughout New Zealand.

7.5. References

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Chapter 8. Concluding remarks

Several transboundary animal diseases constantly threaten the global food supply. A good example is the impact of ASF outbreaks in reducing the pig population of China (Ma et al., 2021) and affecting the livelihood of pig farmers in neighbouring Southeast Asian countries (Mighell & Ward, 2021). More recently, FMD has caused livestock production loss in Indonesia (Susila et al., 2022), previously free from the disease. Thus, preventing disease incursions, reducing the size of an outbreak if it occurs, and rapid control to hasten the return to disease freedom are central to disease readiness in developed countries. The Ministry for Primary Industries is at the forefront of ensuring that New Zealand has a robust biosecurity system to prevent the entry of such high-impact foreign diseases and the capability to detect and contain rapidly if such an animal health emergency occurs. For this reason, a constant overarching purpose of AHL-MPI is to understand the utility of new tools to complement its diagnostic capability. This thesis described developing and validating a pen-side molecular test (mRT-qPCR) system to support in-field clinical FMD diagnosis during index case investigation.

The assay development was aligned with WOAH's recommended pathways (assay development, assay validation, and validation status retention) (WOAH, 2022). Chapters 2, 3, 4, and 5 encompass all the needed work to complete the assay development stage. Defining the assay's intended application was critical at the start to guide the test system design, considering the most suitable sample matrix to detect the three viral targets. Included in the assay design was selecting a fit-for-purpose field-deployable platform, the TCOR-8 (Chapter 2) and a simple sample dilution method for in-field testing (Chapter 3). Crucial for optimising and calibrating the new assay was the availability and construction of a non-infectious RNA-positive standard (R3+) (Chapter 4). Also, an exogenous IC (Qβ phage) was developed as a process control to ensure that a negative result in a PCR amplification reaction is genuinely negative. Lastly, standardising and optimising a multiplex PCR assay was described in Chapter 5 for the simultaneous detection of FMDV and two differential agents (BVDV-1 and BVDV-2) for supporting vesicular disease diagnosis suspected in New Zealand.

After developing the mRT-qPCR assay, the performance of the pen-side test was assessed according to the three stages of the assay validation pathway (WOAH, 2022). During stage 1, the analytical sensitivity of the pen-side PCR assay was examined initially using the R3+ standard (Chapter 5) and then using unextracted virus-spiked templates in a pen-side format (Chapters 6 and 7). Other validation criteria, such as ASp and intra- and inter-run repeatability of the mRT-qPCR, were determined in these chapters. Under stage 2, the pen-side assay's diagnostic characteristic was investigated, specifically the DSe and DSp estimates of the mRT-qPCR in detecting FMDV and BVDV in NZ cattle (Chapter 6 and Chapter 7). New Zealand is free from FMD. Thus, it was also necessary to perform DSe studies in endemic countries where positive outbreak samples were accessible (Lao PDR and Myanmar). Stage 3 of the validation pathway, which requires assessing the reproducibility of the assay by sending a standard set of evaluation panels and pen-side assay set-up to other collaborating laboratories, was not completed due to the lack of a standard panel to mimic an actual sample and time constraints in the study. Since the primary user of the assay will be personnel from AHL-MPI, the precision of the assay between operators was assessed when it was used in various locations (New Zealand, Lao PDR, and Myanmar). Notwithstanding, having completed the WOAH-endorsed evaluation of analytical and diagnostic characteristic criteria, the pen-side mRT-qPCR met the minimum requirement to be considered provisionally "validated" and can be used for the desired purpose. In order to retain the validation status of the pen-side assay within AHL-MPI, it aims to constantly use the system in analysing the proficiency panel from the Pirbright Institute.

Several criteria were considered when developing a fit-for-purpose mRT-qPCR system (Table 2-1) for rapidly ruling in or out a suspected FMD case in New Zealand cattle. One criterion was operational simplicity, with only a few assay steps required to perform the test. The mRT-qPCR consisted only of five easy-to-follow steps, which can produce results within one and a half hours. The pen-side assay can be performed in-field using diluted samples from clinically affected animals without needing the complicated nucleic acid extraction method. Given the simplicity of the technique, the assay is highly transferrable to other AHL-MPI personnel with minimal laboratory background and training. Howson et al. (2018) and Bath et al. (2020) also developed a similar easy-to-use pen-side test concept for FMD to help decision-making at the testing point. Howson et al. (2018) used a commercially produced deployable PCR test in the endemic region of East Africa, similar to the FMD C2T test assessed

in Chapter 6. On the other hand, Bath et al. (2020) described the validation of a simple RT-LAMP assay for in-field detection of FMDV, which state government veterinarians of Australia can use.

Another aspect considered in the pen-side assay development was the optimisation of robust standard reagents that work well in field conditions. The standardised master mix of the mRT-qPCR can be prepared before going to the field and remains unaffected for up to 36 hours in chilled conditions. Although it still lacks the stability offered by lyophilised reagents at ambient temperature, the master mix optimised in this work can be used confidently in outbreak investigation in New Zealand. The field mRT-qPCR assay was robustly evaluated in extreme temperatures in Laos PDR, Myanmar, and the South Island of New Zealand (Chapter 6). Admittedly, the assay could be further improved using shelf-stable reagents to encourage the system's adoption in endemic tropical regions with low resources rather than keeping reagents at cold temperatures. The C2T FMD (Tetracore) are based on lyophilised reagents that are compatible with working at room temperature and could be used in such a situation (Howson et al., 2018).

The pen-side PCR was created with the aim of including differential agents that could interfere with the clinical diagnosis of FMD. Aside from FMDV, the mRT-qPCR contained targets for BVDV-1 and BVDV-2. BVDV-1 is endemic to New Zealand and could mimic FMD lesions. The advantage of such a multiplex format is that it increases the efficiency and hastens disease diagnosis. For instance, when dealing with suspect cattle with vesicular lesions, if the outcome of the mRT-qPCR is BVDV-1 positive but FMDV negative, combined with the pathological lesions observed, it could be construed that it is highly likely that infection could be attributed to the MD form of BVDV-1. Because only PI cattle can develop MD, culling the animal maybe justifiable to prevent the spread of the virus in the herd. However, considering the massive downside of failing to detect FMD, confirmatory laboratory testing is still recommended.

Despite the high DSe and DSp of mRT-qPCR in detecting FMDV, these parameters are impractical in estimating the probability of FMD in suspect cattle (Akobeng, 2006). A better measure of informing the chance of having the disease in sick cattle is using the predictive values of the pen-side test once the result is known. However, PPV and NPV are

greatly influenced by varying disease prevalence (Dohoo et al., 2014). High disease prevalence equates to high PPV, and low disease prevalence equates to low PPV. As exemplified by the BLCM predictive values in this study (Table 6-13), the probability of FMD in suspect cattle with positive mRT-qPCR results is exceptionally high (PPV=100%, 99-100% credible interval) in endemic regions (Lao PDR and Myanmar). In contrast, given that New Zealand is expected to have zero prevalence for FMD, suspect cattle that test positive in the pen-side PCR test will have a lower (PPV = 40%, 10-80% credible interval) chance of having FMD. Indeed, the PPV of an assay, even with high DSe, is inherently limited when applied to animal populations with extremely low disease prevalence. Conversely, the probability of a suspect New Zealand cattle not having FMD is high when the mRT-qPCR result is negative (NPV = 100%, 99-100% credible interval). Nonetheless, the diagnostic value of mRT-qPCR results is greatly enhanced when interpreted in conjunction with clinical presentation, epidemiological background, and additional laboratory testing when arriving at a diagnosis for FMD.

The intended users of the pen-side test are mRT-qPCR-trained personnel that could accompany an MPI veterinary incursion investigator to assist with the field diagnosis of FMD in case an initial investigating veterinarian cannot rule out FMD based on presentation alone. However, a disadvantage of having one machine based in one site (e.g., Upper Hutt) would prevent the ability to respond in case of a multi-pronged outbreak investigation, albeit unlikely. The LAMP pen-side system reported by Bath et al. (2020) was designed to be deployed in different states of Australia that can be used for FMDV exclusion. Adopting and scaling up the pen-side system during the delimitation stage, where we want to identify infected farms rapidly, will also be challenging. The T-COR 8 platform cannot efficiently test the required number of samples per herd to screen farms with no apparent clinical signs. The TCOR-8 only has six sample reactions per run. In order to detect confidently an FMD-affected herd of 1000 cattle, based on a 5 % herd prevalence and test DSe of 95%, sixty samples are required for testing to detect at least one infected animal. Regardless, the mRT-qPCR field assay in this study was fit to reliably detect FMDV in clinically affected cattle in an index case scenario.

One biosecurity risk that needs consideration during field testing is the possible contamination of the PCR machine, which could inadvertently spread the virus to other susceptible animals if used in various sites. The PCR device has to be surface disinfected, for

example, with citric acid (e.g., 0.5 %), before transporting it to separate locations or back to AHL. Additional procedures to mitigate the risk of virus spread include demarcating an imaginary "dirty" and "clean" area on the farm, similar to the method described by Howson et al. (2018). The "dirty" area is where the disease and exposed animals are located. The "clean" area is the area where no animals are allowed. Sample collection, processing and preparation of assay reactions should happen in the "dirty" area. The outside of the reaction tubes should then be wiped with disinfectant before being brought and placed into the T-COR 8 device in the designated "clean" area. In addition, personnel performing the test should be aware of decontamination procedures such as thoroughly cleaning and washing hands, disinfecting boots, and leaving contaminated clothing within the "dirty" area before moving out into the "clean" area. Placing the PCR machine on the back of a vehicle gives the flexibility to park away from a site with infected animals.

Several recommendations identified through the various stages of development can expand its utilisation for other purposes. One example is exploring other field-adaptable methods of preparing samples to improve the diagnostic sensitivity of the assay so that the pen-side test can be used to screen sub-clinical infections or detect carrier animals reliably. As shown in Chapter 6, the current mRT-qPCR had low sensitivity for detecting FMDV in apparently healthy cattle from slaughterhouses compared to collected samples from cattle with clinical signs. Another recommended work is to investigate how to scale-up the field-testing capability by having multiple deployable platforms dispersed immediately across New Zealand and identifying newer deployable technology with higher sample throughput capacity so the rapid detection of infected herds can be made efficiently.

In conclusion, a simple, extraction-free pen-side PCR test (mRT-qPCR) can be deployed around New Zealand for rapid and reliable detection of FMDV in the event of a suspected incursion. The mRT-qPCR field assay also serves as a baseline for establishing future "pen-side" test capability for other infectious agents if needed. The field mRT-qPCR, however, was not designed to replace WOAH (2018)-prescribed laboratory diagnostic tests for confirmation. Samples from sick animals still have to be collected for testing at AHL. Since the project's scope did not include the policy provision, it is recommended to discuss implementation, deployment into the AHL diagnostic algorithm, training, interpretation and reporting of results.

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Appendices

Appendix 1. FMDV 3D 1-Step RT-qPCR

Primers*	Sequence (5' – 3')	Size (bp)	Position	Target	Amplicon
Forward	ACTGGGTTT TACAAACCTGTG A	22	1185	FMDV 3D gene	106 bp
Reverse	GCGAGTCCTGCCACGGA	17	1291		
Probe	FAM-TCCTTTGCACGCCGTGGGAC-TAMRA	20	1236		

Reagent mix	Volume (µL)
Molecular grade water	2.25
2× master mix**	12.5
RT/Taqman enzyme mix**	0.5
10 µM FMDV3DFP.	2.00 (800 nM)
10 µM FMDV3DRP	2.00 (800 nM)
10 µM FMDV3DPro	0.75 (300 nM)
RNA template	5
Reaction volume	25

** PCR kit: Superscript III/Platinum Taq one-step RT-qPCR (Thermo Fisher Scientific)

RT-qPCR controls	Description
Negative	RNA from negative extraction control (molecular grade water)
Positive	FMDV RNA and or R3+ synthetic transcript (10,000 copies/reaction)
NTC	Molecular grade water

Cycling parameters:	Temp (°C)	Time	No. cycles	Channel acquisition
Reverse transcription	60	30 min	1	
Activate polymerase	95	10 min	1	
Denature Anneal/extension	95 60	15 60	45	Anneal/extension FAM (CFX96)

Reference

1. SOP TM-98 Conventional and real time polymerase chain reaction for Immunology. Internal Document. Animal Health Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, 2021.

Appendix 2. BVDV type 1 or BVDV type 2 One-Step RT-qPCR

Primers*	Sequence (5' – 3')	Size (bp)	Position	Target	Amplicon
Forward	CCA TGC CCT TAG GAC TAG C		76-97	5'UTR region	
Reverse	TGA CGA CTA CCC TGT ACT CAG G		176-198		
Probe BVDV-1	FAM- AAC AGT GGT GAG TTC GT - MGB		145-161		92
Probe BVDV-2	FAM - ACT AGC GGT AGC AGT GAG- MGB		109-126		122

Reagent mix	Volume (µL)
Molecular grade water	3.75
2× master mix**	12.5 (1 ×)
RT/Taqman enzyme mix**	1.0
10 µM BVDV forward	1.25 (500 nM)
10 µM BVDV reverse	1.25 (500 nM)
10 µM BVDV 1 or 2 probe	0.25 (100 nM)
RNA template	5
Reaction volume	25

** PCR kit: Superscript III/Platinum Taq one-step RT-qPCR (Thermo Fisher Scientific)

RT-qPCR controls	Description
Negative	RNA from negative extraction control (molecular grade water)
Positive	FMDV RNA and or R3+ synthetic transcript (10,000 copies/reaction)
NTC	Molecular grade water

Cycling parameters:	Temp (°C)	Time	No. cycles	Channel acquisition
Reverse transcription	50	15 min	1	
Activate polymerase	95	2 min	1	
Denature Anneal/extension	95 60	15 s 30 s	45	Anneal/extension FAM (CFX96)

Reference

1. * Willoughby et al. (2006)
2. SOP TM-97Conventional and real time polymerase chain reaction for Virology. Animal Health Laboratory, Biosecurity New Zealand, Ministry for Primary Industries, 2021. Internal document.

Appendix 3. Reaction mix and cycling parameters for FMDV RT-qPCR 3D single-plex assay.

Reaction mix

Component	concentration	volume, µL
RNase free water	-	7.1
4x Taqman Fast Virus* mix	1 ×	5
10 µM FMDV 3D forward	900 nM	1.8
10 µM FMDV 3D reverse	400 nM	0.8
10 µM FMDV 3D probe	150 nM	0.3
PCR template	-	5
Volume per reaction		20

* Taqman Fast Virus 1-step RT-PCR mix - Life Technologies

** T: temperature

*** RT: reverse transcription

Cycling conditions

step	T, ℃ **	Time	cycle no.
RT***	50	5 min	1
Hold	95	20 s	1
Denaturation	95	10 s	
Annealing / Extension	60	30 s	45
FAM channel			

Appendix 4. Primers and probes with corresponding reporters and quenchers are used in the four-plex mRT-qPCR.

Primers / Probe	Target	5' to 3' Sequence	Base Position	Amplic on size	Reference
FMD 3D forward primer	FMDV*	ACT GGG TTT TAC AAA CCT GTG A	6769-6791	106	GenBank AF189157 Callahan et al. 2002
FMD 3D reverse primer		GCG AGT CCT GCC ACG GA	6875-6858		
FMD 3D probe		**FAM-TCC TTT GCA CGC CGT GGG AC-QSY**	6820-6840		
BVDV1_2 F	BVDV 1, BVDV2**	CCA TGC CCT TAG GAC TAG C	76-97	92	NADL genome Willoughby et al., 2006
BVDV1_2R		TGA CGA CTA CCC TGT ACT CAG G	176-198		
BVDV1MGB probe	BVDV 1	**DFO-AAC AGT GGT GAG TTC GT-MGB Eclipse**	145-161		
BVDV2MGB probe	BVDV 2	**Cy5-ACT AGC GGT AGC AGT GAG-MGB Eclipse**	109-126	122	
Qß FJ483843 - 1,574 F1	Qbeta phage***	CAA ACG GTT CTT GTG ACC CA	1574-1594	93	GenBank FJ483843, this study
Qß FJ483843 - 1,666 R2		AAG CTC GTT CCT CAT CGG TA	1666-1646		
Qß FJ483843 - 1,602 Pro1		**Texas red -TCG CCA GGC ATA TGC TGA CGT -BHQ-2**	1602-1623		

* 3D gene DNA polymerase **5'UTR region ***major protein coat gene

Optimised multiplex RT-qPCR parameters and conditions on the T-COR™ 8 PCR machine (Tetracore).

Reaction mix

component	concentration	volume, µL
RNase free water	-	4.2
4x Taqman Fast Virus* mix	1 ×	5.0
50 nM $MgSO_4$	2.5 nM	1.0
10 µM FMDV 3D forward	900 nM	1.8
10 µM FMDV 3D reverse	400 nM	0.8
10 µM FMDV 3D probe	150 nM	0.3
10 µM BVDV-1_2 forward	250 nM	0.5
10 µM BVDV-1_2 reverse	250 nM	0.5
10 µM BVDV-1 probe	50 nM	0.1
10 µM BVDV-2 probe	50 nM	0.1
10 µM QB forward	150 nM	0.3
10 µM QB reverse	150 nM	0.3
10 µM QB probe	50 nM	0.1
PCR template	-	5.0
Volume per reaction		20

* Life Technologies

Cycling conditions

step	T, °C **	Time	cycle no.
RT***	50	5 min	1
Hold	95	20 s	1
Denaturation	95	10 s	45
Annealing / Extension	60	30 s	

** T: temperature

*** RT: reverse transcription

Appendix 5. Comparative test results of two PCR tests, mRT-qPCR (FMDV target) and TPI_FMD (Pirbright Institute, UK) (RT-qPCR) on suspected FMDV-positive pharyngeal swabs from cattle and buffaloes at slaughter in Lao PDR and Myanmar. Animal identification (ID) with the prefix Lao originated in Lao PDR and Mya in Myanmar, respectively. Cq = quantification cycle.

	Animal ID	Species	t1FMD, Cq		r2FMD, Cq	
1	Lao1	cattle	no Cq	-	no Cq	-
2	Lao2	cattle	no Cq	-	no Cq	-
3	Lao3	cattle	no Cq	-	no Cq	-
4	Lao4	cattle	no Cq	-	no Cq	-
5	Lao5	cattle	no Cq	-	no Cq	-
6	Lao6	cattle	no Cq	-	no Cq	-
7	Lao7	cattle	no Cq	-	no Cq	-
8	Lao8	cattle	no Cq	-	no Cq	-
9	Lao9	cattle	no Cq	-	no Cq	-
10	Lao42	buffalo	33.8	+	34.18	+
11	Lao47	buffalo	33.7	+	36.41	+
12	Lao123	cattle	36.0	+	31.61	+
13	Lao124	cattle	no Cq	-	32.83	+
14	Lao114	buffalo	no Cq	-	35.55	+
15	Lao81	cattle	no Cq	-	no Cq	-
16	Lao119	cattle	no Cq	-	no Cq	-
17	Mya1	buffalo	no Cq	-	no Cq	-
18	Mya6	cattle	no Cq	-	no Cq	-
19	Mya8	cattle	no Cq	-	31.48	+
20	Mya10	cattle	no Cq	-	no Cq	-
21	Mya11	cattle	no Cq	-	no Cq	-
22	Mya14	cattle	no Cq	-	no Cq	-
23	Mya21	cattle	no Cq	-	no Cq	-
24	Mya23	cattle	no Cq	-	no Cq	-
25	Mya25	cattle	no Cq	-	no Cq	-
26	Mya30	cattle	no Cq	-	no Cq	-
27	Mya31	cattle	no Cq	-	no Cq	-
28	Mya33	cattle	33.0	+	31.59	+
29	Mya39	cattle	no Cq	-	no Cq	-
30	Mya45	cattle	no Cq	-	no Cq	-
31	Mya49	cattle	no Cq	-	no Cq	-
32	Mya50	cattle	no Cq	-	no Cq	-
33	Mya55	cattle	no Cq	-	no Cq	-
34	Mya58	cattle	no Cq	-	no Cq	-
35	Mya61	cattle	no Cq	-	no Cq	-
36	Mya66	cattle	no Cq	-	30.40	+
37	Mya72	cattle	no Cq	-	29.23	+
38	Mya83	cattle	no Cq	-	no Cq	-
39	Mya84	cattle	no Cq	-	no Cq	-
40	Mya117	cattle	no Cq	-	no Cq	-
41	Mya118	cattle	31.9	+	31.88	+

Appendix 6. Cross tabulation of test results used for Bayesian latent class modelling (refer to Table 6-5 for test abbreviations).

Model 1-FMDV

Lao PDR

	LFD (+)		LFD (-)	
	r1FMD(+)	r1FMD(-)	r1FMD(+)	r1FMD(-)
t1FMD(+)	3	0	2	0
t1FMD(-)	0	0	0	0
	3	0	2	0

Myanmar

	LFD (+)		LFD (-)	
	r1FMD(+)	r1FMD(-)	r1FMD(+)	r1FMD(-)
t1FMD(+)	5	0	1	0
t1FMD(-)	0	0	0	0
	5	0	1	0

Model 2 - FMDV

Lao PDR

	LFD (+)		LFD (-)	
	r1FMD(+)	r1FMD(-)	r1FMD(+)	r1FMD(-)
t2FMD(+)	3	0	1	0
t2FMD(-)	0	0	0	0
	3	0	1	0

Myanmar

	LFD (+)		LFD (-)	
	r1FMD(+)	r1FMD(-)	r1FMD(+)	r1FMD(-)
t2FMD(+)	5	0	0	0
t2FMD(-)	0	0	1	0
	5	0	1	0

Model 3 a and b -FMDV

Lao PDR + Myanmar

	r1FMD	
	(+)	(-)
t1FMD(+)	11	0
t1FMD(-)	0	0
	11	0

New Zealand

	r1FMD	
	(+)	(-)
t1FMD(+)	0	0
t1FMD(-)	0	506
	0	0

Model 1 and 2 - BVDV-1

NI - North Island

	rBVDV-1		
	(+)	(-)	
tBVDV-1a(+)	1	0	1
tBVDV-1a(-)	1	174	175
	2	174	176

SI- South Island

	rBVDV-1		
	(+)	(-)	
tBVDV-1a(+)	0	0	0
tBVDV-1a(-)	1	129	130
	1	129	130

Model 1 - FMD slaughterhouse surveillance

Lao PDR

	r2FMD+	r2FMD-	
t1FMD+	1	0	1
t1FMD-	1	10	11
	2	10	12

Myanmar

	r2FMD+	r2FMD-	
t1FMD+	2	0	2
t1FMD-	3	19	22
	5	19	24

Appendix 7.

FMD Data: t1FMD field PCR, reference r1FMD PCR and t3FMD - 2 populations Lao PDR and Myanmar

```
model{
#Multinomial Model for the Data
x1[1:2,1:2,1:2] ~ dmulti(p1[1:2,1:2,1:2], n1)
x2[1:2,1:2,1:2] ~ dmulti(p2[1:2,1:2,1:2], n2)

#Observed prevalence
p1[1,1,1] <- pi1*Se1*cSe2p*Se3+(1-pi1)*(1-Sp1)*(1-cSp2p)*(1-Sp3) #t3FMD conditionally independent of t1a and
r1FMD
p1[1,2,1] <- pi1*Se1*(1-cSe2p)*Se3+(1-pi1)*(1-Sp1)*cSp2p*(1-Sp3)
p1[2,1,1] <- pi1*(1-Se1)*cSe2n*Se3+(1-pi1)*Sp1*(1-cSp2n)*(1-Sp3)
p1[2,2,1] <- pi1*(1-Se1)*(1-cSe2n)*Se3+(1-pi1)*Sp1*cSp2n*(1-Sp3)
p1[1,1,2] <- pi1*Se1*cSe2p*(1-Se3)+(1-pi1)*(1-Sp1)*(1-cSp2p)*Sp3
p1[1,2,2] <- pi1*Se1*(1-cSe2p)*(1-Se3)+(1-pi1)*(1-Sp1)*cSp2p*Sp3
p1[2,1,2] <- pi1*(1-Se1)*cSe2n*(1-Se3)+(1-pi1)*Sp1*(1-cSp2n)*Sp3
p1[2,2,2] <- pi1*(1-Se1)*(1-cSe2n)*(1-Se3)+(1-pi1)*Sp1*cSp2n*Sp3

p2[1,1,1] <- pi2*Se1*cSe2p*Se3+(1-pi2)*(1-Sp1)*(1-cSp2p)*(1-Sp3)
p2[1,2,1] <- pi2*Se1*(1-cSe2p)*Se3+(1-pi2)*(1-Sp1)*cSp2p*(1-Sp3)
p2[2,1,1] <- pi2*(1-Se1)*cSe2n*Se3+(1-pi2)*Sp1*(1-cSp2n)*(1-Sp3)
p2[2,2,1] <- pi2*(1-Se1)*(1-cSe2n)*Se3+(1-pi2)*Sp1*cSp2n*(1-Sp3)
p2[1,1,2] <- pi2*Se1*cSe2p*(1-Se3)+(1-pi2)*(1-Sp1)*(1-cSp2p)*Sp3
p2[1,2,2] <- pi2*Se1*(1-cSe2p)*(1-Se3)+(1-pi2)*(1-Sp1)*cSp2p*Sp3
p2[2,1,2] <- pi2*(1-Se1)*cSe2n*(1-Se3)+(1-pi2)*Sp1*(1-cSp2n)*Sp3
p2[2,2,2] <- pi2*(1-Se1)*(1-cSe2n)*(1-Se3)+(1-pi2)*Sp1*cSp2n*Sp3

#Derived parameters
Se2 <- Se1*cSe2p + (1-Se1)*cSe2n
Sp2 <- (1-Sp1)*cSp2p + Sp1*cSp2n
C12p <- Se1*(cSe2p-Se2)/sqrt(Se1*(1-Se1)*Se2*(1-Se2))
C12n <- (1-Sp1)*(Sp2-cSp2p)/sqrt(Sp1*(1-Sp1)*Sp2*(1-Sp2))

# Priors
pi1 ~ dbeta(3.26, 3.26)                  #mode=0.5, 95% sure >0.2
pi2 ~ dbeta(3.26, 3.26)                 #mode=0.5, 95% sure >0.2
Se1 ~ dbeta(19.07, 1.75)                 #mode=0.96, 95% sure >0.80
Sp1 ~ dbeta(34.17, 1.34)                 #mode=0.99, 95% sure > 0.90
cSe2p ~ dbeta(151.77, 4.07)              #mode=0.99, 95% sure >0.95
cSp2p ~ dbeta(212.12, 3.13)              #mode=0.99, 95% sure > 0.97
cSe2n ~ dbeta(151.77, 4.07)              #mode=0.98, 95% sure >0.95
cSp2n ~ dbeta(212.12, 3.13)              #mode=0.99, 95% sure > 0.97
Se3 ~ dbeta(13.08, 2.8)                 #mode=0.87, 95% sure > 0.65
Sp3 ~ dbeta(34.17, 1.34)                #mode=0.99, 95% sure > 0.90
}

#Data
list(n1=5,n2=6)

x1[,1,1] x1[,2,1] x1[,1,2] x1[,2,2] x2[,1,1] x2[,2,1] x2[,1,2] x2[,2,2]
 3        0        2        0        5        0        1        0

 0        0        0        0        0        0        0        0
END
#Inits
list(Se1=0.96, Sp1=0.99, cSe2p=0.98, cSe2n=0.98, cSp2p=0.99, cSp2n=0.99, Se3=0.87, Sp3=0.99, pi1=0.5, pi2=0.5)

#use "gen inits" in specification tool for the next two chain initial values
```

Appendix 8

FMD Data: t1FMD (mRT-qPCR) and reference (r1FMD) PCR assuming NO Independence - 2 populations (Laos PDR+Myanmar and New Zealand)

```
model{
#Multinomial Model for the Data
x1[1:2,1:2] ~ dmulti(p1[1:2,1:2], n1)
x2[1:2,1:2] ~ dmulti(p2[1:2,1:2], n2)

#Observed prevalence
p1[1,1] <- pi1*Se1*cSe2p+(1-pi1)*(1-Sp1)*(1-cSp2p)
p1[1,2] <- pi1*Se1*(1-cSe2p)+(1-pi1)*(1-Sp1)*cSp2p
p1[2,1] <- pi1*(1-Se1)*cSe2n+(1-pi1)*Sp1*(1-cSp2n)
p1[2,2] <- pi1*(1-Se1)*(1-cSe2n)+(1-pi1)*Sp1*cSp2n

p2[1,1] <- pi2*Se1*cSe2p+(1-pi2)*(1-Sp1)*(1-cSp2p)
p2[1,2] <- pi2*Se1*(1-cSe2p)+(1-pi2)*(1-Sp1)*cSp2p
p2[2,1] <- pi2*(1-Se1)*cSe2n+(1-pi2)*Sp1*(1-cSp2n)
p2[2,2] <- pi2*(1-Se1)*(1-cSe2n)+(1-pi2)*Sp1*cSp2n

#Derived parameters
Se2 <- Se1*cSe2p + (1-Se1)*cSe2n
Sp2 <- (1-Sp1)*cSp2p + Sp1*cSp2n
C12p <- Se1*(cSe2p-Se2)/sqrt(Se1*(1-Se1)*Se2*(1-Se2))
C12n <- (1-Sp1)*(Sp2-cSp2p)/sqrt(Sp1*(1-Sp1)*Sp2*(1-Sp2))

# Priors
pi1 ~ dbeta(3.26, 3.26)              #mode=0.5, 95% sure >0.2
pi2 ~ dbeta(1.88, 88.32)             #mode=0.01, 95% sure <0.05
Se1 ~ dbeta(19.07, 1.75)             #mode=0.96, 95% sure >0.80
Sp1 ~ dbeta(34.17,1.34)              #mode=0.99, 95% sure > 0.90
cSe2p ~ dbeta(151.77, 4.08)          #mode=0.98, 95% sure >0.95
cSp2p ~ dbeta(212.12, 3.13)          #mode=0.99, 95% sure > 0.97
cSe2n ~ dbeta(151.77, 4.08)
cSp2n ~ dbeta(212.12, 3.13)

PPV1 <-pi1*Se1/(pi1*Se1+(1-pi1)*(1-Sp1))
NPV1<-(1-pi1)*Sp1/(pi1*(1-Se1)+(1-pi1)*Sp1)
PPV2 <-pi2*Se2/(pi2*Se2+(1-pi2)*(1-Sp2))
NPV2<-(1-pi2)*Sp2/(pi2*(1-Se2)+(1-pi2)*Sp2)

Diff<-Se2-Se1
Pval<-step(Diff)
}

#Data
list(n1=11,n2=506)

x1[,1] x1[,2] x2[,1] x2[,2]
11          0          0          0
0           0          0          506
END

#Inits
list(Se1=0.85, Sp1=0.99, cSe2p=0.97, cSe2n=0.97, cSp2p=0.99, cSp2n=0.99, pi1=0.1,pi2=0.08)
# and 2 from "gen inits"
```

Appendix 9

BVDV-1 Data: tBVDV-1a (mRT-qPCR) and reference (rBVDV-1) PCR assuming NO Independence - 2 populations (North Island and South Island) - serum (not heat treated)

```
model{
#Multinomial Model for the Data
x1[1:2,1:2] ~ dmulti(p1[1:2,1:2], n1)
x2[1:2,1:2] ~ dmulti(p2[1:2,1:2], n2)

#Observed prevalence
p1[1,1] <- pi1*Se1*cSe2p+(1-pi1)*(1-Sp1)*(1-cSp2p)
p1[1,2] <- pi1*Se1*(1-cSe2p)+(1-pi1)*(1-Sp1)*cSp2p
p1[2,1] <- pi1*(1-Se1)*cSe2n+(1-pi1)*Sp1*(1-cSp2n)
p1[2,2] <- pi1*(1-Se1)*(1-cSe2n)+(1-pi1)*Sp1*cSp2n

p2[1,1] <- pi2*Se1*cSe2p+(1-pi2)*(1-Sp1)*(1-cSp2p)
p2[1,2] <- pi2*Se1*(1-cSe2p)+(1-pi2)*(1-Sp1)*cSp2p
p2[2,1] <- pi2*(1-Se1)*cSe2n+(1-pi2)*Sp1*(1-cSp2n)
p2[2,2] <- pi2*(1-Se1)*(1-cSe2n)+(1-pi2)*Sp1*cSp2n

#Derived parameters
Se2 <- Se1*cSe2p + (1-Se1)*cSe2n
Sp2 <- (1-Sp1)*cSp2p + Sp1*cSp2n
C12p <- Se1*(cSe2p-Se2)/sqrt(Se1*(1-Se1)*Se2*(1-Se2))
C12n <- (1-Sp1)*(Sp2-cSp2p)/sqrt(Sp1*(1-Sp1)*Sp2*(1-Sp2))

# Priors
pi1 ~ dbeta(10.63, 151.79)          #mode=0.06, 95% sure <0.10
pi2 ~ dbeta(20.71, 262.96)          #mode=0.07, 95% sure <0.10
Se1 ~ dbeta(4.6, 1.9)               #mode=0.8, 95% sure >0.40
Sp1 ~ dbeta(34.17,1.34)             #mode=0.99, 95% sure > 0.90
cSe2p ~ dbeta(42.11, 1.84)          #mode=0.98, 95% sure >0.90
cSp2p ~ dbeta(34.17, 1.34)           #mode=0.99, 95% sure >0.90
cSe2n ~ dbeta(42.11, 1.84)
cSp2n ~ dbeta(34.17, 1.34)

PPV1 <-pi1*Se1/(pi1*Se1+(1-pi1)*(1-Sp1))
NPV1<-(1-pi1)*Sp1/(pi1*(1-Se1)+(1-pi1)*Sp1)
PPV2 <-pi2*Se2/(pi2*Se2+(1-pi2)*(1-Sp2))
NPV2<-(1-pi2)*Sp2/(pi2*(1-Se2)+(1-pi2)*Sp2)

Diff<-Se2-Se1
Pval<-step(Diff)
}

#Data
list(n1=176,n2=130)

x1[,1] x1[,2] x2[,1] x2[,2]
1          0          0          0
1          174       1          129
END

#Inits
list(Se1=0.85, Sp1=0.99, cSe2p=0.97, cSe2n=0.97, cSp2p=0.99, cSp2n=0.99, pi1=0.1,pi 2=0.08)
# and 2 from "gen inits"
```

Appendix 10. Performance of the mRT-qPCR as indicated by the positive R3+ synthetic control (10,000 copies/reaction) during actual field testing in three countries with three prevailing temperatures (Lao PDR= 29°C, Myanmar=35°C and New Zealand=3°C). CI = confidence interval

	mRT-qPCR			
TCOR-8 PCR run no.	FMD	BVDV-1	QBIC	BVDV-2
New Zealand				
165	30.20	31.90	30.50	32.20
166	30.50	31.60	30.10	31.70
167	30.50	32.50	30.80	32.80
168	30.20	31.30	30.20	31.30
169	30.50	32.20	30.60	32.60
170	28.30	30.20	28.40	30.50
171	28.20	30.30	28.20	30.60
172	28.20	29.80	28.10	30.10
173	28.20	29.80	26.50	30.10
175	28.40	30.30	26.90	30.60
176	28.50	30.40	26.90	30.80
177	28.30	30.10	26.70	30.60
n=12				
mean	29.17	30.87	28.66	31.16
SD	1.079	0.973	1.691	0.949
CV, %	3.70	3.15	5.90	3.05
95% CI	28.48-29.86	30.25-31.49	27.59-29.73	30.56-31.76
Laos PDR				
-	27.8	28.6	28	29
-	28.7	29.3	28.4	29.8
-	29.1	29.7	28.5	30.1
-	29.5	30	29	30.4
-	28.3	29.9	28.4	30.5
219	31.4	31.9	30.7	32.2
223	30.5	30.7	no Cq	30.9
224	30.2	30.9	30	31.2
227	30.2	31.2	29.2	31.5
232	29.9	31.6	29.8	31.5
232	29.7	31.2	29.8	31.2
232	30	31.6	30.1	31.8
n=12				
mean	29.61	30.55	29.26	30.84
SD	1.001	1.037	0.873	0.916
CV, %	3.38	3.39	2.98	2.97
95% CI	28.97-30.25	29.89-31.21	28.71-29.81	30.26-31.42

Appendix 9 continuation

Myanmar	FMD	BVDV-1	QBIC	BVDV-2
333	29.90	31.30	29.90	31.50
335	30.80	32.90	30.60	33.10
336	28.50	30.00	28.20	30.10
338	28.60	30.70	28.50	31.10
339	28.50	30.10	28.30	30.40
341	28.90	30.90	28.90	31.20
342	28.90	30.80	28.90	31.10
344	29.00	31.10	28.80	31.60
347	28.7	30.6	28.9	30.9
348	28.6	30.4	28.4	30.4
349	28.7	30.6	28.5	31.1
n=11				
mean	29.01	30.85	28.90	31.14
SD	0.712	0.781	0.729	0.802
CV, %	2.45	2.53	2.52	2.58
95% CI	28.53-29.49	30.33-31.37	28.41-29.39	30-60-31.68

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