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Development of a reverse transcription loop-mediated isothermal amplification assay for rapid detection of foot-and-mouth disease virus

By

Duaa' Muhammad Ghaith

In Partial Fulfillment of the Requirements for the Degree

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Development of a reverse transcription loop-mediated isothermal amplification assay for rapid detection of foot and mouth disease virus

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ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease caused by foot-and-mouth disease virus (FMDV), which infects cloven-hoofed animals resulting in severe economic losses. Because very early detection of the virus is considered a cornerstone for minimization of the spread of the disease, the development of sensitive on-site testing techniques is urgently needed. Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification technique that can be performed using basic equipment and its results can be readily observed. Thus it can be used as a rapid and sensitive on-site testing technique. The aim of this study was to develop an RT-LAMP assay as a potential on-site testing technique for the rapid detection of FMDV. Evaluation of LAMP additives to enhance the specificity of the reaction to allow for reliable diagnosis was performed; the optimized RT-LAMP assay using 1.5M formamide/N,N-dimethylformamide additives and 6mM $MgSO_4$ was shown to detect FMDV RNA using the enhanced reverse transcriptase and strand displacement activities of the *Bst* 3.0 DNA polymerase in less than 1 hour. SYBR Green I dye was used for detection of amplification products and the results were further validated by agarose gel electrophoresis and turbidity observation. As compared to conventional PCR, the RT-LAMP assay was ten times more sensitive when using 10-fold serially diluted cDNA as a target, and while the intensity of bands in PCR became successively fainter as the target was diluted, the SYBR Green I detection of LAMP products was uniform and unambiguous over the complete range of detection making this assay a good candidate for point of care testing and screening. The LAMP assay showed a 90% detection rate, compared to 69% for PCR, when tested on cDNA prepared from 29 samples taken from animals with clinical signs of FMDV from 2014-2018, which is another indication of the greater sensitivity of the developed RT-LAMP assay. Furthermore, all positive samples detected by PCR were also detected by RT-LAMP. These results suggest a potential use of the visually inspected RT-LAMP as a more rapid and sensitive tool for simple diagnosis of FMDV.

ملخص

مرض الحمى القلاعية هو مرض فيروسي شديد العدوى يصيب المواشي مسببا خسائر اقتصادية فادحة. يعتبر الكشف المبكر عن المرض العامل الأهم في الحد من انتشار الفيروس، لذلك فإن تطوير تقنيات تشخيصية يمكن تطبيقها في الحقل يعد حاجة ملحة. تقنية (LAMP) هي من تقنيات مضاعفة الحموض النووية التي يتم إجراؤها باستخدام أدوات بسيطة و بذلك فهي تعد من التقنيات التشخيصية السريعة و الحساسة التي يمكن إجراؤها في الحقل. هدف هذه الدراسة هو تطوير هذا الفحص كتقنية يمكن استخدامها في الحقل للكشف السريع عن فيروس الحمى القلاعية و تقييم أثر بعض المواد المضافة على التفاعل. من خلال الفحص الذي تم تطويره، و باستخدام 1.5 مول/لتر من الفورماميد و ثنائي ميثل فورماميد و 6 مللي مول/لتر من كبريتات المغنيسيوم، تم الكشف عن المادة الوراثية (RNA) لفيروس الحمى القلاعية في وقت لا يتعدى الساعة باستخدام خاصيتي النسخ العكسي و بلمرة DNA اللتان يوفرهما انزيم *Bst* 3.0، و تم الكشف عن نتائج التفاعل باستخدام صبغة SYBR Green I. بالمقارنة مع ال PCR العادي و عند استخدام التخفيف المتسلسل لل cDNA، كان الفحص الذي تم تطويره أكثر حساسية بعشرة مرات في الكشف عن المادة الوراثية، و قد أعطت الصبغة نتائج واضحة فيما يخص ذلك مما يجعل هذه التقنية مرشحة جيدة للتشخيص الحقلي مستقبلا. لتقييم الفحص الذي تم تطويره، تم استخدام 29 عينة تم جمعها ما بين عامي 2014-2018 من حيوانات تظهر عليها أعراض مرض الحمى القلاعية و تمت مقارنة النتائج مع نتائج ال PCR. تمكن ال PCR من الكشف عن المادة الوراثية للفيروس في 69% من العينات بينما تمكن LAMP من الكشف عن 90% من العينات بما فيها تلك التي تم الكشف عنها بوساطة ال PCR و هذا يعد مؤشرا أخرا على حساسية الفحص. هذه النتائج تشير إلى أن الفحص الذي تم تطويره يمكن أن يكون بديلا واعدا للكشف السريع عن فيروس الحمى القلاعية.

DECLARATION

I declare that the master thesis entitled "Development of a reverse transcription loop-mediated isothermal amplification assay for rapid detection of foot-and-mouth disease virus" is my own original work, and thereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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DEDICATION

I would like to dedicate this work to my loving parents who have always supported me. I would also like to dedicate it to my brothers and sister who have encouraged and taught me to work hard for the things that I aspire to achieve.

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Abbreviations

B3	Backward outer primer
BIP	Backward inner primer
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
Bst	*Bacillus stearothermophilus*
°C	Celsius degree
cDNA	Complementary DNA
CPE	Cytopathic effect
DMF	N,N-dimethylformamide
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
DW	Distilled water
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
F3	Forward outer primer
FA	Formamide
FIP	Forward inner primer
FMD	Foot and mouth disease
FMDV	Foot and mouth disease virus
HNB	Hydroxynaphthol blue
IRES	Internal ribosome entry site
KCl	Potassium chloride
LB	Backward loop primer
LF	Forward loop primer
L	Liter
LAMP	Loop-mediated isothermal amplification
LFD	Lateral flow device

M	Molar
MAFFT	Multiple Alignment using Fast Fourier Transform
MG	Malachite green
$MgSO_4$	Magnesium sulfate
$MgCl_2$	Magnesium chloride
min	Minute
ml	Milliliter
mM	Millimolar
µl	Microliter
µM	Micromolar
MSA	Multiple sequence alignment
NAAT	Nucleic acid amplification technique
NCBI	National Center for Biotechnology Information
ND	Newcastle Disease
NDV	Newcastle disease virus
NEB	New England BioLabs
NTC	No template control
OIE	World Organization for Animal Health
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
POCT	Point-of-care testing
RT	Reverse transcriptase
RT-LAMP	Reverse transcription loop-mediated isothermal amplification
RT-PCR	Reverse transcription polymerase chain reaction
SAT	South African Territories
TBE	Tris/Borate/EDTA
TE	Tris/EDTA
UTR	Untranslated region
VNT	Virus Neutralization Test

VP	Viral protein
VPg	Viral protein genome linked
WRLFMD	World Reference Laboratory for FMD
w/v	Weight by volume

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Chapter one

1 Introduction

1.1 Foot-and-Mouth disease

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease of cloven-hoofed animals and is one of the listed diseases that should be reported to the World Organization for Animal Health (Office International des Épizooties, OIE) (OIE, 2008). It is caused by Foot-and-mouth disease virus (FMDV), which is a positive sense, single stranded RNA virus that belongs to the Aphthovirus genus in the Picornaviridae family of the order Picornavirales (Sanfaçon et al., 2012). The disease is characterized by low mortality rates (Domingo et al., 1990), yet it is considered the most economically serious disease of livestock (James and Rushton, 2002), because it causes severe losses that arise from reduced productivity, trade restrictions, and expensive control measures (Knight-Jones and Rushton, 2013).

The earliest description of the disease dates back to the 16th century in Italy, but its causative agent was not identified until the end of the 19th century, when it became the first animal virus to be discovered (Grubman and Baxt, 2004). The virus exists as 7 different serotypes that mainly affect cattle, pigs, sheep, goats, and buffalos. It could also infect more than 70 species of animals including deer, camels, giraffes and elephants (Alexandersen and Mowat, 2005). The disease is currently endemic in several parts of the world including Africa and parts of Asia and South America. Palestine, as an endemic region, suffers from annual episodes of FMD outbreaks with 216 outbreaks reported to the OIE between 2006 and 2017 (OIE, 2018).

1.2 FMDV structure and genome organization

FMDVs are non-enveloped viruses that have the icosahedral capsid structure typical of the picornaviruses (Sanfaçon et al., 2012). The icosahedral capsid is composed of 60 protomeric units (Figure1.1a) each consisting of 4 different proteins termed VP1, VP2, VP3,and VP4 (Knowles et al., 2012) . Loop structures extend from the surface structural proteins, which are the major determinants of antigenicity and are the sites that mediate interaction with cellular receptors (Knowles et al., 2012, Racaniello, 2007). The genomic RNA (Figure 1.1b) is protected

inside the capsid and possesses a single, long open reading frame (ORF) flanked by a 5'-UTR, covalently linked to the VPg protein, which primes RNA synthesis, and a polyadenylated 3'-UTR (Racaniello, 2007).

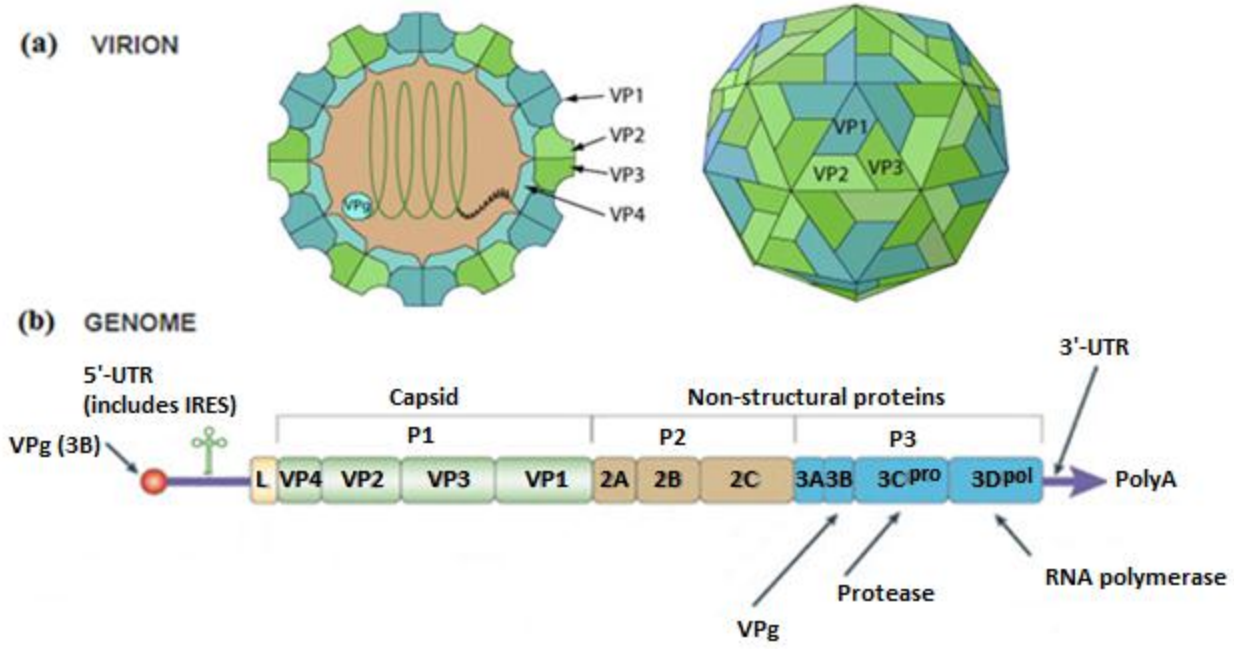

Figure 1.1: Schematic representation of FMDV and its genome. (a) FMDV capsid showing the iscosahedral structure (ViralZone, Swiss Institute of Bioinformatics, 2008). (b) FMDV genome organization showing the VPg protein at the 5' end, the 5'-UTR containing the IRES, the protein coding region, the 3'-UTR, and the poly(A) tail (Whitton et al., 2005).

The virus enters target cells in culture by endocytosis upon attachment to integrin receptors through the Arg-Gly-Asp (RGD) specific sequence in the VP1 protein (Baxt and Becker, 1990, Fox et al., 1989). Once in the acidic compartment of the endosome, the virus dissociates through a low-pH mediated loss of capsid integrity releasing genomic RNA, which is translated upon release into the cytoplasm by cytosolic ribosomes giving a single polyprotein, which is cleaved co-transnationally by the action of the encoded viral proteinases ($2A^{pro}$, $3C^{pro}$, $3CD^{pro}$) (Racaniello, 2007). The translation event is initiated by binding of the genomic RNA to ribosomes through a special sequence in the 5'-UTR known as the internal ribosome entry site (IRES). The resulting polyprotein consists of 3 regions (P1, P2, and P3) preceded by the L

protein which is involved in the inhibition of cellular protein synthesis (Devaney et al., 1988). The P1 region contains the structural proteins VP1 through VP4, while P2 and P3 regions contain non-structural proteins involved in protein processing and replication (Figure 1.1b).

1.3 Serotypes

Seven immunologically distinct serotypes that cause indistinguishable clinical signs have been identified for FMDV: A, O, C, SAT1, SAT2, SAT3, and Asia1. The major antigenic determinants of the different serotypes are found in the VP1 protein, namely in the G-H loop and the C terminus (Borrego et al., 1995). The most widely prevalent serotypes are O and A, which are spread throughout the endemic areas in Asia, Africa, and South America (Knowles and Samuel, 2003, Samuel and Knowles, 2001). The other serotypes, on the contrary, appear to be generally restricted to specific geographic regions. Serotype Asia 1, for example, is restricted to Southern Asia and the Middle East, and SAT serotypes are restricted to Africa (Knowles and Samuel, 2003)), although in recent years some have been detected in the Middle East (Valdazo-González et al., 2012). Serotype C appears to be disappearing from circulation with the last outbreaks being reported in 2004 (Rweyemamu et al., 2008). The unequal distribution of the serotypes has been manifested in their classification into 7 viral pools (Figure 1.2) (Paton et al., 2009) each containing viruses that evolve independently (Sumption et al., 2012).

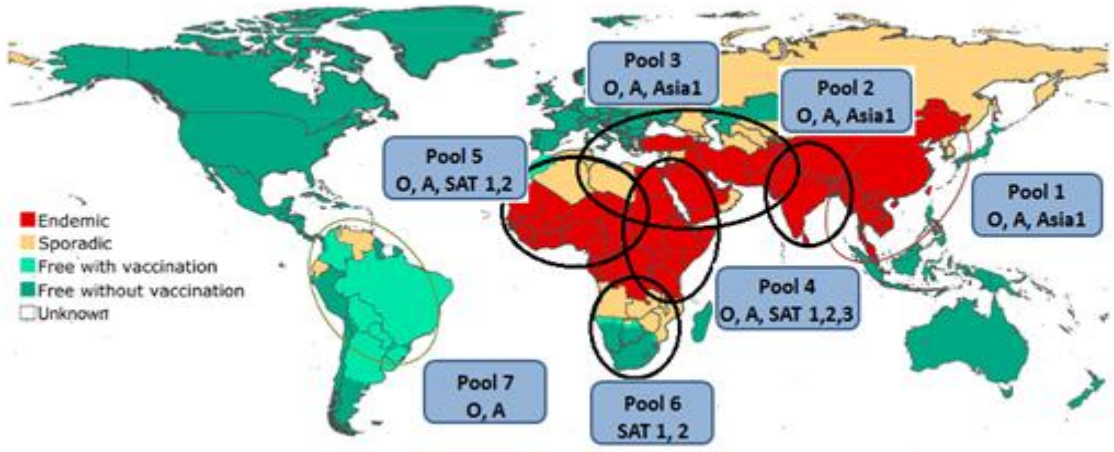

Figure 1.2: Distribution of the seven endemic pools of FMDV during 2016 (OIE/FAO Foot-and-Mouth Disease Reference Laboratory Network Annual Report 2016)

The high mutation rate of FMDVs leads to rapid evolution within the different geographic locations giving rise to antigenic variation even within the same serotype. Based on this fact, Samuel and Knowles (2001) classified serotype O viruses into 8 genotypes that reflect distinct geographic distribution and then used the term 'topotypes' to relate genotype with geographic location. These topotypes were identified by comparing the nucleotide sequence of the 3' end of the VP1 gene, which is highly variable between serotypes. There are currently 11 known topotypes in serotype O, while using a similar approach; serotype A was classified into 3 topotypes, C into 8 topotypes, Asia1 into 1 topotype, SAT1 into 8 topotypes, SAT2 into 14 topotypes (Vosloo et al., 2004), and SAT3 into 6 topotypes (Bastos et al., 2003, Vosloo et al., 2004).

1.4 Clinical signs

The clinical signs of FMD are similar to those of other vesicular diseases such as, swine vesicular disease, vesicular stomatitis and vesicular exanthema. The signs are usually obvious in cattle and pigs but are less apparent and may pass undiagnosed in sheep and goats (Barnett and Cox, 1999, Gibson and Donaldson, 1986). The typical clinical sign of FMD is the development of vesicles on the mouth, tongue, feet, nose, and teats (Figure 1.3) (Alexandersen et al., 2003). These vesicles result in lameness and reluctance to walk and eat, which causes weight loss, in addition to secondary infections due to their rupture. Other signs of the disease include fever, excessive salivation, loss of appetite, abortion, reduction in milk production, and sudden death of young animals because of myocarditis (Alexandersen et al., 2003).

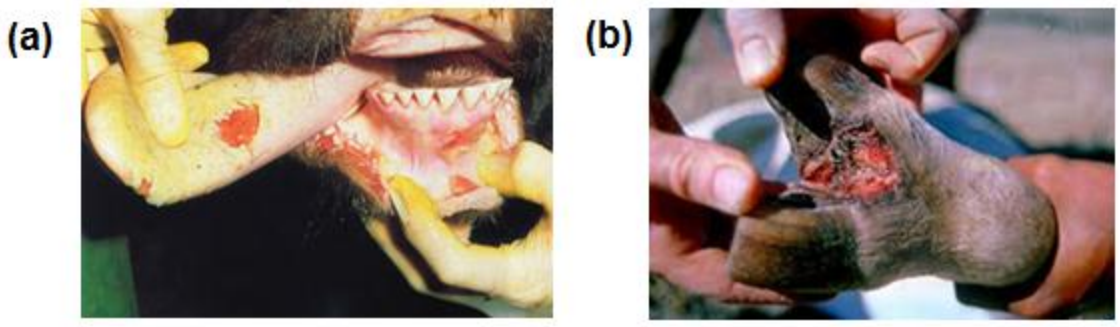

Figure 1.3: Typical vesicles of FMD. (a) Ruptured vesicles on the tongue, lip, and gum. (b) Ruptured vesicle in the inter-digital region.

1.5 Host response

The host responds to the virus by producing antibodies that can be detected 3-4 days after the onset of clinical signs, which coincides with a sharp decline in virus shedding (Alexandersen et al., 2002b). Usually, the antibodies can effectively remove the virus from circulation, but they are less efficient in removing it from the esophageal-pharyngeal region (Alexandersen et al., 2003). Therefore, the virus may be detected in this area weeks after of recovery, and a proportion of animals could become carries of the disease (Burrows, 1966).

1.6 Transmission and spread

FMDV is transmitted via respiratory and oral routes and through cuts and abrasions in the skin (Alexandersen et al., 2003). The virus can be found in the fluid and epithelia of vesicles, in exhaled air, and is normally found in all body secretions of animals in the acute phase of infection including blood, milk, saliva, urine and semen, and could be isolated from the heart and other organs of the body in fatal cases (Hyde et al., 1975, OIE, 2008).

The principal means of transmission of FMDV is via direct contact between infected and susceptible animals especially when the animals are kept in close proximity, which enhances the inhalation of aerosols carrying the virus; particularly in cattle and sheep (Grubman and Baxt, 2004). Pigs are less susceptible to infection via the respiratory route (Alexandersen et al., 2002a, Alexandersen and Donaldson, 2002) and the virus is usually transmitted by consumption of contaminated food and through damaged skin (Alexandersen et al., 2003). Transmission through wind, although less common, has contributed to the spread of the disease (Henderson, 1969) and usually entails the transmission from pigs to cattle because pigs secrete huge amounts of the virus in exhaled air (Donaldson et al., 2001). FMDV could be also transmitted indirectly through contact with contaminated surfaces of transport vehicles, milking machines, and the hands of farmers and veterinarians (Alexandersen and Mowat, 2005).

1.7 Disease control

In most FMD-free countries, outbreaks are controlled by restriction of animal movement and culling of infected and in-contact susceptible animals when outbreaks appear. In endemic countries and a minority of FMD-free countries (Figure 1.2), annual vaccination campaigns with inactivated virus particles are performed in addition to culling and trade restrictions (Grubman and Baxt, 2004). Since vaccines do not provide cross-protection between different serotypes, the

vaccine selected should match the serotype that circulates in the region of interest. Many FMD vaccines are multivalent and combine a number of different serotypes or strains that are most likely to be found in a specific geographic region in order to confer protection against as many as possible of them (OIE, 2008).

1.8 Laboratory diagnosis

Laboratory tests are required for definitive diagnosis and effective control of FMD because the clinical signs of FMD are sometimes difficult to distinguish from those of other vesicular disease, and virus serotype, which is essential for the selection of appropriate vaccines, cannot be identified by FMD signs. Laboratory diagnosis of FMD involves demonstration of the presence of the virus or virus specific antibodies, or detection of its genomic material by molecular based methods followed by serotype identification if required.

1.8.1 Detection of viable virus

Virus detection in clinical samples is commonly achieved by virus isolation in primary cultures of bovine thyroid cells or pig, calf or lamb kidney cells, or in cell lines like BHK-21 and IBRS-2 cells (OIE, 2008). Observation for FMDV is done by examining cultures for cytopathic effect (CPE) after 2 days of incubation.

1.8.2 Detection of viral proteins

The use of lateral flow devices (LFDs) is another way for virus detection. LFDs are designed to capture virus particles on strips, pre-adsorbed with antibody coated beads, and positive results are interpreted by a simple color change. LFDs are suitable for field diagnosis of FMD because of their low cost, simplicity, and the minimal requirement for expertise and special facilities. LFDs were reported to have a sensitivity comparable to that of ELISA with the advantage of being faster; requiring only 30 minutes for completion (Ferris et al., 2009).

1.8.3 Detection of virus specific antibodies

Antibodies produced against FMDV can be detected using virus neutralization test (VNT) or by enzyme-linked immunosorbent assay (ELISA). VNT is used for the detection of antibodies produced against FMDV structural proteins (Golding et al., 1976), so it is mainly used to confirm effective vaccination or infection in unvaccinated herds (OIE, 2008). ELISA, on the other hand, can be used for the detection of antibodies produced against structural or non-structural proteins.

ELISAs specific for non-structural proteins, which are conserved, have the advantage of distinguishing between vaccinated and infected animals (OIE, 2008).

These methods, succeeded in providing reliable diagnoses, but they do have some drawbacks. For example, VNT relies on growing virus stocks in cell cultures which requires well-equipped bio-containment facilities and experienced personnel, making it a tedious and time consuming test. ELISA and also LFDs, are hindered by the requirement of high virus or antibody titers which requires collecting samples in the acute clinical phase of infection or an additional step of virus isolation in cell culture which takes a minimum of 2-4 days if primary cell cultures are already prepared, in addition to requiring high quality samples and intact viruses. These drawbacks may cause a delay in diagnosis making these tests suitable for the confirmation of clinical diagnosis, but not for early and rapid detection of the disease which are cornerstones for rapid intervention and control of outbreaks (Anderson, 2002, Charleston et al., 2011, Nelson et al., 2017). Molecular based methods are, therefore, preferable.

1.8.4 Detection of the genomic material by molecular methods

Reverse transcription polymerase chain reaction (RT-PCR) assays have been designed for pan-serotype detection through targeting conserved sequences in the FMDV genome, namely the 3D gene (Laor et al., 1992, Meyer et al., 1991), the IRES (Reid et al., 2000), and the 2B gene (Vangrysperre and De Clercq, 1996). However, these methods are not sufficiently sensitive to replace ELISA and virus isolation, and were just used to support them. Pan-serotype-specific real-time RT-PCR assays were later developed, also targeting the conserved regions of the 3D gene (Callahan et al., 2002) and the IRES (Reid et al., 2002). These assays have been automated (Reid et al., 2003, Shaw et al., 2007) and extensively validated (King et al., 2006, Reid et al., 2009, Shaw et al., 2004) using a wide range of clinical samples and proved to offer a high degree of sensitivity, which is superior to that obtained by the traditionally used methods of ELISA and virus isolation. These automated assays are currently adopted by the World Reference Laboratory for FMD (WRLFMD) as principal means for diagnosis of FMD (OIE, 2008).

Serotype specific assays, on the other hand, target the 1D gene which encodes VP1 . This gene is a suitable serotyping target because it, as mentioned previously, shows sequence variability between the different serotypes and even between topotypes; especially in its 3’ end. Several serotype specific assays have been designed (Callens and De Clercq, 1997, Rodríguez et al.,

1992, Vangrysperre and De Clercq, 1996), but the evaluation of these assays on a wide range of field clinical samples showed that they do not offer a satisfactory degree of sensitivity and specificity compared to traditional methods (Reid et al., 1999, Reid et al., 2001).

Because of the high mutation rate of FMDVs, especially in the region targeted by serotype-specific assays, and owing to the fact that viruses belonging to the different virus pools tend to evolve independently, the specificity of primers designed to target an entire serotype remains an issue. Alternatively, designing primers based on the topotypes and lineages that circulate in specific geographic regions seems to be a better choice. This concept was successfully applied to the lineages of the serotypes that circulate in India (Giridharan et al., 2005), the Middle East (Reid et al., 2014), West Eurasia (Jamal and Belsham, 2015), and East Africa (Bachanek-Bankowska et al., 2016). It was also applied for the specific detection of lineage VII of SAT2 serotype viruses that have been circulating in Egypt (Ahmed et al., 2012).

In spite of the successful implementation of real-time RT-PCR assays for rapid and highly sensitive disease confirmation, in addition to their ability to be performed utilizing multiple sample types in the different phases of infection, these tests usually require sample transmission to distant reference laboratories where these tests are usually carried out, or to local laboratories that may lack the infrastructure. This major constraint causes a delay in diagnosis and eliminates the potential for early and preclinical diagnosis, which are the most important steps for the control and minimization of the spread of the disease (Nelson et al., 2017, Charleston et al., 2011). Portable real-time PCR platforms have been developed in an attempt to overcome this constraint and were evaluated as point-of-care testing (POCT) platforms and proved to be applicable under field conditions (Hearps et al., 2002, Howson et al., 2018, Madi et al., 2012). These platforms are, however, expensive and unaffordable for low-income countries, which makes them unsuitable for POCT (Niemz et al., 2011).

Isothermal nucleic acid amplification techniques which could be performed using basic equipment, have been suggested as a better choice for POCT (Paz et al., 2014).

1.9 Isothermal nucleic acid amplification techniques

There has been a growing interest over the last 30 years in developing nucleic acid amplification techniques that can be performed isothermally using basic equipment, thus paving the way for on-site-testing. Isothermal amplification techniques employ different methods for pursuing the

amplification reaction, without temperature shifting used in PCR, thus eliminating the need for thermal cyclers. However, most of these methods are either compromised in their specificity because of the low reaction temperature (around 40 $^{\circ}$C) and require probes to enhance their specificity, or they require labor-intensive detection methods. In addition, most of these techniques have complex experimental designs and require the use of either multiple enzymes or specially modified reagents (Guatelli et al., 1990, Compton, 1991, Walker et al., 1992a, Walker et al., 1992b, Vincent et al., 2004).

Loop-mediated isothermal amplification (LAMP) is an isothermal technique that overcomes the shortcomings of the other isothermal methods (Notomi et al., 2000). It uses only one enzyme, does not require specially modified reagents or probes to enhance specificity, and its products are easily detected. In addition, LAMP was shown to be less affected by contaminants and inhibitors than PCR (Kaneko et al., 2007). This property allowed the possibility of performing the reaction with less sample processing, which is ideal for point of care applications. There are reports of performing LAMP directly from samples without DNA or RNA extraction and with non-processed or minimally processed samples (Boehme et al., 2007, Poon et al., 2006, Yamada et al., 2006).

1.9.1 Loop-mediated isothermal amplification (LAMP)

LAMP relies on the use of a DNA polymerase with strand displacement activity such as *Bst* polymerase from *Bacillus steorothermophillus* and a set of 4 to 6 primers (Nagamine et al., 2002, Notomi et al., 2000). Besides having the DNA polymerization activity, *Bst* polymerase also has reverse transcription activity which makes it suitable for both DNA and RNA targets. LAMP primers include two loop-generating primers (forward inner primer (FIP) and backward inner primer (BIP)), two outer or displacement primers (F3 and B3), and two loop primers (LF, LB) (Figure 1.4). These primers correspond to 6 regions in the target gene amplifying sequences not longer than 300 bases, and preferably 130-200 bases including F2 and B2 (Notomi et al., 2000).

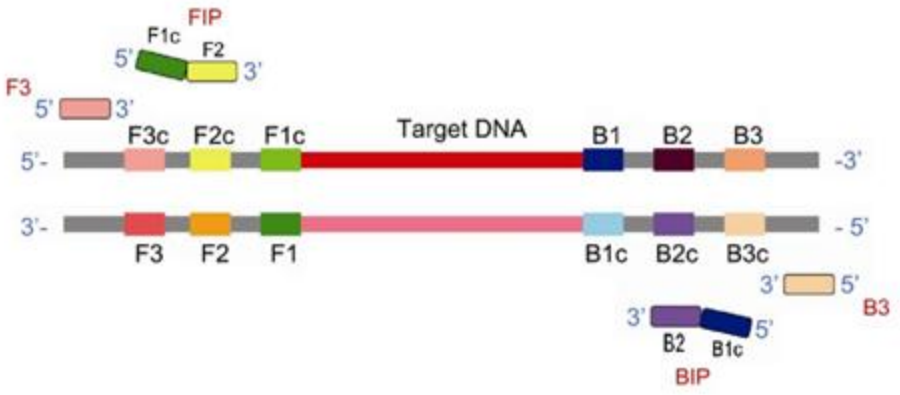

Figure 1.4: Schematic representation of LAMP primers. FIP and BIP are the forward and the backward inner primer. F1c and B1c are complementary sequences of F1 and B1 respectively. F3 and B3 are the outer or displacement primers (Eiken chemical Co. Ltd.).

The LAMP reaction starts by hybridization and amplification through the inner primers (FIP, BIP) and displacement of the generated complementary strands by the outer primers (F3, B3), which results in the release of a single stranded segment with a stem and two loops at the ends (dumbbell structure). This structure acts as the seed for exponential LAMP reaction which ends up with the production of segments with various stem lengths and multiple loops (Notomi et al., 2000) (Figure 1.5).

1.9.2 Detection of LAMP products

LAMP products, just as PCR products, can be detected by gel electrophoresis but instead of producing a distinct band with a specific size, LAMP produces a ladder-like pattern because of the production of the different sized fragments (Notomi et al., 2000). Besides gel electrophoresis, other visual detection methods that have been developed to simplify the process are commonly used. These include turbidity, which results from the precipitation of magnesium pyrophosphate (Mori et al., 2001), color change or fluorescence due to the addition of DNA intercalating dyes post amplification such as ethidium bromide, SYBR green, or propidium iodide (Hill et al., 2008, Iwamoto et al., 2003), and color change due to the addition of metal ion indicators to the reaction before amplification; such as calcein (Tomita et al., 2008), hydroxynaphthol blue (HNB) (Goto et al., 2009), and malachite green (MG) (Nzelu et al., 2014), and pH indicators like phenol red, cresol red, and neutral red (Tanner et al., 2015). Lateral flow devices in which LAMP products

are captured on nitrocellulose strips labeled with antibodies, or probes are also used for detection (Ge et al., 2013, Roskos et al., 2013, Waters et al., 2014).

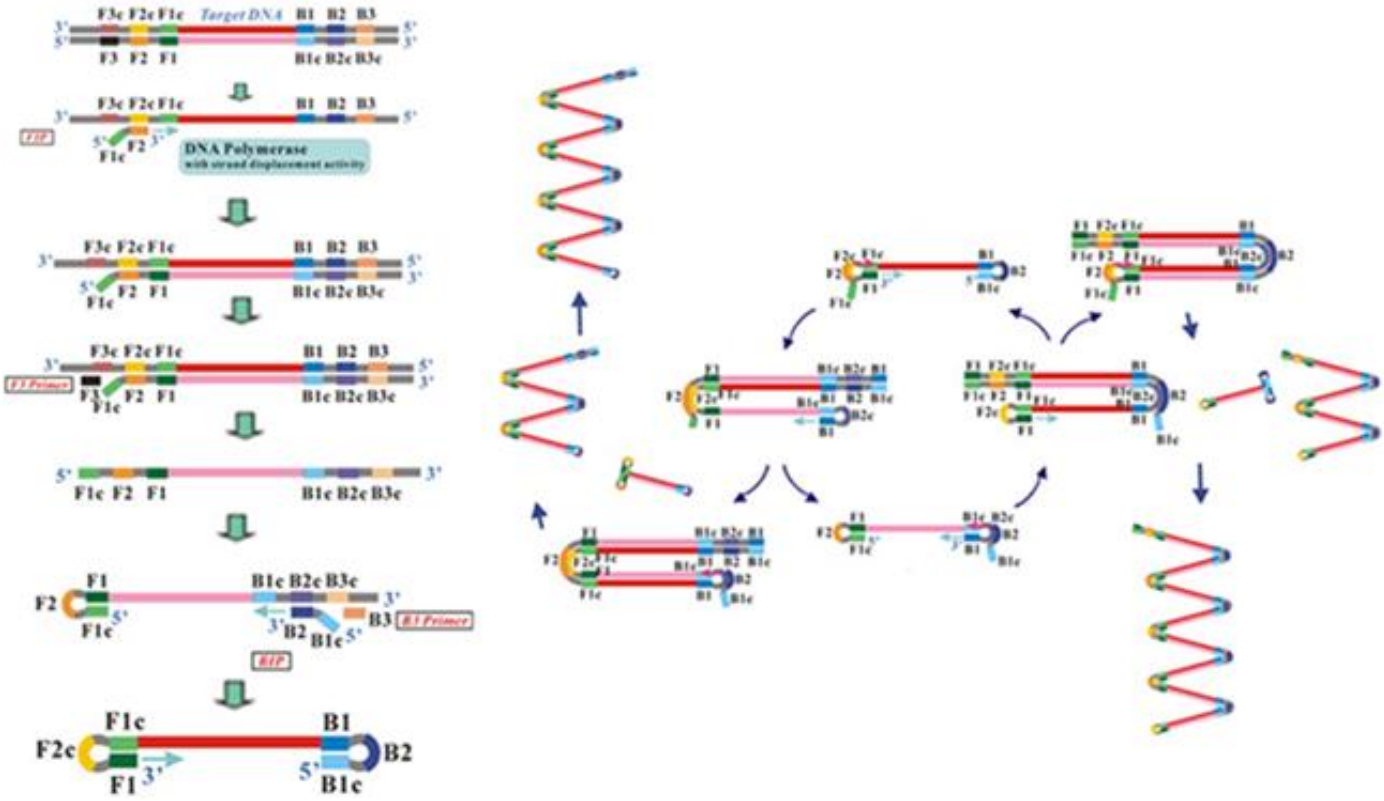

Figure 1.5: Mechanism of loop-mediated isothermal amplification. The amplification starts when F2 of FIP hybridizes to F2c region of the template and *Bst* polymerase initiates strand displacement amplification. F3 then hybridizes to F3c region and *Bst* generates a new strand while displacing the old one, which results in the release of a single stranded DNA that contains the FIP, thus it immediately forms a loop at its 5' end. The displaced single stranded DNA serves as a template for the BIP primer and the B3 displacement primer. At the end of this step, the original DNA structure is reproduced along with a single stranded segment with a stem and two loops at the ends (dumbbell structure), which enters a cyclic reaction where the inner primers anneal to the loop regions and start strand displacement amplification. While this segment is being amplified from the inner primers annealing sites it is also self-primed at the 3' end of F1 and B1 producing both the original dumbbell structure and other segments with various stem lengths and multiple loops. (Eiken chemical Co. Ltd.)

1.9.3 LAMP assays for detection of FMDV

Various LAMP assays have been developed for the diagnosis of FMDV. A pan-serotype specific RT-LAMP assay targeting 3D gene was developed (Dukes et al., 2006), but several isolates were found to escape detection by this assay. Another assay, also targeting the 3D gene, was developed with a special focus on serotypes O, A, Asia1, and C (Shao et al., 2010) and showed a higher sensitivity than that's of Dukes et al. (2006). A complex multiplex RT-LAMP with multiple primer sets was later developed to cover all 7 serotypes of the virus (Yamazaki et al., 2013).

Serotype specific LAMP assays were also developed. For example, serotype specific primers which targeted 1D gene were designed to specifically detect serotypes Asia1, C, O, and A (Chen et al., 2011b, Ding et al., 2014, Madhanmohan et al., 2013), in addition to another serotyping antigen-capture RT-LAMP with primers targeting 3D gene (Guan et al., 2013).

However, because of the high mutation rate of FMDVs (Elena and Sanjuán, 2005, Carrillo et al., 1990), modifications should be introduced regularly to accommodate for the high sequence variability. In addition, because of the use of multiple primers with high concentrations in LAMP, non-specific amplification is a frequently encountered problem especially when the assay requires targeting specific regions.

Chapter two

2 Problem statement and objectives

FMD is a devastating disease of livestock that causes serious problems in many parts of the world. The long-lasting limited productivity associated with FMD threatens food security particularly in low income endemic areas where people rely directly on livestock and livestock products. Minimization of the spread of FMDV is therefore of utmost importance.

A recent study evaluated the potential for diagnosis during the different phases of infection to reduce FMDV transmission and concluded that clinical inspection alone is not sufficient to limit FMD spread and only preclinical diagnosis has the potential to reduce between-farm disease transmission by allowing for implementing early control measures (Nelson et al., 2017). Preclinical diagnosis of FMD is possible since infected animals shed the virus in secretions and excretions like exhaled air, blood, nasal fluid, saliva, and esophageal-pharyngeal fluid during the incubation period (Charleston et al., 2011, Nelson et al., 2017, Pacheco et al., 2017). This is in agreement with the recommendations of previous studies which stressed the importance of investment in the development of preclinical diagnostic tools that could be performed on site for effective control of FMD (Anderson, 2002, Charleston et al., 2011).

There is a need therefore to have a diagnostic method that is sensitive enough to detect preclinical virus shedding and simple enough to be performed under field conditions to help in implementing control measures at early stages. Loop-mediated isothermal amplification, as a nucleic acid amplification technique that can be performed using basic equipment, offers such characteristics in terms of sensitivity and rapidity.

The aim of this study is therefore to contribute to the local diagnostic capacity for relevant FMDV serotypes and topotypes, which threaten West Bank livestock.

Objectives:

- To develop a visually inspected RT-LAMP assay for the detection of FMDV RNA.
- To use the developed RT-LAMP assay to test suspected FMDV clinical samples and to compare its performance with that of conventional PCR.

Chapter three

3 Materials and methods

3.1 Samples

A field isolate of FMDV from our laboratory was used as a positive control in PCR and as a template for LAMP assay development. The sample was obtained from a sheep farm in Bethlehem in September 2015 and was confirmed by PCR (section 3.5) and Sanger sequencing.

To validate the developed LAMP assay, 29 clinical samples suspected for FMDV infection were used. The clinical samples were obtained from 19 different sheep and cattle farms from the West Bank in the period from 2014 to 2018. The samples consisted of swabs, vesicular epithelia, and heart tissue and were initially tested by PCR (section 3.6).

3.2 Sampling approach

Sampling was performed as recommended by the OIE manual of diagnostic tests and vaccines for terrestrial animals (OIE, 2008). Tissue samples were collected from un-ruptured or recently ruptured vesicles or from the hearts of dead animals and were placed in 2 ml of transport medium composed of a 1:1 ratio of glycerol and phosphate buffered saline (PBS) with antibiotics (100 unit/ml Penicillin, 0.1 mg/ml Streptomycin). The pH of the transport medium was adjusted to 7.2-7.6 before use because FMDV is labile under acidic conditions. Swab samples were either left to dry or placed in 2 ml of PBS with antibiotics and the pH was adjusted to 7.2-7.6.

3.3 Sample preparation

- **Tissue samples:** a maximum of 20 mg tissue samples were homogenized in PBS to prepare 10% (w/v) suspensions. The suspensions were then centrifuged at 10000 g for 2 minutes, and the supernatants were used for RNA extraction.
- **Swabs:** the swabs were placed into 1.5 ml eppendorf tubes containing 2ml PBS, if not originally placed in PBS, and incubated for 15 minutes at room temperature. Afterward the swabs were shaken vigorously and squeezed against tube wall and the resulting solutions were used for RNA extraction.

3.4 RNA extraction

Total RNA was extracted from 150-200 µl swab specimens or epithelial suspension supernatants using innuPREP RNA Virus kit (AnalytikJena, Jena, Germany) according to the manufacturer's instructions. Briefly, 450 µl RL/carrier mix and 20 µl Proteinase K were added to 150-200 µl specimen and incubated for 10 minutes at room temperature. 600 µl of binding solution B were then added and the resulting mixture was filtered by centrifugation at 10000 g for 1 minute. RNA was then washed with HS and LS solutions and eluted in 60 µl of RNase free water and stored at -80°C until required.

3.5 cDNA synthesis

cDNA was synthesized in 20 µl reaction volumes using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. A 5 µl sample of eluted RNA was mixed with 5µM random hexamer primer, and nuclease free water was added up to 12 µl. The resulting mixture was incubated at 65°C for 5 minutes to eliminate secondary structures, then chilled on ice. 1 X reaction buffer (50 mM tris-HCl pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT), 1 U/µl RiboLock RNase inhibitor, 1 mM dNTPs mixture (DNTp 100, Sigma), and 10 U/µl RevertAid M-MuLV reverse transcriptase were then added to the mixture. The reverse transcription reaction was performed by incubating reaction tubes at 25°C for 5 minutes followed by 60 minutes at 42°C and terminated by heating at 70°C for 5 minutes. cDNA was stored at -20°C until required.

3.6 Laboratory testing by PCR

The primers used for the initial testing of clinical samples are listed in Table (3.1). The pan-serotype specific primer pair 1F/1R is described by Reid et al. (2000), and corresponds to a conserved region in the IRES in the 5'UTR. P1/P2 primer pair was designed based on serotype O as described by Amaral-Doel et al. (1993). This pair flanks the region between the 3' end of the 1D gene and the beginning of the conserved 2A gene. cDNA produced from each sample was tested with at least one primer pair individually.

Table 3.1: Primers used for the primary laboratory testing of FMDV clinical samples

Primer	Sequence (5'-3')	Region	Product length	Reference
1F	GCCTGGTCTTTCCAGGTCT	IRES	328 bp	(Reid et al., 2000)
1R	CCAGTCCCCTTCTCAGATC			
P1	CCTACCTCCTTCAACTACGG	1D/2A	216 bp	(Amaral-Doel et al., 1993)
P2	GAAGGGCCCAGGGTTGGACTC			

PCR for each primer pair was performed separately using the Taq DNA polymerase kit (Hy Labs cat # HTD0078) according to the manufacturer's instructions. All PCRs were performed in 25 µl reaction volumes containing 1X reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 0.1% Triton X-100 , 0.1 mg/ml BSA), 2 mM $MgSO_4$, 0.2 mM dNTPs mixture (DNTp 100, Sigma), 0.2 µM each primer, 0.6 unit Taq DNA polymerase, 1 µl cDNA and distilled water to bring the final reaction volume up to 25 µl. A no template control (NTC) was included in all reactions by mixing all reaction components and replacing template cDNA with distilled water.

The reactions were carried out using MJ mini Bio-Rad PTC 1148 thermocycler (BioRad, USA) with the following temperature profile:

Step	Temperature	Time	Number of cycles
Initial denaturation	94 °C	5 minutes	1
Denaturation	94 °C	30 seconds	35
Annealing	55 °C	30 seconds	
Extension	72 °C	45 seconds	
Final extension	72 °C	10 minutes	1
Hold	4 °C	∞	1

PCR products were detected by agarose gel electrophoresis stained with 0.2-0.5 µg/ml ethidium bromide. The products were electrophoresed on a 1.5% (w/v) agarose gel prepared using TBE buffer (5X stock TBE buffer was prepared by mixing 54 g Tris base (Amresco/ 0826), 27 g boric acid (Sigma/ 078K0037), 20 ml of 0.5 M EDTA (Alfa Aesar/ 10122546), and distilled water up to 1 L). 1X dilution of the buffer was used for agarose gel preparation and 0.5X for running buffer in gel electrophoresis. Amplified products were mixed with 3 µl of 6X loading dye (composed of 0.25% (w/v) bromophenol blue (Fluka/ 417639/1), 0.25% (w/v) xylene cyanol FF (Amresco/ 1897B006), and 30% glycerol (v/v) (Amresco/ 0176B017)) and loaded into the gel.

3.7 Reverse transcription Loop-mediated isothermal amplification (RT-LAMP)

3.7.1 Primers

The 3D gene was chosen as a target for LAMP primer design, as it provides a relatively conserved segment of about 200 bases. The selection was based on the results of multiple sequence alignment (MSA) of the full 3D gene consensus sequences of serotypes O, A, Asia1, and SAT2 (Figure 3.1). To create the consensus sequences, all the full length 3D gene sequences were retrieved from the National Center for Biotechnology Information (NCBI) database (as of February 2018), classified based on the serotype, and aligned using MAFFT under the default conditions. The consensus sequences were then created using BioEdit Sequence Alignment Editor v.7.2.6 (Hall, 1999).

A set of 6 primers (two outer primers F3/B3, two inner primers FIP/BIP, and two loop primers LF/LB) that was described previously (Shao et al., 2010), and found to target a relatively conserved region in the 3D gene based on MSA, was chosen and its specificity was evaluated *in silico* by using the Basic Local Alignment Search Tool (BLAST). The selected primers were modified based on the MSA (Figure 3.1) by introducing degenerate sites to certain sites to accommodate for sequence variability and by designing a new LB primer using PrimerExplorer version 5 program (Eiken Chemical Co., Tokyo, Japan). The sequences and lengths of primers are shown in Table 3.2.

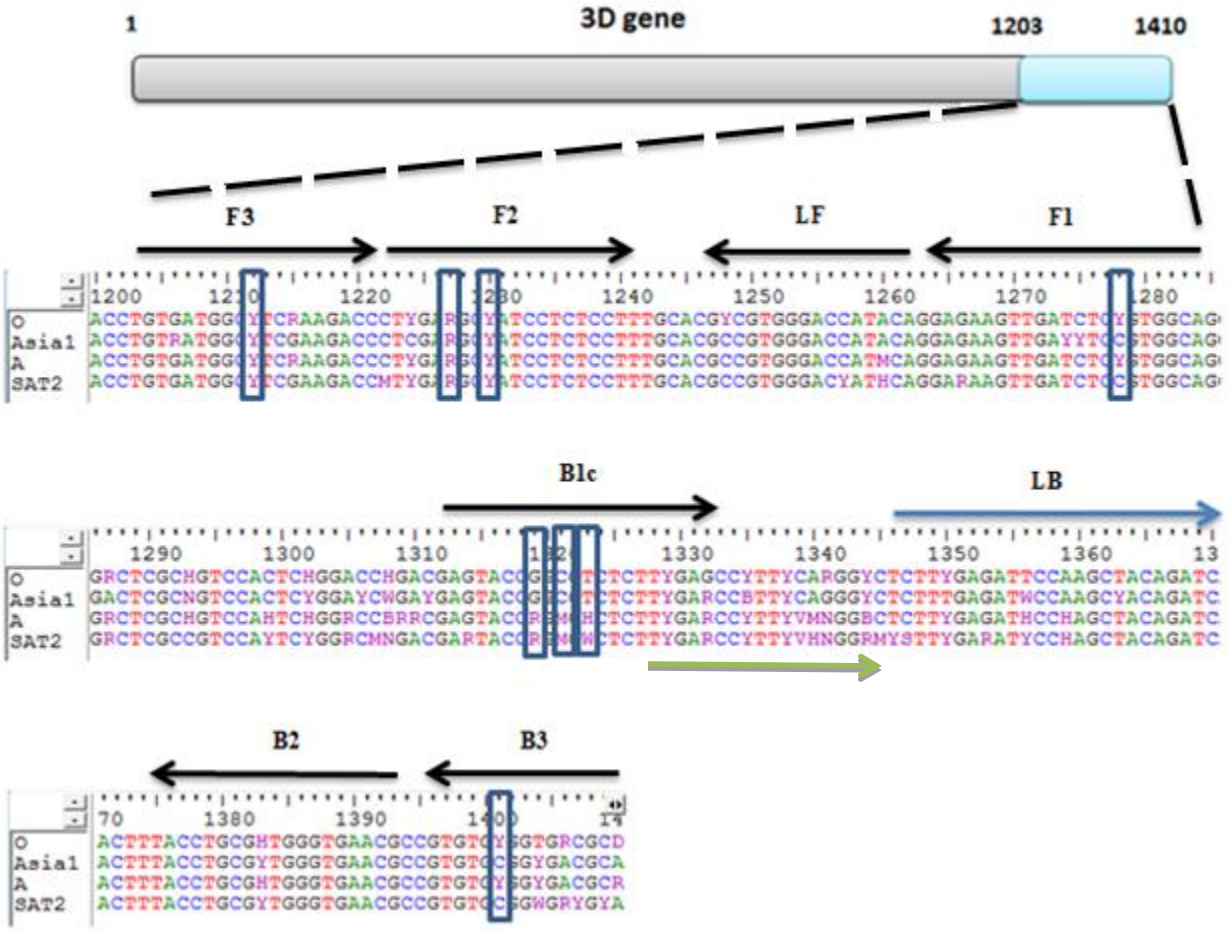

Figure 3.1: Locations of RT-LAMP primers targeting the 3D gene. Partial MSA of the 3D gene consensus sequences of serotypes O, A, Asia1, and SAT2 showing the locations of the 6 primers: F3, B3, FIP (F1c-TTTT-F2), BIP (B1c-TTTT-B2), LF, and LB as arrows that indicate the direction of extension. Rectangles mark the degenerate sites introduced. The blue arrow represents the newly designed LB primer and the green arrow represents the old LB primer.

Table 3.2: sequences, positions, and lengths of primers used for LAMP

Primer	Sequence (5'-3')	Position in 3D gene	Length
F3	TGTGATGGCYTCGAAGACC	1203-1221	19
B3	TGCGTCACCRCACACG	1410-1395	16
FIP	TGCCACRGAGATCAACTTCTCC**TTTT**C TCGARGCYATCCTCTCCTT	**(F2)** 1222-1241 **(F1c)** 1263-1284	42
BIP	GAGTACCRGMGWCTCTTTGAGC**TTTT** CGTTCACCCAACGCAGGTAA	**(B2)** 1393-1374 **(B1c)** 1312-1333	42
LF	TGTATGGTCCCACGGCG	1246-1262	17
LB	TCTTTGAGATTCCAAGCTACAGATC	1346-1370	25

3.7.2 Primary LAMP protocol

The basic conditions of the LAMP reaction were adopted from the New England BioLabs (NEB) protocol (M0374). The LAMP reaction was performed using the *Bst* 3.0 DNA polymerase kit (New England BioLabs, cat # M0374) in 25 µl reaction volume containing 1X isothermal amplification buffer II (20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 150 mM KCl, 2 mM $MgSO_4$, 0.1 % Tween 20), 8 mM $MgSO_4$, 1.4 mM dNTPs mixture (DNTp 100, Sigma), 1.6 µM each of the primers FIP and BIP, 0.2 µM each of the primers F3 and B3, and 0.4 µM each of the primers LF and LB, 8 units *Bst* 3.0 DNA polymerase, 1 µl cDNA, and distilled water to bring the final reaction volume up to 25 µl. A no template control (NTC) was included in all reactions by mixing all reaction components and replacing template cDNA with distilled water. The reaction was carried out using Applied Biosystems thermocycler (2720) by incubating reaction tubes at 65°C for 1 hour followed by polymerase inactivation at 80°C for 5 minutes and cooling at 5°C for 5 minutes.

3.7.3 Optimization of the LAMP reaction

To optimize and completely eliminate non-target amplification in the established RT-LAMP assay, reaction temperature, $MgSO_4$ concentration, reaction time, and LAMP additives were examined. All optimization reactions were carried out in duplicate in 10 µl reactions.

3.7.3.1 Effect of reaction temperature

To examine the effect of temperature on target amplification and background non-specific amplification, the basic RT-LAMP reaction was performed at temperatures ranging from 65-72°C.

3.7.3.2 Effect of $MgSO_4$ concentration

In order to determine the optimal concentration of $MgSO_4$ in the RT-LAMP reaction, four different concentrations in the final reaction mixture were used while keeping the concentrations of other components constant; 2 mM, 4 mM, 6 mM, and 8 mM.

3.7.3.1 Effect of betaine

Using the temperature and the concentration determined to be optimal for $MgSO_4$, betaine (Sigma, B0300) was added to the reaction to final concentrations ranging from (0.5-2 M).

3.7.3.2 Effect of reaction time

Using the concentrations determined to be optimal in the previous sections, the RT-LAMP reaction was conducted for 15, 30, 45, and 60 minutes.

3.7.3.3 Effect of carboxamide and N-alkylcarboxamide additives

In order to eliminate non-specific amplification in the NTC, a mixture of carboxamide/N-alkylcarboxamide was added to the RT-LAMP reaction as recommended by (Tanner and Evans, 2013). A range of 0.3-1.8 M total concentration of formamide (FA) (Fluka/ 47680) and N,N-dimethylformamide (DMF) (Sigma/ 15440); additives were added to the optimized LAMP reaction. The concentrations of additives were calculated using a (1: 0.3) molar ratio of FA:DMF as recommended.

3.7.4 Optimized RT-LAMP method for the detection of FMDV

The optimized RT-LAMP reaction was carried out using *Bst* 3.0 DNA polymerase kit (New England BioLabs, cat # M0374) in 25 µl reaction mixture containing 1X isothermal

amplification buffer II , 6 mM $MgSO_4$, 1.4 mM dNTPs mixture (DNTp 100, Sigma), 1.6 µM each of FIP and BIP, 0.2 µM each of F3 and B3, 0.4 µM each of LF and LB, 8 units *Bst* 3.0 DNA polymerase, 1.5M formamide/ N,N-dimethylformamide (1: 0.3), 1 µl cDNA, and distilled water to bring the final reaction volume up to 25 µl. The reaction was carried out by incubating reaction tubes at 65°C for 1 hour followed by polymerase inactivation at 80°C for 5 minutes and cooling at 4°C for 5 minutes.

3.7.5 Detection of LAMP products

LAMP products were detected by 3 methods: agarose gel electrophoresis, turbidity observation, and addition of either malachite green (MG) dye or SYBR green I for colorimetric detection.

3.7.5.1 Agarose gel electrophoresis:

LAMP products were run on a 2% (w/v) agarose gel prepared using 1X TBE buffer stained with 0.2-0.5 µg/ml ethidium bromide. The gel was imaged using BioRad molecular imager® gel doc XR+.

3.7.5.2 Turbidity observation:

LAMP reaction tubes were visually inspected against light for turbidity after reaction completion relative to no template control.

3.7.5.3 Malachite green (MG) dye:

Malachite green dye (Sigma, M9015) was added to the LAMP reaction mixture before amplification as indicated by (Nzelu et al., 2014). MG was prepared as a 0.1% stock solution in distilled water, and added to the optimized LAMP reaction to 0.004% final concentration. Reaction tubes were inspected for color change after reaction completion. Positive reactions remain blue, while negative reactions become colorless.

3.7.5.4 SYBR Green I :

A working solution of 1000 X of SYBR green I dye (Sigma, S9430) was prepared in TE buffer and 2 µl were added per 25 µl LAMP reaction after amplification. The tubes were visually inspected for color change immediately after dye addition. The color of SYBR green I becomes bright yellow-green when added to positive reactions and retains its orange color in negative reactions.

3.7.6 Comparison of the detection limit of RT-LAMP and conventional PCR

Following the establishment of the optimized RT-LAMP assay, its detection limit was compared to that of conventional PCR using a 10-fold serially diluted positive control cDNA. F3 and B3 LAMP primers were used for the conventional PCR assay. PCR was performed in 25 µl reaction volume containing 1X reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 0.1% Triton X-100 , 0.1 mg/ml BSA), 2 mM $MgSO_4$, 0.2 mM dNTPs mixture, 0.3 µM each of F3 and B3 primers, 0.6 units Taq DNA polymerase, 1 µl of 10-fold serially diluted cDNA, and distilled water to bring the final reaction volume up to 25 µl. The reactions were carried out using MJ mini Bio-Rad PTC 1148 thermocycler (BioRad, USA) with the following program:

Step	Temperature	Time	Number of cycles
Initial denaturation	94 °C	5 minutes	1
Denaturation	94 °C	30 seconds	35
Annealing	55 °C	30 seconds	
Extension	72 °C	45 seconds	
Final extension	72 °C	10 minutes	1
Hold	4 °C	∞	1

The 10-fold serially diluted cDNA was also used as a template for the RT-LAMP assay and its performance was compared with that of PCR. Detection of amplification products was performed by turbidity observation and by addition of 2 µl of SYBR Green I to the RT-LAMP reaction tubes, followed by agarose gel electrophoresis of both PCR and LAMP products (2% w/v).

3.7.7 Confirmation of RT-LAMP products

To confirm that the LAMP reaction with the selected primer set has amplified the targeted FMDV gene segment, the fastest migrating band obtained from positive RT-LAMP reaction was excised after agarose gel electrophoresis. The amplified product was purified from the gel slice using the Gel/PCR DNA Fragment Extraction Kit (Geneaid, cat # DF100) according to the manufacturer's instructions and the purified product was eluted in 35µl of elution buffer. The

purified LAMP product was sequenced in Augusta Victoria Hospital (Jerusalem) using B3 primer.

3.7.8 Application of RT-LAMP to RNA targets

The optimized RT-LAMP reaction was used to amplify FMDV RNA targets directly without cDNA synthesis using the *Bst* 3.0 polymerase which has an enhanced reverse transcriptase activity. The reactions were conducted by adding 2 µl of RNA to the RT-LAMP reaction mixture. An NTC was included in parallel.

3.7.9 Evaluation of the established RT-LAMP assay

To evaluate the established RT-LAMP, the assay was used to test 29 samples from 19 different farms collected previously between 2014 and 2018. The samples were collected from animals showing clinical signs of FMD and were initially tested by PCR (section 3.6).

3.7.10 Utility of the LAMP additives to other targets and primer sets

To investigate whether the additives used for elimination of non-specific amplification in the NTC could be used with the same efficiency when using other primer sets and targets, FA and DMF additives were used in another assay that targeted Newcastle Disease virus (NDV). A set of 6 primers was designed based on the nucleotide sequence of the F gene of the commercially available VH vaccine strain (Biovac) using PrimerExplorer version 5 program (Eiken Chemical Co., Tokyo, Japan). The sequences and lengths of primers are shown in Table (3.3).

Table 3.3: Primers used for NDV-LAMP

Primer	Sequence (5'-3')	Length
ND-F3	CAGCATTTTGTTTGGCTTG	19
ND-B3	CCCTTGGATGCATACAACA	19
ND-FIP	ATAGGCGCCATTATTGGCGG**TTTT**CGCTGTTATTTGTGCGG	41
ND-BIP	CCCTCCAGATGTAGTCACAGAC**TTTT**CATTGACCACTTTGCTCAC	45
ND-LF	TGTGGCTCTTGGGGTTG	17
ND-LB	GTATCCTACGGATAGAGTCACC	22

The RT-LAMP reaction was performed in 25 µl reaction volume containing 1X isothermal amplification buffer II (20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 150 mM KCl, 2 mM $MgSO_4$, 0.1 % Tween 20), 1.4 mM dNTPs mixture, 1.6 µM each FIP and BIP, 0.2 µM each F3 and B3, 0.4 µM each LF and LB, a range of 4 mM $MgSO_4$, 8 units *Bst* 3.0 DNA polymerase, 1.5M final concentration of FA and DMF (1: 0.3 molar ratio), 1 µl cDNA or 2 µl RNA, and up to 25 µl distilled water. A no template control (NTC) was included in parallel. The reaction was carried out using Applied Biosystems thermocycler (2720) by incubating reaction tubes at 65°C for 1 hour followed by polymerase inactivation at 80°C for 5 minutes and cooling at 4°C for 5 minutes.

RT-LAMP products were detected by turbidity observation, addition of 2 µl of 1000 X SYBR green I, and running on 2% (w/v) agarose gel.

Chapter four

4 Results

4.1 Optimization of the RT-LAMP reaction

The basic RT-LAMP assay conducted for the detection of FMDV 3D gene successfully produced the ladder-like pattern characteristic of LAMP in positive reactions, but there was a high level of non-specific amplification in the NTC (Figure 4.1 a). This was accompanied by the accumulation of magnesium pyrophosphate precipitate, which increased reaction turbidity, in both positive and no template control reaction tubes (Figure 4.1 b).

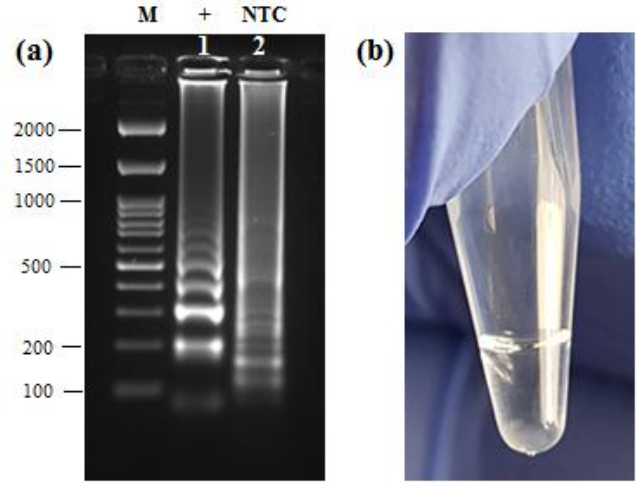

Figure 4.1: RT-LAMP products of the initial RT-LAMP assay. (a) Agarose gel electrophoresis of the initial RT-LAMP products. M= 100 bp DNA marker. Lane 1: Positive reaction (with target FMDV cDNA). Lane 2: NTC. (b) Turbidity observation of the initial RT-LAMP assay representing both positive and NTC reaction products, at the bottom magnesium pyrophosphate precipitate.

In order to completely eliminate non-specific amplification while maintaining the amplification of target nucleic acid, a number of reaction parameters were optimized including temperature, $MgSO_4$ concentration, betaine concentration, reaction time, and carboxamide/N-alkylcarboxamide additives.

4.1.1 Effect of reaction temperature

In order to examine the effect of reaction temperature on RT-LAMP, the reaction was performed at temperatures ranging from 65-72°C and the results were observed by reaction turbidity and by gel electrophoresis. As shown in Figure 4.2, FMDV cDNA was successfully amplified at temperatures up to 70°C, as apparent by reaction turbidity and on the gel. As the figure shows, increasing reaction temperature did not affect the non-specific amplification as its products were still evident even at these elevated temperatures. At 71°C and 72°C, inconsistent results started to appear, therefore temperatures above 70°C were excluded and 65°C temperature was set for all subsequent reactions.

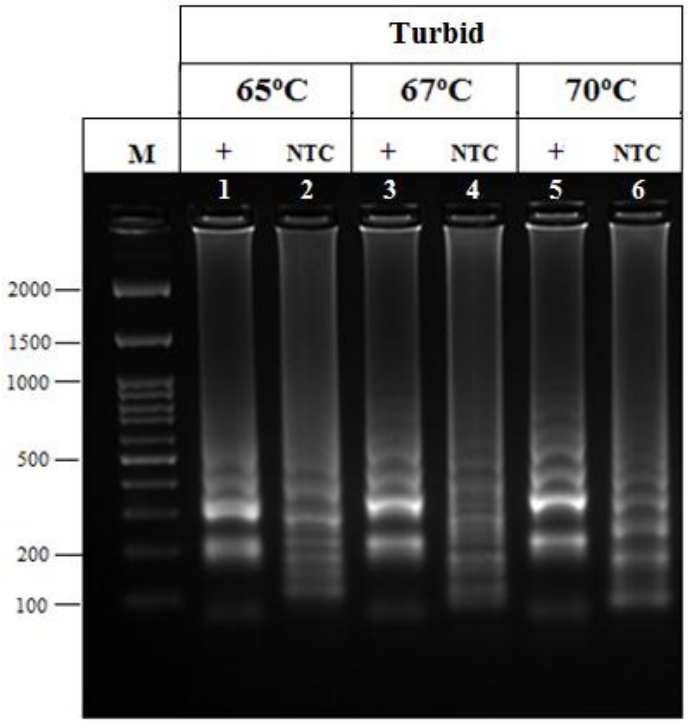

Figure 4.2: Effect of temperature on the RT-LAMP reaction as apparent by reaction turbidity and by agarose gel electrophoresis. M= 100 bp DNA marker. Lanes 1, 3, and 5 are the products of reactions on FMDV cDNA conducted at 65°C, 67°C and 70°C respectively. Lanes 2, 4, and 6, are the products of NTCs conducted at 65°C, 67°C and 70°C respectively.

4.1.2 Effect of $MgSO_4$ concentration

Figure 4.3 shows the effect of using 2, 4, 6, and 8 mM final concentration of $MgSO_4$. A concentration of 2 mM of $MgSO_4$ resulted in clear reaction tubes and failed to produce any visible band on the gel in both positive and negative reactions. Concentrations of 4-8 mM resulted in the precipitation of magnesium pyrophosphate in both positive and negative reaction tubes and produced the non-specific pattern in the NTCs. At 4 mM the amplification of the target cDNA was suboptimal, while 6 and 8 mM concentrations were suitable for amplification. A concentration of 6 mM of $MgSO_4$ was set for use in subsequent reactions as there was no difference in the intensity of bands as compared with 8 mM $MgSO_4$.

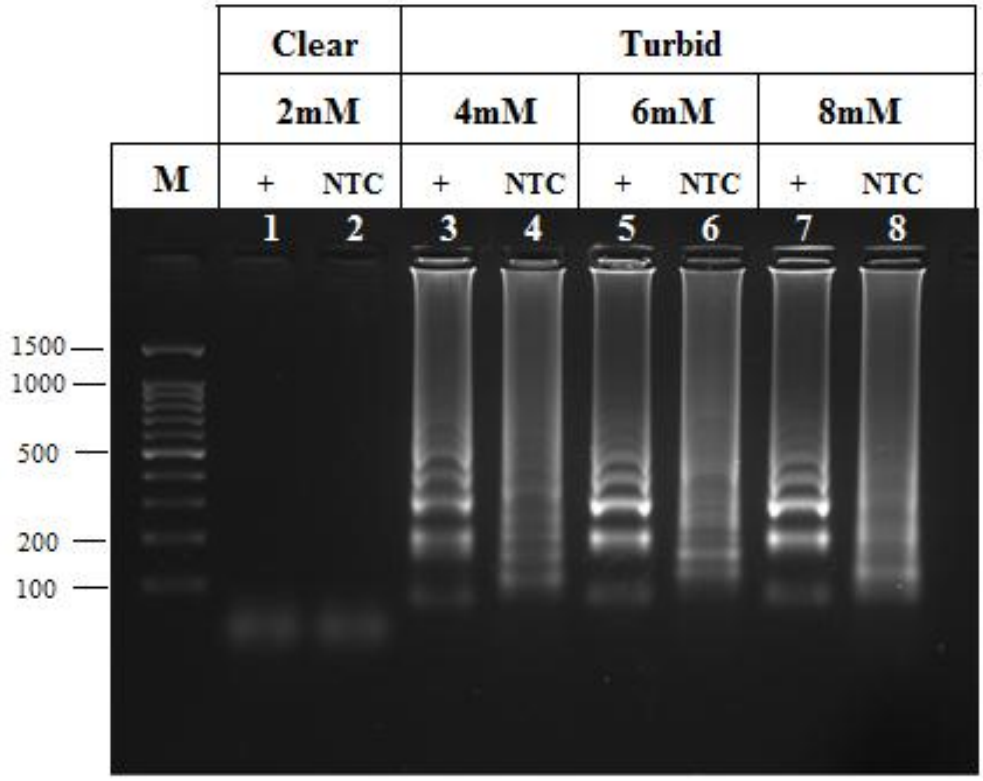

Figure 4.3: Effect of $MgSO_4$ concentration on the RT-LAMP reaction as apparent by reaction turbidity and by agarose gel electrophoresis. M= 100 bp DNA marker. Lanes 1, 3, 5, and 7 are the products of reactions on FMDV cDNA conducted using 2, 4, 6, and 8 mM final concentration of $MgSO_4$ respectively. Lanes 2, 4, 6, and 8 are the products of NTCs conducted using 2, 4, 6, and 8 mM final concentration of $MgSO_4$.

4.1.3 Effect of betaine

Addition of betaine to the RT-LAMP reaction at concentrations up to 2M did not improve the reaction with FMDV template cDNA or reduce false positive turbidity or bands in the NTC (Figure 4.4), and therefore, it was excluded from all further reactions.

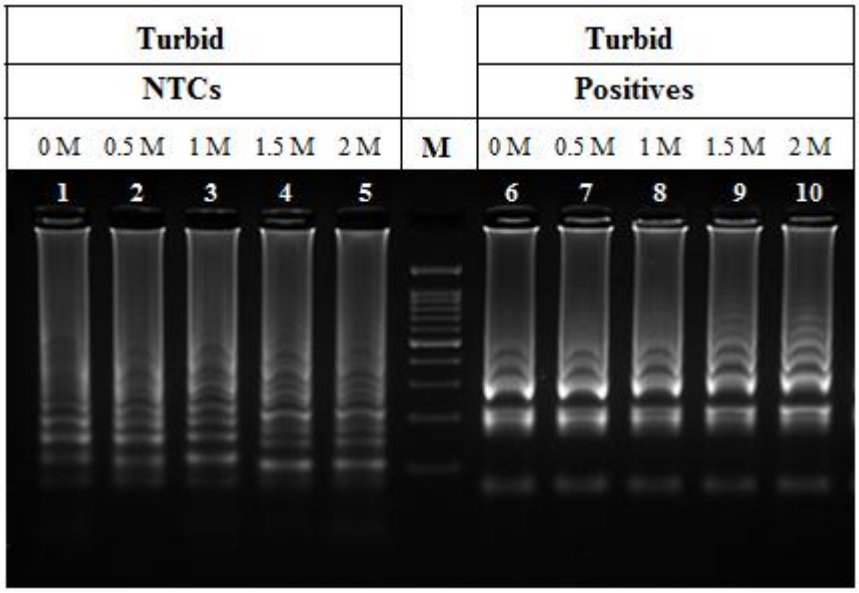

Figure 4.4: Effect of betaine concentration on the RT-LAMP reaction as apparent by reaction turbidity and by agarose gel electrophoresis. Lanes 1-5 are the NTCs conducted using 0, 0.5, 1, 1.5, and 2M final concentration of betaine respectively. M= 100 bp DNA marker. Lanes 6-10 are the products of reactions on FMDV cDNA conducted using 0, 0.5, 1, 1.5, and 2M final concentration of betaine respectively.

4.1.4 Effect of reaction time

The effect of reaction time is shown in Figure 4.5. The positive control was successfully amplified when conducting the reaction for all four times examined (15, 30, 45, and 60 minutes) as apparent on the gel, but there was a clear difference in the pattern observed in the NTCs. At 15 and 30 minutes reaction times, the non-specific amplification in the NTC was not apparent on the gel, while at 45 and 60 minutes the non-specific bands appeared. In terms of reaction turbidity, at 15 minutes reaction time both positive and negative reaction tubes were clear, while at 30 minutes turbidity was apparent in positive but not in negative reaction tubes. At 45 and 60 minutes reaction times, turbidity appeared in both positive and negative reaction tubes.

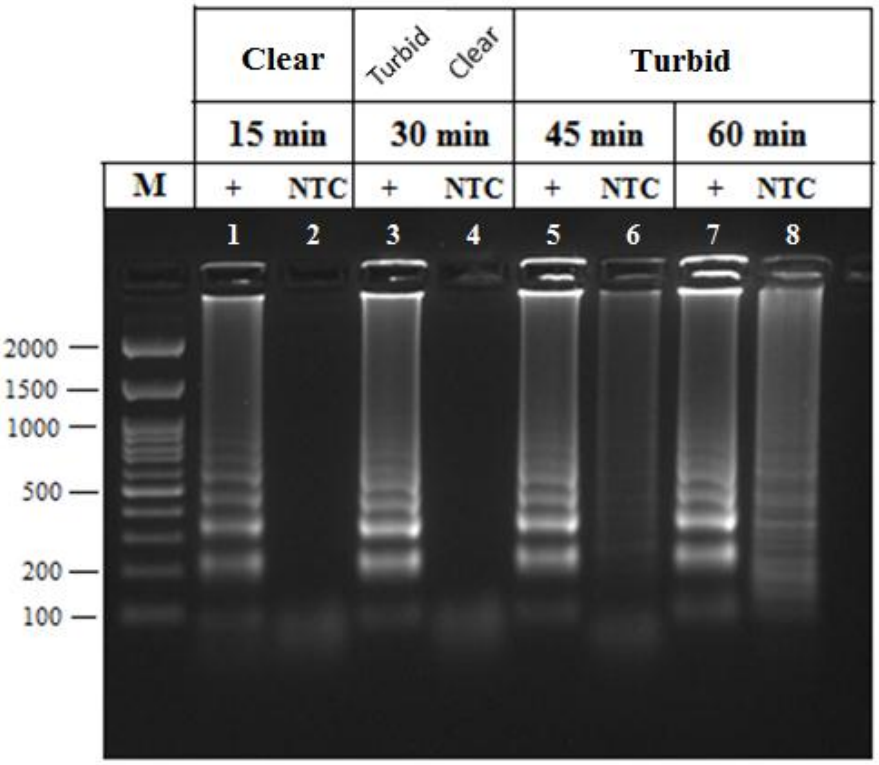

Figure 4.5: Effect of reaction time on RT-LAMP as apparent by reaction turbidity and by agarose gel electrophoresis. M= 100 bp DNA marker. Lanes 1, 3, 5, and 7 are the products of reactions on FMDV cDNA conducted for 15, 30, 45, and 60 minutes respectively. Lanes 2, 4, 6, and 8 are the NTC reactions conducted for 15, 30, 45, and 60 minutes respectively.

After the successful elimination of the non-specific amplification in the NTC, we sought to use malachite green and SYBR Green I dyes and to compare their performance for visual inspection to provide a means for a simpler and clearer interpretation of the results. To evaluate the performance of malachite green, the RT-LAMP reaction was conducted for 30 minutes at 65^{o}C. Malachite green added to the reaction mixture at a final concentration of 0.004%, as recommended by Nzelu et al. (2014), retained a degree of blue color that is less intense than expected in positive reaction tubes (Figure 4.6). However, it failed to produce a clear result in the NTC, which also retained a faint blue color, although lighter than that observed in the positive reaction (Figure 4.6).

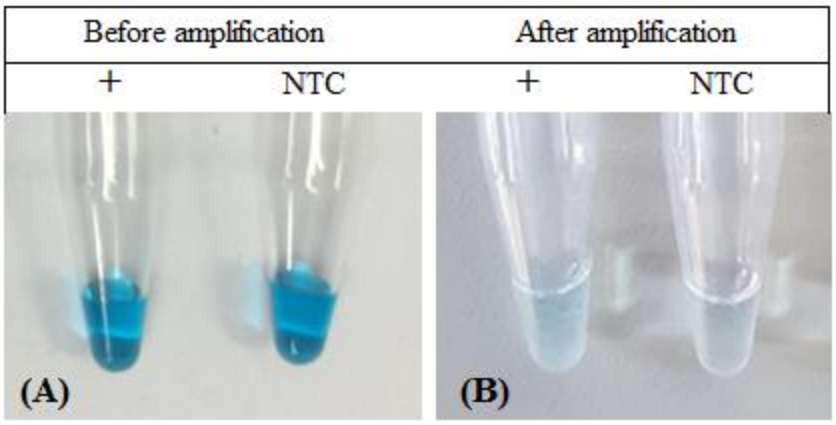

Figure 4.6: Visual inspection of the malachite green-based RT-LAMP reaction before and after amplification. (A) Positive and negative reaction tubes before amplification with MG added at a final concentration of 0.004%. (B) Positive and negative reaction tubes after amplification with MG added at a final concentration of 0.004%.

SYBR Green I, contrary to malachite green dye, produced a clear visual distinction between positive and negative reactions when added to the reactions conducted for 15 and 30 minutes; positive reaction tubes turned bright yellow-green upon addition of SYBR Green I while the NTCs remained orange (Figure 4.7). However, when added after conducting the reactions for 45 and 60 minutes, both positive and negative reactions turned yellow-green (Figure 4.7).

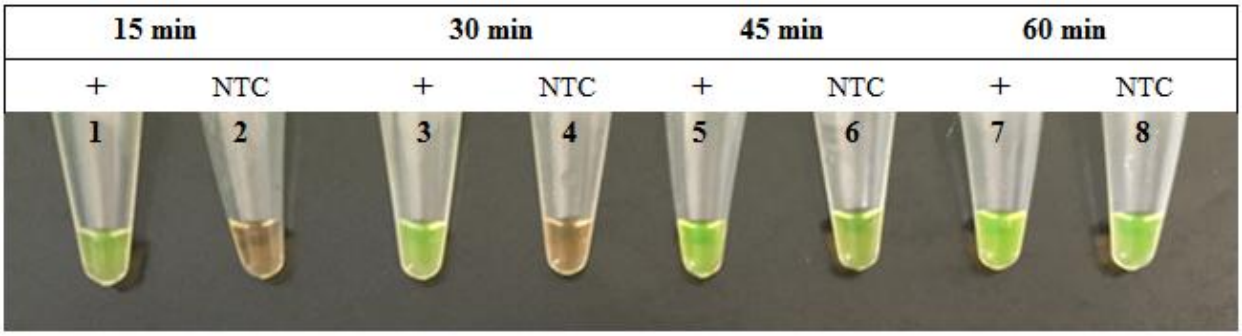

Figure 4.7: Visual inspection of the SYBR Green I-based RT-LAMP reaction. Tubes 1, 3, 5, and 7 are the positive RT-LAMP reaction tubes after reactions conducted for 15, 30, 45, and 60 minutes respectively and addition of SYBR Green I. Tubes 2, 4, 6, and 8 represent negative LAMP reaction tubes after reactions conducted for 15, 30, 45, and 60 minutes respectively and addition of SYBR Green I.

However, performing the reaction for 30 minutes may not be sufficient for weak positive samples, which may require longer reaction time to produce a detectable yield. Therefore another solution was searched to allow for performing the reaction for 1 hour while suppressing the non-specific amplification. For this purpose, the effect of LAMP additives was evaluated.

4.1.5 Effect of carboxamide and N-alkylcarboxamide additives

Figure 4.8 shows the effect of adding FA/DMF additives to the reaction mixture at concentrations ranging from 0.3-1.8 M. Concentrations lower than 1.2M did not have any effect neither on positive nor on negative reactions as the non-specific amplification was evident in the NTCs as indicated by reaction turbidity and by agarose gel electrophoresis (Figure 4.8). Higher concentrations (1.5M and above) completely eliminated the non-specific amplification pattern in the NTCs while maintaining the amplification of the target as evident on the gel and by reaction turbidity (Figure 4.8). Therefore, 1.5 M total concentration of FA/DMF additives was used in all subsequent reactions.

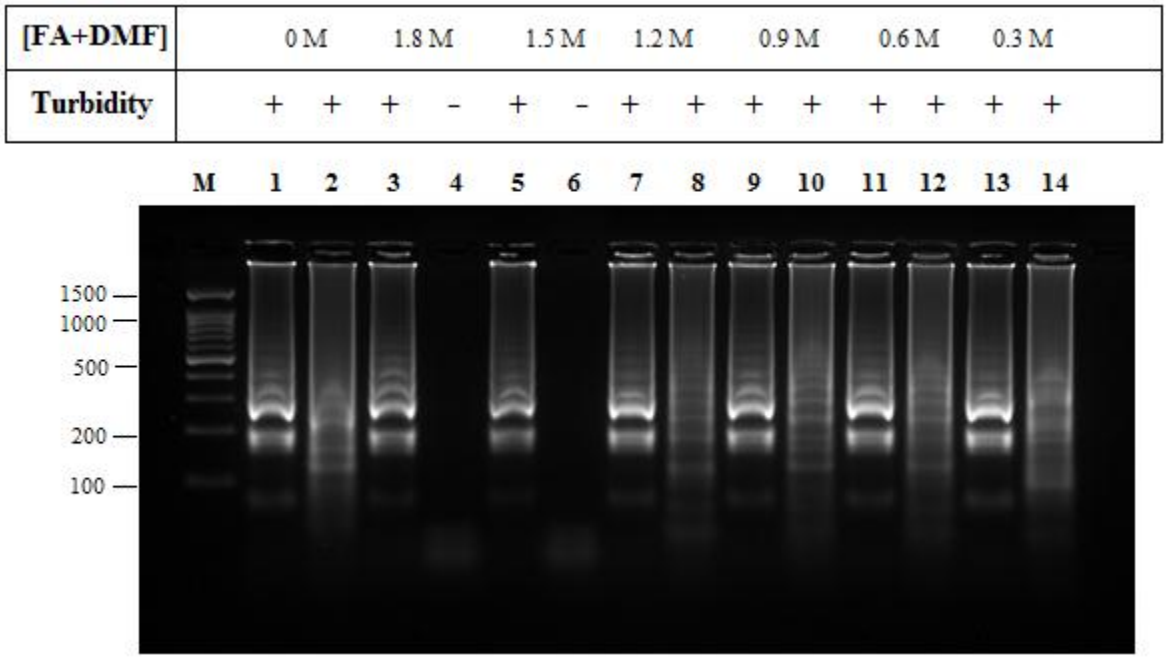

Figure 4.8: Effect of FA/DMF additives on the RT-LAMP reaction as apparent by reaction turbidity and by agarose gel electrophoresis. Turbidity (+/-): (+) indicates turbid reaction tubes while (-) indicates clear reaction tubes. M= 100 bp DNA marker. Lanes 1, 3, 5, 7, 9, 11 and 13 are the products of reactions on FMDV cDNA conducted using 0, 1.8, 1.5, 1.2, 0.9, 0.6, and 0.3 M final concentration of FA/DMF additives respectively. Lanes 2, 4, 6, 8, 10, 12, and 14 are the products of NTCs conducted using 0, 1.8, 1.5, 1.2, 0.9, 0.6, and 0.3 M final concentration of FA/DMF additives respectively.

Figure 4.9 shows the visual inspection results of the RT-LAMP reaction using 1.5M of FA/DMF additives.

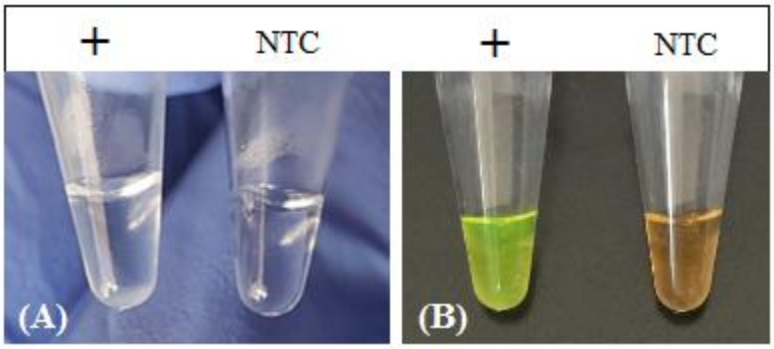

Figure 4.9: visual inspection of RT-LAMP reaction tubes after using 1.5M total concentration of FA/DMF additives. (A) RT-LAMP products detected by inspection of turbidity following the reaction, the first tube shows a positive reaction tube with magnesium pyrophosphate at the bottom, the second tube shows a clear NTC. (B) RT-LAMP products detected by adding SYBR Green I dye to the tubes after the completion of the reaction. The first tube is a positive reaction tube that turned yellow/green after the addition of SYBR Green I, the second tube is an NTC that remained orange after the addition of SYBR Green I.

4.2 Detection limit of the RT-LAMP and PCR assays

The detection limit of LAMP with the FA/DMF additives was assessed using 10-fold serial dilution of positive control cDNA and compared to that of conventional PCR. Figure 4.10 shows that LAMP is 10 times more sensitive than conventional PCR as detectable by SYBR Green and by agarose gel electrophoresis. LAMP detected cDNA down to 10^{-4} dilution (Figure 4.10 a,b) while conventional PCR detected cDNA down to 10^{-3} dilution (Figure 4.10 c).

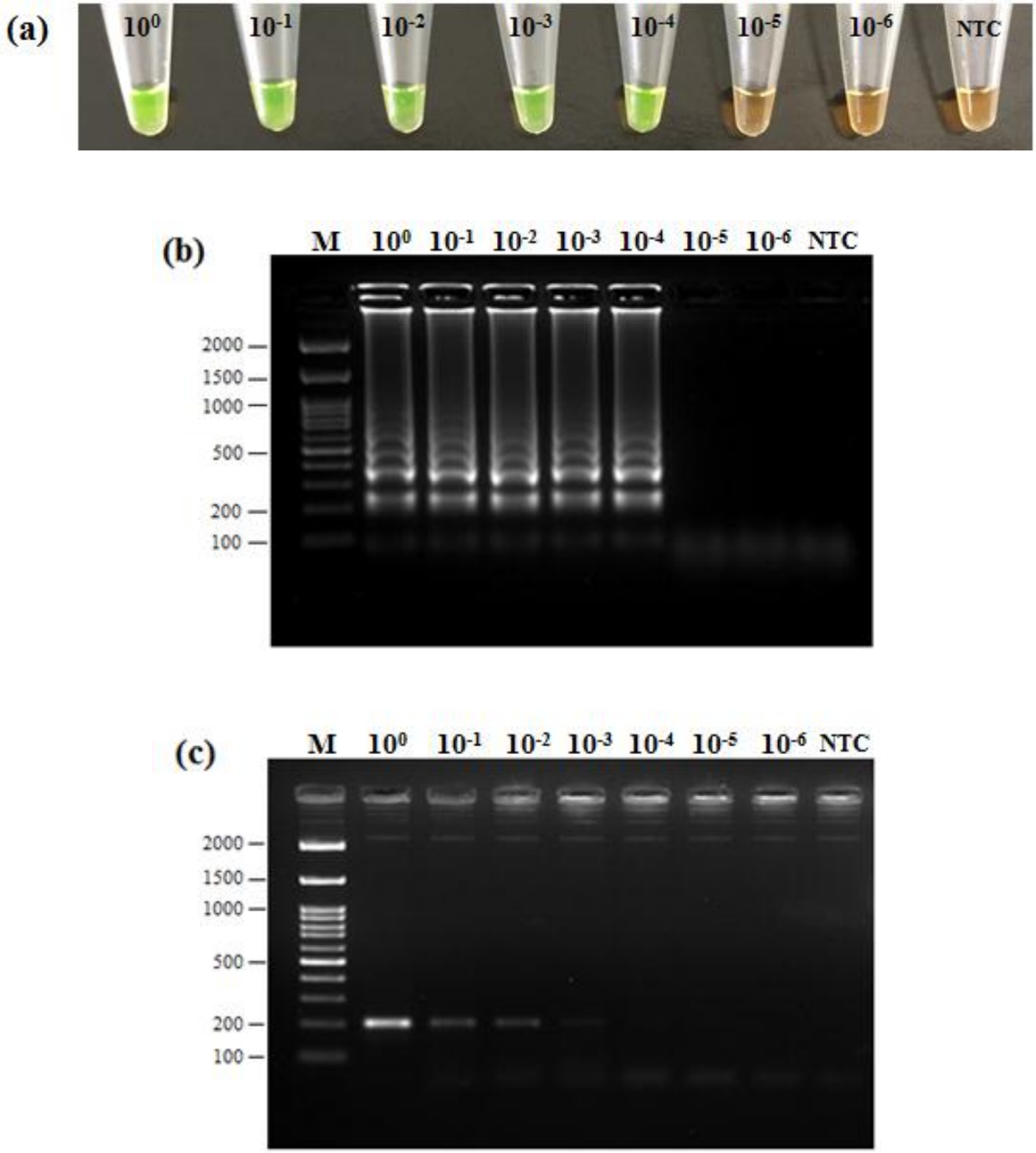

Figure 4.10: Detection limit of LAMP vs. conventional PCR for detection of 10-fold serially diluted FMDV cDNA. (a) SYBR Green I-based visual detection of the optimized LAMP assay products. (b) Agarose gel electrophoresis of the products of the optimized LAMP assay. (c) Agarose gel electrophoresis of the products of conventional PCR using F3 and B3 primers.

4.3 Application of RT-LAMP to RNA targets

The established LAMP assay successfully amplified FMDV genomic RNA without cDNA synthesis or the addition of reverse transcriptase. Figure 4.11 shows the amplification products of FMDV RNA.

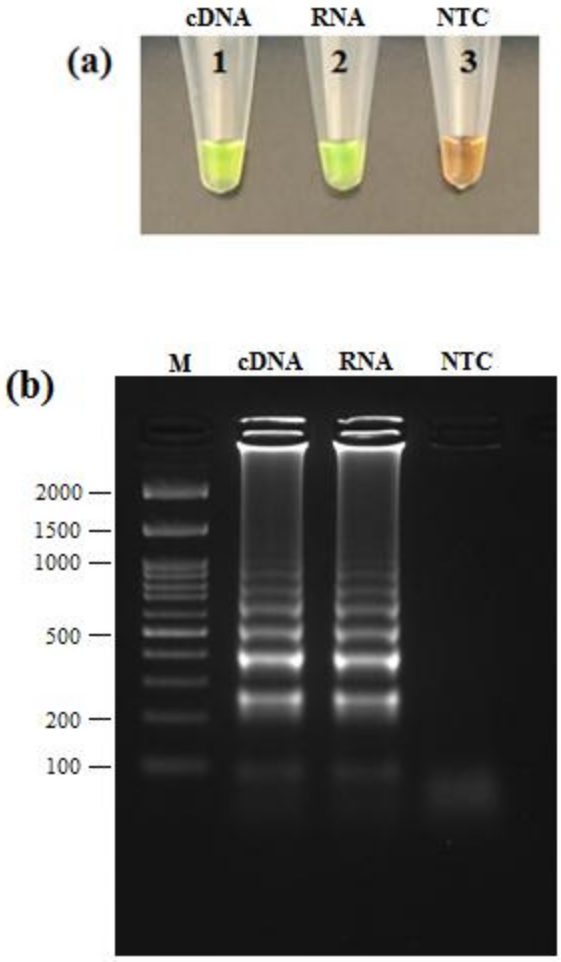

Figure 4.11: Application of the optimized RT-LAMP assay to RNA targets. (a) visual inspection of the RT-LAMP amplification products by SYBR Green I, tube 1: positive control FMDV cDNA, tube 2: FMDV RNA, tube 3: NTC. (b) Agarose gel electrophoresis of the LAMP amplification products, M= 100 bp DNA marker, lane 1 positive control cDNA, lane 2 FMDV RNA, lane 3 NTC.

4.4 Validation of the established RT-LAMP assay

To evaluate the established RT-LAMP assay, we tested it using 29 isolates collected from animals showing clinical signs of FMD. The samples were collected from 19 different farms and were tested by PCR (section 3.6). Of the tested samples 26 (90%) were found to be positive by RT-LAMP and 20 (69%) by PCR.

4.5 Utility of the additives for other targets and sets of primers

To test whether the FA/DMF LAMP additives, used for the suppression of non-specific amplification, can be applied with the same efficiency when using other targets and primer sets, a completely different target and a completely different primer set was tested. Newcastle Disease virus (NDV), which is an RNA virus that belongs to the Paramyxoviridae family, was selected and a set of 6 primers targeting its F gene was designed.

When performing the LAMP reaction for 1 hour without using the additives, NDV cDNA was successfully amplified and a similar non-specific amplification pattern to that observed in the FMDV-LAMP assay was observed (Figure 4.12).

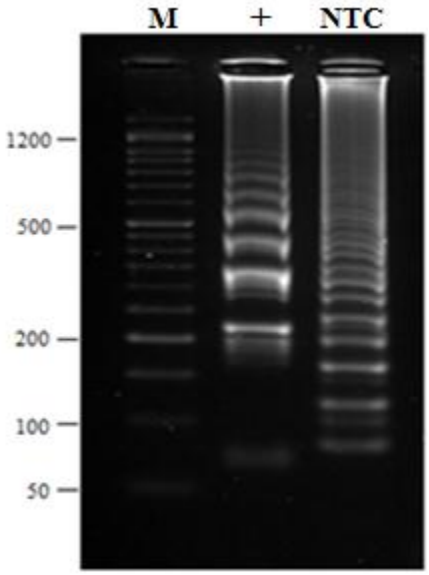

Figure 4.12: Agarose gel electrophoresis of the NDV-LAMP without additives. M = 50 bp DNA marker. Lane1: Positive reaction (with target NDV cDNA). Lane 2: NTC.

When using 1.5 M concentration of FA/DMF additives at 65^{o}C, the positive control was still amplified but the nonspecific amplification was completely eliminated (Figure 4.13). The performance of the NDV-LAMP assay using RNA was also tested. Figure 4.13 shows the results of NDV RNA amplification.

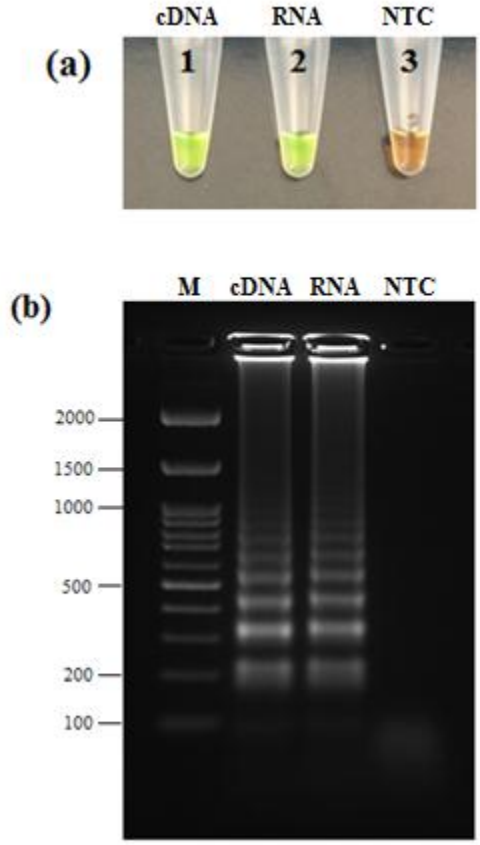

Figure 4.13: RT-LAMP products of the NDV-LAMP assay. (a) Visual inspection of the RT-LAMP amplification products by SYBR Green I, tube 1: positive control NDV cDNA, tube 2: NDV RNA, tube 3: NTC. (b) Agarose gel electrophoresis of the RT-LAMP amplification products, M= 100 bp DNA marker, lane 1 positive control cDNA, lane 2 NDV RNA, lane 3 NTC.

Chapter five

5 Discussion

Foot and mouth disease is a highly contagious disease of livestock that causes severe economic losses and threatens food security of low income countries that rely directly on livestock and livestock products. Since control depends upon culling infected animals at the earliest stage of infection, accurate and rapid diagnostic techniques are considered the keys to effectively minimize the spread of the disease (Anderson, 2002, Charleston et al., 2011). Nucleic acid amplification techniques offer such characteristics in terms of accuracy and rapidity, but these usually require sample transport to diagnostic laboratories. This may cause difficulties in laboratory diagnosis if the samples were inadequately submitted or at least cause a delay in diagnosis. The development of on-site testing techniques is, therefore, strongly recommended to provide timely and accurate diagnosis (Anderson, 2002, Charleston et al., 2011).

Loop-mediated isothermal amplification is a nucleic acid amplification technique that can be performed using basic equipment. This technique offers the advantages of NAATs while obviating the need for being performed in the lab. The aim of this study was, therefore, to develop an RT-LAMP assay that can be visually inspected for the detection of FMDV. The developed assay successfully detected FMDV RNA using only one enzyme and allowed for naked-eye interpretation of the results by turbidity observation or by the addition of SYBR green I nucleic acid dye, thus fulfilling the requirements for POCT. The optimized RT-LAMP assay was 10 times more sensitive than conventional PCR and succeeded in detecting 26 out of 29 clinical samples suspected of FMDV infection as compared to conventional PCR which detected 20 samples.

For establishment of the LAMP assay, the relatively conserved 3D gene was chosen as a target and a set of 6 primers that was described previously was selected and modified based on the results of MSA. The IRES, besides the 3D gene, are the two genome sites usually targeted for pan-serotype detection of FMDV. However, the preliminary comparison of the MSA results of the IRES (data not shown) and the 3D gene sequences belonging to the different serotypes showed that the 3D gene was more suitable for LAMP primer design as it provides a relatively

conserved segment of about 200 bases, which is within the range of the length preferred for LAMP amplification (Notomi et al., 2000). This is in agreement with the conclusion of a previous study, which stated that LAMP oligonucleotides designed to target the IRES had poor diagnostic sensitivity (Yamazaki et al., 2013).

The original primer set described by Shao et al. (2010) was based on the 3D gene sequences of serotypes O, A, Asia1, and C. In Palestine, FMD outbreaks are most commonly caused by serotype O and to a lesser extent by serotypes A and Asia1 while serotype C has never been reported in the region (OIE, 2018). Furthermore, sporadic outbreaks caused by serotype SAT2 were reported in Palestine in 2012 and recently in 2017 in addition to SAT2 outbreaks reported in Egypt in around the same period of time (OIE, 2018). In order, therefore, to be relevant to the regional situation and especially to allow for effective screening with the LAMP assay for any re-incursions of SAT2, the previously described primers were modified based on MSA of the 3D gene sequences of serotypes O, A, Asia1, and SAT2, particularly the topotypes detected in Palestine. In addition, modifications were introduced to accommodate for sequence variability. The modifications included the introduction of degenerate sites to certain positions in the primers and the design of a new LB primer to target a more conserved region (Figure 3.1). Degenerate sites were introduced at the positions that showed sequence variability, as shown in Table 3.2, with special emphasis on the viruses that circulate in the Middle East.

The primer set used successfully amplified the target FMDV 3D gene, but there was initially a level of non-specific amplification in the NTC. Non-specific amplification is a frequently encountered problem in LAMP, which arises because of the use of multiple primers with high concentrations, which increases the probability of the formation of primer dimers and secondary structures (Tanner and Evans Jr, 2014, Wang et al., 2015) This, in combination with the high concentration of Mg^{2+} and dNTPs used in LAMP enhance non-specific amplification and false positive interpretation of the results; particularly when indirect methods such as turbidity or dyes are used for the detection of secondary LAMP products. The non-specific amplification products were observed both by agarose gel electrophoresis and by reaction turbidity (Figure 4.1). The pattern observed for the non-specific amplification on the gel is distinct from the ladder-like pattern characteristic for LAMP products. However, in terms of reaction turbidity, positive and negative reactions cannot be distinguished because magnesium pyrophosphate produced as a

byproduct in the LAMP reaction precipitated similarly in both positive and negative reaction tubes. Since the developed LAMP assay is intended to be used as an on-site testing technique, non-specific amplification should be completely eliminated to allow for visual interpretation of the results without the step of agarose gel electrophoresis. Therefore, a number of reaction parameters were optimized to completely eliminate non-specific amplification including reaction temperature, $MgSO_4$ and betaine concentrations, reaction time, and the use of carboxamide/N-alkylcarboxamide additives.

The LAMP reaction was primarily performed at 65°C, but because of the non-specific amplification observed at this temperature, the effect of higher temperatures up to 72°C, which is the upper limit at which *Bst* 3.0 polymerase functions optimally, were examined. Higher reaction temperatures are recommended to minimize non-specific amplification since they destabilize non-specific priming (Tanner and Evans Jr, 2014). Within the temperature range examined, target cDNA was amplified efficiently at temperatures up to 70°C, but non-specific amplification was still evident even at these elevated temperatures. Beyond 70°C, inconsistent results were observed. Therefore, temperatures above 70°C, which are close to the limit beyond which *Bst* 3.0 polymerase loses activity, were excluded, and 65°C was set for performing all subsequent reactions.

$MgSO_4$ concentration is another reaction parameter that was reported to have a profound effect on the sensitivity and specificity of LAMP (Lee et al., 2016, Liu et al., 2013). Mg^{+2} is a cofactor for DNA polymerases and is a component of the reaction mixture that binds to the dNTPs, primers, and templates (Markoulatos et al., 2002). Mg^{+2} is required at high concentrations for maximum activity of the *Bst* polymerase (Rittié and Perbal, 2008), but high concentrations of $MgSO_4$ were reported to increase the level of non-specific amplification (Lee et al., 2016, Liu et al., 2013, Chandra et al., 2015). This is because Mg^{+2}, as a divalent cation, decreases the electrostatic repulsion between the negatively charged phosphodiester backbones thus stabilizing mis-priming and enhancing non-specific amplification (Markoulatos et al., 2002). Therefore, $MgSO_4$ concentration should be adjusted to minimize non-specific amplification while maintaining the amplification of the target. For this purpose, $MgSO_4$ concentrations ranging from 2-8 mM were tested (Figure 4.3). The concentration already available in the standard buffer, 2 mM, was insufficient for amplification of the target, while higher concentrations increased the

intensity of the ladder-like products of the amplified target and the non-specific amplification products as well. A final concentration of 6 mM $MgSO_4$ was set for performing all subsequent LAMP reaction because this concentration provided comparable amplification efficiency to 8 mM, but better efficiency than 4 mM concentration of $MgSO_4$.

Betaine, is an enhancer of nucleic acid amplification reactions and an additive used to increase reaction specificity (Frackman et al., 1998, Tanner and Evans Jr, 2014). Betaine was used in the original LAMP assay developed by Notomi et al (2000), and was reported to elevate amplification efficiency by facilitating DNA strand separation and to reduce amplification of irrelevant sequences. However, more recent reports stated that betaine is necessary only for amplifying GC-rich templates (Zhou et al., 2014). The effect of betaine was examined on the developed RT-LAMP assay, and under the conditions used, no difference was observed on amplification of both positive and no template controls, so it was excluded from further experiments.

The effect of reaction time was then examined and target amplification was evident even at 15 minutes. Non-specific amplification bands were detectable on the gel, and by turbidity, when the reactions were conducted for extended periods of time (45-60 minutes), but when conducting the reactions for 30 minutes or less, non-specific amplification was eliminated (Figure 4.5). After successful elimination of the non-specific amplification in the NTC when conducting the reaction for 30 minutes, the use of dyes for colorimetric detection of LAMP products was investigated. The use of dyes is preferred for the detection of LAMP products because reaction turbidity is sometimes difficult to detect specially for weak positive samples (Fischbach et al., 2015, Venkatesan et al., 2016), and as it was observed at 15 minutes reaction time, reaction turbidity was not apparent although the ladder like pattern was apparent on the gel (Figure 4.5).

Malachite green dye was next used for the detection of LAMP products. Malachite green dye is added to the LAMP reaction before amplification giving it an intense blue color. This dye is supposed to remain blue in positive reactions and to turn colorless in negative reactions. However, when used in our LAMP assay, conducted for 30 minutes, a faint blue color was retained in the NTC, which could be confused with a weak positive amplification of a diluted target. In addition, the intensity of the blue color retained in the positive reactions was not as supposed to be (Nzelu et al., 2014). Therefore, it was concluded that MG dye is not a reliable

colorimetric indicator of LAMP products, and SYBR Green I nucleic acid dye was tested instead.

SYBR Green I is a nucleic acid dye that intercalates between the bases of double stranded DNA (Dragan et al., 2012). It serves in the LAMP reaction as a colorimetric indicator because it changes color upon binding to the amplified DNA from orange to bright yellow-green, thus providing a means for detection of the products without agarose gel electrophoresis. The results showed that SYBR Green I dye provided excellent visual discrimination between positive and negative samples that are in agreement with the results of agarose gel electrophoresis. As shown in Figure 4.7, SYBR Green I showed higher sensitivity than turbidity observation as it was able to detect amplification products at 15 minutes reaction time that were not detectable by turbidity.

Although performing the reaction for 30 minutes produced good results, the reaction does not reach saturations within this time when using diluted templates, and extending reaction time is sometimes required to increase the yield (data not shown). Therefore, a method that allows for performing the LAMP reaction for longer periods of time, while preventing non-specific amplification is required. Amides have traditionally been used to increase the specificity of PCRs (Chakrabarti and Schutt, 2001), where they exert their action through binding in the major and minor grooves of DNA thus destabilizing the double helix. Tanner and Evans (2013) suggested the use of mixtures amides (a carboxamide and an N-alkyl carboxamide) at specific ratios that depend on the compounds used to enhance the specificity of isothermal amplification techniques. Such additives were reported to act synergistically to eliminate background non-specific amplification without reducing the amplification of the target, thus allowing for performing the LAMP reactions for longer periods of time to increase the sensitivity in case of diluted templates. This is the first report of the application of this LAMP chemistry to an animal virus.

When used at suitable concentrations, the compounds suggested were reported to act similarly with no preference for specified pairs. Therefore, formamide from the carboxamides, and N,N-dimethylformamide from the N-alkyl carboxamides, were chosen for testing in the FMDV-LAMP reactions. The additives were mixed at a ratio of (1: 0.3) molar concentration of FA: DMF as recommended (Tanner and Evans, 2013), and combined mixture concentrations ranging from 0.3-1.8M were tested. This range was selected based on the individual additive concentration required for suppression of non-template amplification (Tanner and Evans, 2013).

FA concentration required individually for complete suppression of non-specific amplification was reported to be 1.8M, while DMF was reported to be 0.77M. Since the additives used as a mixture were claimed to act synergistically to prevent non-specific amplification at lower concentrations, an upper limit of 1.8 M of additives mixture at 65°C was used. Of the concentrations tested, 1.5M and 1.8M succeeded in completely suppressing non-specific amplification, while efficiently amplifying the target at 65°C. A 1.5M concentration was therefore set for performing all subsequent reactions. It is noteworthy to mention that we have also tested the effect of this concentration at temperatures up to 68°C and found a complete suppression of non-specific amplification while maintaining target amplification at temperatures up to 67°C.

Under the optimized conditions of the LAMP assay (6 mM $MgSO_4$, 1.5 M FA/DMF additives, 65°C) and using FMDV cDNA as a target, the reported visually inspected RT-LAMP assay was 10 times more sensitive than conventional PCR using F3 and B3 primers. Relative to conventional PCR, which requires an average of 2-2.5 hours for thermal cycling and 1-1.5 hours for agarose gel electrophoreses, the developed RT-LAMP assay is faster requiring 1 hour or less for amplification and its results are readily evaluated.

FMDV is an RNA virus, but the preceding optimization reactions were performed using cDNA as a template in order to prevent the possibility of adventitious RNase contamination influencing some of the results, which would cause a reproducibility problem over the course of a lengthy series of experiments. RNA is much less stable than DNA.

However, most of the LAMP assays that have been developed so far for the detection of FMDV targeted RNA in a one-step RT-LAMP with the addition of reverse transcriptase in addition to *Bst* DNA polymerase (Dukes et al., 2006, Shao et al., 2010, Yamazaki et al., 2013, Ranjan et al., 2014, Madhanmohan et al., 2013, Farooq et al., 2015, Ding et al., 2014, Chen et al., 2011b, Chen et al., 2011a). *Bst* polymerases in general have a weak reverse transcriptase activity that is not sufficient to replace the activity of reverse transcriptases (Tanner and Evans Jr, 2014). Recently, *Bst* 3.0 polymerase, which is an *in-silico* designed homolog of *Bst* DNA polymerase I large fragment, was commercialized. This enzyme has an enhanced reverse transcription activity in addition to DNA polymerizing activity, so it is a good choice for LAMP reactions with RNA viruses, as it means that there is no need to add reverse transcriptase. To date, however, very few

published studies have employed the dual activity of *Bst* 3.0 polymerase for the amplification of RNA targets; these include Zika virus, Newcastle disease virus, and Japanese encephalitis virus (Lee et al., 2016, Tian et al., 2016, Li et al., 2018). The optimized LAMP assay discussed above, which uses *Bst* 3.0, was used for the amplification of FMDV RNA directly and it succeeded in amplifying it, but further work to compare the sensitivity of the reaction using RNA with that using cDNA as a template is suggested. However, it is worth mentioning that a number of studies reported the observation of higher background non-specific amplification (Lee et al., 2016, Wang et al., 2017) and lower specificity of SNP-LAMP (Mohon et al., 2018) when using the *Bst* 3.0 polymerase, which could be attributed to the higher amplification efficiency of this enzyme as compared to the other *Bst* polymerases.

Since non-specific amplification is a frequently encountered problem in LAMP, modifications that can help in increasing their specificity would be of considerable value. Amide additives, which now have been successfully applied to suppress the non-specific amplification in RT-LAMP for FMDV, have received little attention since they were patented in 2013. To date, only one group has applied these additives (Valeramide and N,N,-diethylformamide), and they reported a significant enhancement of specificity with negligible effect on sensitivity (Poole et al., 2015). Therefore, to gauge whether these additives could be of more general value to researchers in the LAMP field, another RNA virus target and another primer set was chosen and used at the same additives concentration and temperature as before. Newcastle disease virus (NDV) RNA was chosen as a target and a set of 6 primers was designed for this purpose. Initially, the NDV-LAMP assay also had a high level of non-specific amplification, but when using FA/DMF additives at 1.5M concentration a complete inhibition of non-specific amplification was observed. So, it was confirmed that these additives exert their effect regardless of the target and primers sequences used, in support of the claims of Tanner and Evans (2013), and the value of these additives to the development of LAMP assays for animal virus detection is demonstrated.

We recommend that *Bst* 3.0 and amide mixtures be more widely used by those seeking to develop future LAMP assays. Also, although a visually inspected LAMP assay for the detection of FMDV and NDV using RNA as a target, without the addition of reverse transcriptase, has been developed, it is desirable that an on-site-testing technique be performed using unprocessed

samples to achieve the full promise of the technique, which requires further research and implementation.

Chapter five

6 Conclusion

In conclusion, the established LAMP assay combined with SYBR Green I visual inspection method is a simple, rapid, and sensitive detection technique that can provide results in less than an hour. The assay developed can be used for the amplification of RNA targets through using *Bst* 3.0 polymerase without the addition of reverse transcriptase or a prior step of cDNA synthesis. The reaction and the detection of amplification products do not require sophisticated equipment, therefore it can be potentially used as a POCT technique for the early detection of FMDV. This study also proved that mixtures of amide additives, which have been rarely utilized before, can be used at certain concentrations to suppress non-specific amplification frequently encountered in isothermal amplification techniques, thus avoiding the need for testing multiple sets of primers. The system used for suppression of non-specific amplification was also proved to be applicable for different targets and different sets of primers, which could impact upon the application of the LAMP technique for the detection of other diseases that are of veterinary importance. Further work is now required to include the appropriate controls and to test the performance of the developed assay using unprocessed or minimally processed samples so that it could serve as a POCT technique on the farm.

7 References

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