ORIGINAL ARTICLE

Field-Deployable Reverse Transcription-Insulated Isothermal PCR (RT-iiPCR) Assay for Rapid and Sensitive Detection of Foot-and-Mouth Disease Virus

A. Ambagala[1,*], M. Fisher[1,*], M. Goolia[2], C. Nfon[2], T. Furukawa-Stoffer[1], R. Ortega Polo[1] and O. Lung[1,*]

[1] Canadian Food Inspection Agency, National Centres for Animal Disease, Lethbridge Laboratory, Lethbridge, AB, Canada
[2] Canadian Food Inspection Agency, National Centre for Foreign Animal Diseases, Canadian Science Centre for Human and Animal Health, Winnipeg, MB, Canada

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Correspondence:
A. Ambagala. Canadian Food Inspection Agency, National Centre for Foreign Animal Diseases, 1015 Arlington Street, Winnipeg, MB R3E 3M4, Canada. Tel.: +1 204 789 2013; Fax: +1 204 789 2038;
E-mail: Aruna.Ambagala@inspection.gc.ca

*Current address: Canadian Food Inspection Agency, National Centre for Foreign Animal Diseases, Canadian Science Centre for Human and Animal Health, Winnipeg, MB, Canada

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Summary

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals, which can decimate the livestock industry and economy of countries previously free of this disease. Rapid detection of foot-and-mouth disease virus (FMDV) is critical to containing an FMD outbreak. Availability of a rapid, highly sensitive and specific, yet simple and field-deployable assay would support local decision-making during an FMDV outbreak. Here we report validation of a novel reverse transcription-insulated isothermal PCR (RT-iiPCR) assay that can be performed on a commercially available, compact and portable POCKIT™ analyser that automatically analyses data and displays '+' or '−' results. The FMDV RT-iiPCR assay targets the 3D region of the FMDV genome and was capable of detecting 9 copies of *in vitro*-transcribed RNA standard with 95% confidence. It accurately identified 63 FMDV strains belonging to all seven serotypes and showed no cross-reactivity with viruses causing similar clinical diseases in cloven-hoofed animals. The assay was able to identify FMDV RNA in multiple sample types including oral, nasal and lesion swabs, epithelial tissue suspensions, vesicular and oral fluid samples, even before the appearance of clinical signs. Clinical sensitivity of the assay was comparable or slightly higher than the laboratory-based real-time RT-PCR assay in use. The assay was able to detect FMDV RNA in vesicular fluid samples without nucleic acid extraction. For RNA extraction from more complex sample types, a commercially available taco™ mini transportable magnetic bead-based, automated extraction system was used. This assay provides a potentially useful field-deployable diagnostic tool for rapid detection of FMDV in an outbreak in FMD-free countries or for routine diagnostics in endemic countries with less structured laboratory systems.

Introduction

Foot-and-mouth disease (FMD) is considered the most contagious infectious disease of cloven-hoofed animals. Factors including excretion of large quantities of virus during infection, low infectious dose, multiple routes of transmission and ability to spread via aerosols for a long distance contribute to the highly contagious nature of this disease (Alexandersen et al., 2003). FMD causes severe economic damages due to production losses (decrease milk production, poor growth rate and calf mortality) in endemic countries and trade restrictions in FMD-free countries. FMD is caused by foot-and-mouth disease virus (FMDV), a non-enveloped, single-stranded, positive-sense RNA virus belonging to the *Picornaviridae* family. FMDV occurs as seven distinct serotypes: A, O, C and Asia 1 and South

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African Territories (SAT) serotypes SAT1, SAT2 and SAT3. There is little or no cross-protection between serotypes and even between different strains of the same serotype (Brooksby, 1982; Paton et al., 2005, OIE Terrestrial Manual, 2012).

FMD is characterized by vesicular lesions and ulcerations on the tongue, mouth, nasal region and coronary bands of infected animals (Alexandersen and Mowat, 2005). The clinical signs of FMD are not pathognomonic. A number of viral diseases including vesicular stomatitis (VS), swine vesicular disease (SVD), idiopathic vesicular disease (IVD), vesicular exanthema of swine (VES), mucosal disease (MD) and papular stomatitis clinically mimic FMD. In addition, bacteria, chemicals and mechanical trauma also cause erosions and ulcerations in mouth and feet of pigs and ruminants. Therefore, laboratory confirmation is essential for identification of FMDV-infected animals. Current laboratory assays for FMDV detection include virus isolation, antigen ELISA and molecular assays [conventional and real-time RT-PCR (RRT-PCR)]. Virus isolation takes several days, is labour-intensive and can only be performed in specialized biocontainment facilities. Antigen ELISAs show limited sensitivity compared with molecular assays and also require skilled technicians to perform and interpret the assays. Conventional RT-PCR and RRT-PCR assays are highly sensitive and specific; however, sophisticated instrumentation, post-amplification sample/data analyses and skilled technicians are required (Rasmussen et al., 2003; Oem et al., 2005; Moniwa et al., 2007; Goris et al., 2009; Longjam et al., 2011; McKillen et al., 2011; Madi et al., 2012).

FMD outbreaks have to be quickly detected and contained, and therefore, onsite detection tools are highly desirable. To date, a number of field-deployable molecular assays have been developed and evaluated for their potential to use in the field to support local decision-making (Dukes et al., 2006; Chen et al., 2011a,b; Madi et al., 2012; Abd El Wahed et al., 2013; Yamazaki et al., 2013; Kasanga et al., 2014; Ranjan et al., 2014; Waters et al., 2014; Howson et al., 2017), and some of them have been successfully tested in the field (Madi et al., 2012; Abd El Wahed et al., 2013; Howson et al., 2017). To our knowledge, none of those assays are commercially available yet. Here we report laboratory validation of a commercially available *TaqMan* probe-based, field-deployable reverse transcription-insulated isothermal PCR (RT-iiPCR) assay for rapid detection of all FMDV serotypes. The assay was performed on a commercially available compact POCKIT™ Nucleic Acid Analyser (GeneReach USA, Lexington, MA, USA). The assay, POCKIT™ FMDV Reagent Set (GeneReach USA), is available as lyophilized single-tube reactions that can be performed on a field-deployable instrument, POCKIT™ Nucleic Acid Analyser (GeneReach USA). The POCKIT™ analyser is a relatively inexpensive (~US$ 5000.00) compared with currently available thermocyclers. It is a compact (28 × 25 × 8.5 cm), lightweight (~2.1 kg) and user-friendly instrument specially designed to perform PCR and RT-PCR assays based on Rayleigh–Bénard convection (Chang et al., 2012; Tsai et al., 2012; Balasuriya, 2014; Ambagala et al., 2017; Chua et al., 2016; Go et al., 2016). It measures the fluorescence intensity of each sample once prior to and once after the PCR or RT-PCR amplification is complete, automatically calculates the ratio between initial and final fluorescence intensities or signal-to-noise ratio (R1) and therefore requires no data interpretation. If the R1 value is above the manufacturer's default threshold (1.3), a '+' result is displayed, and if it is below 1.2, a '−' result is displayed on the LED screen built into the machine. Rarely, a '?' result is displayed if the R1 value is equal to or between 1.2 and 1.3 ($1.2 \leq R1 \leq 1.3$), indicating an unreliable result and this requires retesting the sample. The POCKIT™ analyser processes up to 8 samples at a time with running time of around 60 min from sample addition to results.

Many complex sample types require nucleic acid extraction to remove potential PCR inhibitors prior to using them in PCR-based assays, and it is one of the major challenges for using PCR-based molecular assays in the field. In this study, we also evaluated a field-deployable, automated nucleic acid extraction system taco™ mini (GeneReach USA) for easy, onsite extraction of nucleic acid from complex clinical samples.

Materials and Methods

Nucleic acid extraction from laboratory isolates

An archived collection of laboratory-extracted genomic RNA from 63 FMDV strains encompassing all seven FMDV serotypes and 19 related non-targeted viruses were used to determine the specificity of the FMDV RT-iiPCR assay (Table 1). FMDV RNA was extracted from virus-infected cell culture supernatant using TriPure® (Roche, Indianapolis, IN, USA) reagent as recommended by the manufacturer. Bluetongue virus 11 (BTV-11) and SVDV POR 1/2003 RNA was extracted from virus-infected cell culture supernatant using Trizol® LS (Life Technologies, Grand Island, NY, USA) reagent as per the manufacturer's instructions. Classical swine fever virus (CSFV) Alfort/187 RNA was kindly provided by the EU Reference Laboratory for CSF, Hanover, Germany. Porcine circovirus (PCV) DNA was obtained from Dr. Markus Czub (University of Calgary, Calgary, AB, Canada). Nucleic acid from the remaining viruses was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions. Before use in the FMDV RT-iiPCR assay, the presence of viral RNA in each extraction was confirmed

Table 1. FMDV and non-target viral RNA used to determine analytical specificity of the FMDV RT-iiPCR assay. Number of strains used for each serotype is given in parenthesis.

FMDV		
Serotype A (16)	O/IRN/8/2005	**Serotype Asia (2)**
A/EGY/3/2009	O/KEN/62/2009	Asia/NKR/2/2007
A/ERI/2/1998	O/KRG/2/2006	Asia/VIT/8/2006
A/ETH/12/2009	O/MAI/15/2006	**Serotype SAT1 (4)**
A/IRN/36/2010	O/MAY/1/2005	SAT1/BOT/12/2006
A/IRQ/21/2009	O/MAY/1/2010	SAT1/ETH/3/2007
A/KEN/7/2008	O/NEP/3/2010	SAT1/KEN/88/2010
A/MAI/12/2006	O/NIG/15/2009	SAT1/ZAM/9/2008
A/MAY/1/2007	O/PAK/1/2010	**Serotype SAT2 (10)**
A/NIG/38/2009	O/SAU/1/2009	SAT2/ETH/2/2007
A/PAK/12/2010	O/SAU/4/2005	SAT2/KEN/122/2009
A/SAU/24/1995	O/SEN/8/2006	SAT2/KEN/122/2009
A/SUD/1/2006	O/SKR/4/2010	SAT2/KEN/122/2009
A/TAI/5/2009	O/SOM/1/2007	SAT2/KEN/2/2007
A/TUR/1/2008	O/SUD/3/2008	SAT2/MOZ/1/2010
A/TUR/25/2007	O/TAN/5/2009	SAT2/SEN/27/2009
A/VIT/8/2009	O/UAE/2/2010	SAT2/SUD/1/2008
Serotype O (27)	O/UAE/9/2009	SAT2/TAN/43/2009
O/BUL/3/2011	O/VIT/7/2008	SAT2/ZAM/8/2008
O/ECU/4/2010	O/VIT/9/2005	**Serotype SAT3 (2)**
O/EGY/8/2006	O/ZAM/1/2010	SAT3/SAR/1/2006
O/ETH/39/2009	**Serotype C (2)**	SAT3/UGA/10/1997
O/HKN/1/2010	C/ETH/6/2005	
O/IRN/31/2010	C/KEN/1/2004	
Non-targets		
VSV-IND	BPI-3 SB	PRRSV YNL
VSV-NJ	RPV Kabete O	PRCV Kiva
BTV-11	SVDV POR 1/2003	PCV1
EHDV Type 2	SVDV UK27/72	PCV2 B
BHV-1 Type 1.1 Colorado #34	CSFV Alfort 902	ERAV
BVDV Type 2 24515	VESV	
BCoV BRCV-OK-0514-2	ASFV Georgia 2007	

Codes for country of origins associated with FMD strains are based on abbreviations published by the FAO World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) -http://www.wrlfmd.org/fmd_serotyping/fmd_cntry_codes.htm. BOT: Botswana, BUL: Bulgaria, ECU: Ecuador, EGY: Egypt, ERI: Eritrea, ETH: Ethiopia, HKN: Hong Kong, IRN: Iran, IRQ: Iraq, KEN: Kenya, KRG: Kyrgyzstan, MAI: Mali, MAY: Malaysia, MOZ: Mozambique, NEP: Nepal, NIG: Niger, NKR: North Korea, PAK: Pakistan, POR: Portugal, SAR: Republic of South Africa, SAT: South African Territory, SAU: Saudi Arabia, SEN: Senegal, SKR: South Korea, SOM: Somalia, SUD: Sudan, TAI: Thailand, TAN: Tanzania, TUR: Turkey, UAE: United Arab Emirates, UK: United kingdom, UGA: Uganda, VIT: Vietnam, ZAM: Zambia. Non-target viruses: ASFV: African swine fever virus, BCoV: bovine coronavirus, BHV-1: bovine herpesvirus 1, BPI: bovine parainfluenza virus, BTV: bluetongue virus, BVDV: bovine viral diarrhoea virus, CSFV: classical swine fever virus, EHDV: epizootic haemorrhagic disease virus, ERAV (from NVSL): equine rhinitis A virus, PCV: porcine circovirus, PRCV: porcine respiratory coronavirus virus, PRRSV: porcine reproductive and respiratory syndrome virus, RPV: rinderpest virus, SVDV: swine vesicular disease virus, VESV: vesicular exanthema of swine virus, VSV-IND: vesicular stomatitis virus – Indiana strain, VSV-NJ: VSV – New Jersey strain.

using published (Forsyth and Barrett, 1995; Tsunemitsu et al., 1999; Paton et al., 2000; Pasick et al., 2001; King et al., 2003; Deregt et al., 2006; Toussaint et al., 2007; Hindson et al., 2008; Lung et al., 2011, 2017; Lu et al., 2012; Klima et al., 2014) or unpublished but laboratory-validated (for PCV-1) pathogen-specific real-time or conventional RT/PCR assays (data not shown).

To compare the analytical sensitivity of the FMDV RT-iiPCR assay to the FMDV RRT-PCR assay, cell culture grown FMDV strains (Table 2B) belonging to each FMDV serotype were used. Each virus culture was serially diluted (10^{-1}–10^{-9}) in sterile phosphate-buffered saline (PBS) and subjected to MagMAX™ extraction (MagMAX™-96 Total RNA Isolation Kit, Life Technologies) according to the manufacturer's recommendations. The extracted nucleic acid was tested in the FMDV RRT-PCR assay, and three of the dilutions around the limit of detection for FMDV RRT-PCR assay were selected and tested in the FMDV RT-iiPCR assay.

In vitro transcription of FMDV 3D RNA

According to the manufacturer, the FMDV RT-iiPCR assay detects as low as 22 copies of *in vitro*-transcribed (IVT) FMDV Asia 1 strain ZB/CHA/58 3D RNA (GenBank accession # DQ533483.2) with 95% confidence. In order to confirm the limit of detection (LOD) of the assay, IVT RNA of FMDV O1 Manisa/69 3D polymerase was generated. Briefly, 861 bp (7175–8036) of the FMDV O1 Manisa/69 3D polymerase gene (GenBank accession number AY593823) was synthesized (Integrated DNA Technologies, Coralville, IA, USA) and cloned into pGEM-3Zf(+) cloning vector (Promega, Madison, WI, USA) and the resulting plasmid was used to generate RNA using the MEGAscript® T7 Kit (Life Technologies) as recommended by the manufacturer. After residual DNA was removed using TURBO™ DNase (Thermo Fisher Scientific, Mississauga, ON, Canada), IVT RNA was purified using RNeasy® mini columns (Qiagen), concentration was measured by the Qubit® RNA assay kit, Life Technologies), and single-use aliquots were frozen down at −80°C until used. Prior to use, serial dilutions of IVT RNA were made in nuclease-free water containing 40 ng/μl yeast tRNA.

Clinical samples & nucleic acid extraction

A large number of archived clinical samples (swabs, epithelial tissues and vesicular and oral fluids) collected from cattle, sheep and pigs infected with different FMDV strains were used for clinical validation of the FMDV RT-iiPCR assay (Table 3).

Nasal and oral swabs: Nasal and oral swabs collected from FMDV-infected 5-month-old calves (n = 2, Holstein), 6-month-old sheep (n = 2, Suffolk) and 3-week-old piglets (n = 3, Landrace) were used to determine the clinical sensitivity of the FMDV RT-iiPCR assay. The calves

Table 2. Detection of the analytical sensitivity of the FMDV RT-iiPCR assay

(A)

Copy #	RT-iiPCR Results (#positive/total)	R1 (Average ± SD)
100 000	5/5	4.81 ± 0.12
10 000	5/5	4.69 ± 0.12
1000	5/5	4.68 ± 0.08
100	6/6	4.62 ± 0.31
50	7/7	4.10 ± 0.80
20	5/5	4.36 ± 0.73
5	2/6	2.87 ± 2.05
0	0/7	0.99 ± 0.04

(B)

FMDV		RT-iiPCR		RRT-PCR
Isolate (Serotype)	Dilution	Result	R1	C_T
A22/IRQ/24/64 (A)	10^{-5}	+	4.96	34.91
	10^{-6}	+	4.94	39.53
	10^{-7}	–	1.00	No C_T
O/UKG/11/2001 (O)	10^{-6}	+	4.92	28.01
	10^{-7}	+	4.77	31.84
	10^{-8}	–	1.03	38.13
C1/Noville (C)	10^{-7}	+	4.97	33.81
	10^{-8}	+	4.94	No C_T
	10^{-9}	–	1	No C_T
Asia 1/Shamir (Asia)	10^{-4}	+	4.86	34.61
	10^{-5}	+	4.95	No C_T
	10^{-6}	–	0.98	No C_T
SAT1/KEN/4/98 (SAT1)	10^{-7}	+	4.97	31.55
	10^{-8}	+	4.8	35.83
	10^{-9}	–	1.09	No C_T
SAT2/SAU/1/2000 (SAT2)	10^{-6}	+	4.95	32.08
	10^{-7}	+	4.84	38.73
	10^{-8}	–	0.99	No C_T
SAT3/ZIM/4/81 (SAT3)	10^{-7}	+	4.91	34.61
	10^{-8}	+	4.93	No C_T
	10^{-9}	–	0.97	No C_T

(A) Using *in vitro*-transcribed (IVT) FMDV O1 Manisa/69 3D RNA. The IVT RNA was serially diluted in nuclease-free water, and 5 μl of each dilution was tested in RT-iiPCR assay. The assay was repeated a minimum of 5 times. (B) Using FMD viral RNA representing all 7 serotypes. R1 = R1 (signal/noise ratio) value. SD = standard deviation. In RRT-PCR assay, a sample was considered positive when the threshold cycle (C_T) value was lower than 36. UKG = Channel Islands, ZIM = Zimbabwe.

(#1 and #2) and sheep (#525 and #566) were inoculated intradermal lingually with the FMDV O/UKG/11/2001 strain at $10^{6.5}$ $TCID_{50}$. The piglets (#78, #79 and #80) were inoculated subdermally into the heel bulb of the left hind leg with the FMDV A/IRAN/1/2009 at $10^{7.0}$ $TCID_{50}$. Nasal and oral swabs were collected from each animal on −2, 1, 2, 3 and 4 days post-infection (dpi). A swab from a ruptured vesicle on the left hind foot of pig #80 was also collected on 3 dpi. Total nucleic acid was extracted from nasal and oral swabs using the column-based PetNAD™ Nucleic Extraction System (GeneReach USA) (Ambagala et al., 2017) and MagMAX™-96 Total RNA Isolation Kit (Life Technologies) according to the manufacturer's instructions. PetNAD™ System is recommended for nucleic extractions for the POCKIT™ analyser, and MagMAX extraction is used for routine nucleic acid extractions for the FMDV RRT-PCR assay at the CFIA-NCFAD. The baseline samples for cattle #1, #2 and sheep #535 and #566 were not available, and therefore, nasal and oral samples collected from 10 healthy sheep and cattle were evaluated.

A selected number of swab samples (oral swabs from cattle #1, #2 and sheep #566 and oral and nasal swabs from pig #80) was also used to evaluate the taco™ mini extraction system.

Oral fluid: For collection of oral fluids, 4-week-old piglets (n = 24, all Landrace) were randomly assigned to 4 pens (6 animals per pen). Two piglets from each group were arbitrarily selected and inoculated with cell culture supernatant containing $10^{6.0}$ $TCID_{50}$ FMDV O/UKG/11/2001 in one of the heel bulbs of hindlimb. Starting from day 0, sterile cotton ropes (5/8-inch) were suspended in each pen at shoulder height for 20–30 min. Oral fluid was obtained by wringing the rope in a ziplock plastic bag. Then, the corner of each bag was cut to drain the oral fluid into a 50-ml Falcon tube. The oral fluid samples were clarified at 2000 ***g*** for 3 min using Beckman GS-6R centrifuge GH3.8 rotor at 4°C, aliquoted and stored at −80°C until use. Total RNA was extracted from 55 μl of each sample using MagMAX™-96 Total RNA Isolation Kit (Life Technologies).

Epithelial tissues: Epithelial tissues were collected from freshly ruptured vesicles from 4-week-old piglets (n = 4, Landrace) experimentally inoculated with the FMDV A/IRN/1/2005 strain (animal #77), FMDV SAT1/ZAM/9/2000 strain (Animal #76) or FMDV O/UKG/11/2001 strain (animals #14 and #16) in one of the heel bulbs of the right front limb (100 μl) and the left hindlimb (100 μl). FMDV-negative epithelial tissue was collected from a freshly euthanized FMDV-negative animal at necropsy from an unrelated animal experiment conducted at the laboratory. The samples were placed in virus transport medium with added antibiotics and stored at 4°C until used. Prior to nucleic acid extraction, 10% tissue suspensions in 0.04 M phosphate buffer (pH 7.4 ± 0.05) were prepared using a hand-held mortar and pestle, and total nucleic acids were extracted from each sample using the MagMAX™-96 Total RNA Isolation Kit or the taco™ mini. The extracted nucleic acids were used immediately or stored at −80°C.

Vesicular fluid: Vesicular fluid was collected from unruptured vesicles from 4-week-old piglets (n = 5, Landrace) inoculated with the FMDV O/UKG/11/2001 (#4, #15 and #24), the FMDV SAT1/ZAM/9/2008 (#81) or the SVDV Por 1/2003 (#5) intradermally in the heel bulb of one hindlimb. For sample collection, the animals were physically

Table 3. Clinical samples used for validation of the FMDV RT-iiPCR assay

Species	Animal #/Pen #	Virus strain	DPI	Sample type	Extraction methods
Cattle	1	FMDV O/UKG11/2001	1,2,3	Nasal Swabs	P/M
				Oral swabs	P/M/t
	2	FMDV O/UKG11/2001	1,2,3	Nasal swabs	P/M
				Oral swabs	P/M/t
Sheep	535	FMDV O/UKG11/2001	1,2,3	Nasal and Oral swabs	P/M
	566	FMDV O/UKG11/2001	1,2,3	Nasal Swab	P/M
				Oral swabs	P/M/t
Pig	3	Uninfected	–	Tongue and soft palate epithelium	t
	4	FMDV O/UKG11/2001	4	Vesicular fluid	None
	5	SVDV PO1/2003	17	Vesicular fluid	None
	14	FMDV O/UKG11/2001	3	Tongue epithelium	t
	15	FMDV O/UKG11/2001	4	Vesicular fluid	None
	16	FMDV O/UKG11/2001	3	Tongue epithelium	t
	24	FMDV O/UKG11/2001	7	Vesicular fluid	None
	76	FMDV SAT1/ZAM9/2000	1	Coronary band and interdigital epithelium	M
	77	FMDV A/IRN1/2005	1	Coronary band and interdigital epithelium	M
	78	FMDV A/IRN1/2009	−2, 1, 2, 3, 4	Nasal and oral swabs	P/M
	79	FMDV A/IRN1/2009	−2, 1, 2, 3, 4	Nasal and oral swabs	P/M
	80	FMDV A/IRN1/2009	−2, 1, 2, 3, 4	Nasal swab	P/M
				Oral swab	P/M/t
				Lesion swab	t
	81	FMDV SAT1/ZAM9/2008	6	Vesicular fluid	None
	Pen #1	FMDV O/UKG11/2001	0, 1, 2, 3	Oral Fluid	M
	Pen # 2	FMDV O/UKG11/2001	0, 1, 2, 4	Oral Fluid	M
	Pen # 3	FMDV O/UKG11/2001	0, 1, 2, 5	Oral Fluid	M
	Pen # 4	FMDV O/UKG11/2001	0, 1, 2, 6	Oral Fluid	M

A large collection of archived clinical samples (swabs, epithelial tissues and vesicular and oral fluids), collected from cattle, sheep and pigs infected with different FMDV strains, was used for clinical validation of the FMDV RT-iiPCR assay. Nucleic acid was extracted from the samples using routine laboratory-based MagMAX™ (M) extraction system, or PetNAD™ (P) column-based or automated taco™ mini extraction system (t) recommended for the portable POCKIT analyser. Vesicular fluid was diluted in nuclease-free water and used directly in the FMDV RT-iiPCR assay. In addition to the samples listed, 10 additional nasal and oral samples from heathy cattle and sheep were used as negative controls for PetNAD™ and MagMAX™ extraction followed by testing in FMDV RT-iiPCR and FMDV RRT-PCR assays.

restrained, surface of vesicles were wiped with a cotton wool containing 70% ethanol, and vesicular fluid was aspirated using sterile 1-CC intradermal insulin syringes. The vesicular fluid collected was used immediately or stored at −80°C until use.

All animal experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care. The design of the experiment was approved by the Institutional Animal Care Committees, and all efforts were made to minimize the number of animals used and animal discomfort. All the virus stocks used were free from bacterial and other viral contaminants.

Automated nucleic acid extraction using taco™ mini

Total nucleic acid was extracted from a selected number of clinical samples using a fully automated taco™ mini extraction system (GeneReach USA) as per the manufacturer's instructions. The taco™ mini system uses magnetic bead-based extraction method and has a throughput of 8 samples/45 min (total run time not-including sample processing). Briefly, 200 μl of each sample (10% epithelial tissue suspension, scarified oral, nasal or lesion swab material) was added to individual wells in the first row of the ready-to-use reagent cartridge (taco™ mini Preloaded DNA/RNA Extraction Set, GeneReach USA). The cartridge was loaded on to the instrument, and the 'start' button was pressed. The extraction time was 40 min, and the eluted nucleic acid was available in the last row of the reagent cartridge. The nucleic acid was used immediately in the POCKIT™ FMDV assay or stored at −80°C until use.

FMDV RRT-PCR assay

The in-house FMDV TaqMan RRT-PCR was carried out according to a published protocol (Moniwa et al., 2007). This assay used primers (FMDV1186F 5′ACTGGGTTTTA YAAACCTGTGATG3′ and FMDV 1237R 5′TCAACTTC TCCTKGATGGTCCCA3′) and a TaqMan probe (5′FAM-ATCCTCTCCTTTGCACGC-MGB3′) that specifically

targeted a conserved region of the FMDV 3D gene resulting in the amplification of an 88-bp product. The AgPath-ID™ One-Step RT-PCR reagents (Life Technologies) were used for all tests, and the test was performed in 96-well plates on ABI SDS7900ht with a final volume of 25 μl per well containing 1 × RT-PCR buffer, 0.5 μM forward primer, 0.5 μM reverse primer, 0.2 μM probe, 1 × RT enzyme mix and 5 μl RNA template. The thermal cycler was as follows: 50°C for 30 min, 95°C for 15 min followed by 45 cycles of 95°C for 10 s, 60°C for 1 min. The threshold cycle (C_T) values <36 were considered positive. The samples were run in an Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific).

POCKIT™ FMDV RT-iiPCR assay

The FMDV RT-iiPCR assay (POCKIT™ FMDV Reagent Set, Cat # apls-033), a lyophilized single-tube reaction kit that targets the FMDV 3D RNA polymerase gene, was performed on the POCKIT™ instrument as described previously (Ambagala et al., 2017; Lung et al., 2016). The sequence information of the iiPCR primers and probes, and the components of the assay were proprietary to GeneReach USA and therefore were not available. Briefly, 50 μl of Premix Buffer A (GeneReach USA) was added to individual FMDV Premix tubes containing the lyophilized reagents, the tubes were briefly spun in a cubee™ mini centrifuge, 5 μl of extracted nucleic acid or diluted vesicular fluid was added to each tube, and 50 μl of the mixture was transferred to pre-labelled R-tubes (GeneReach USA). The R-tubes were then spun in a cubee™ for 10 s and placed in the POCKIT™ analyser and the 'Run' button was pressed.

The POCKIT™ analyser was run on a default program set by the manufacturer, with a total run time of 1 h. In all experiments, except the one for the determination of analytical sensitivity, the samples were run in singles to increase the throughput.

Statistical analysis

To determine the limit of detection (LOD) of the FMDV RT-iiPCR assay, probit analysis (a nonlinear regression model) was performed using commercial software SPSS 14.0 (SPSS Inc., Chicago, IL, USA).

Results

Analytical specificity and sensitivity of the FMDV RT-iiPCR assay

The analytical specificity of the FMDV RT-iiPCR assay was evaluated using RNA extracted from 63 FMDV isolates (C_T values ranged from 12 to 16 in the FMDV RRT-PCR assay) belonging to all seven FMDV serotypes, and 19 clinically relevant, non-target viruses. The assay was able to detect all FMDV isolates (R1 values ranged from 4.82 to 4.90), and no cross-reactivity was observed with equine rhinitis A virus (ERAV, a closely related aphthovirus), and other viruses that cause vesicular lesions [VSV, SVDV, VESV] or mucosal erosions [Epizootic hemorrhagic disease virus (EHDV), bovine herpesvirus-1 (BHV-1), rinderpest virus (RPV)] in farm animals (data not shown). The assay also did not cross-react with other high-consequence pathogens of swine [African swine fever virus (ASFV) and CSFV], and viral pathogens commonly encountered in cattle [bovine viral diarrhoea virus (BVDV), BHV-1) and pig [porcine respiratory and reproductive syndrome virus (PRRSV), porcine respiratory coronavirus (PRCV) and porcine circovirus (PCV)] farms (data not shown).

The analytical sensitivity of the assay was evaluated using *in vitro*-transcribed FMDV 3D RNA representative of the FMDV O1/Manisa/TUR/69 strain. The FMDV RT-iiPCR assay was able to detect 9 copies of the FMDV-3D IVT RNA with 95% confidence (Table 2A). The limit of detection of the assay was further analysed using a serially diluted panel of viral RNA representing all seven FMDV serotypes (Table 2B), and the results were compared to that of the FMDV RRT-PCR used in the laboratory. The sensitivity of the two assays was comparable for the FMDV O/UKG/11/2001 and SAT1/KEN/4/98 strains; however, the FMDV RT-iiPCR assay was slightly more sensitive for the A22/IRQ/24/64, C1/Noville, Asia 1/Shamir and SAT2/SAU/1/2000 and SAT3/ZIM/4/81 strains.

Clinical sensitivity and specificity of the FMDV RT-iiPCR assay

Clinical sensitivity of the RT-iiPCR assay was determined using total RNA extracted from nasal and oral swabs collected from FMDV-infected cattle ($n = 2$), sheep ($n = 2$) and pigs ($n = 3$) (Table 4). The cattle started showing clinical signs (fever) as early as 1 dpi, and the RT-iiPCR assay was able to detect FMDV RNA in both nasal and oral swabs collected from calves #1 and #2 on 1 dpi. The laboratory-based RRT-PCR assay also detected FMDV RNA in the nasal and oral swabs from calf #1 on 1 dpi, but failed to do so in the nasal swab from calf #2 on 1 dpi. Both assays detected FMDV RNA in nasal and oral swabs collected from calves on 2 and 3 dpi.

The sheep inoculated with FMDV were identified by RT-iiPCR assay as early as 1 dpi, although both animals did not develop any clinical signs. Meanwhile, the RRT-PCR assay was only able to identify one of the infected sheep (#566) on 3 dpi.

The baseline samples from cattle and sheep were not available, and therefore, nasal and oral samples collected from 10 healthy sheep and cattle were evaluated. All the

Table 4. Detection of FMDV by RT-iiPCR in nasal and oral samples collected from experimentally infected animals

Species-Animal #		Sample	DPI −2	1	2	3	4
Cattle	#1	Nasal	NA	+ (30.51)	+ (33.29)	+ (22.59)	NA
		Oral	NA	+ (24.13)	+ (21.41)	+ (20.65)	NA
	#2	Nasal	NA	+ (No C_T)	+ (29.34)	+ (25.33)	NA
		Oral	NA	+ (14.50)	+ (21.79)	+ (22.38)	NA
	C. signs		N	Y	Y	Y	
Sheep	#535	Nasal	NA	+ (No C_T)	+ (No C_T)	+ (No C_T)	NA
		Oral	NA	− (No C_T)	− (No C_T)	− (No C_T)	NA
	#566	Nasal	NA	+ (37.96)	+ (No C_T)	+ (29.50)	NA
		Oral	NA	+ (44.62)	+ (No C_T)	+ (35.89)	NA
	C. signs		N	N	N	N	
Pig	#78	Nasal	− (No C_T)	− (No C_T)	+ (29.64)	ND (No C_T)	ND (No C_T)
		Oral	NA	− (No C_T)	+ (34.41)	ND (31.52)	ND (32.62)
	#79	Nasal	− (No C_T)	− (No Ct)	− (No C_T)	ND (No C_T)	ND (No C_T)
		Oral	− (No C_T)	− (No C_T)	− (No C_T)	ND (No C_T)	+ (27.98)
	#80	Nasal	− (No C_T)	− (No C_T)	− (No C_T)	ND (No C_T)	+ (No C_T)
		Oral	− (No C_T)	− (No C_T)	− (No C_T)	ND (No C_T)	+ (24.53)
	C. signs		N	N	B	Y	Y

FMDV RT-iiPCR results and RRT-PCR C_T values (in parentheses) for each sample are presented. In the RRT-PCR assay, a sample was considered positive when the threshold cycle (C_T) value was lower than 36. ND = not done, C. signs = clinical signs, N = not present; B = begin to appear; Y = present. DPI = days post-infection, NA = samples not available. No oral swabs were available from pig #78 for -2 dpi, and no oral or nasal swabs were available from calves and sheep for -2 dpi and 4 dpi. Nucleic acid extracted from nasal and oral samples from all piglets on 3 dpi, nasal and oral samples from piglet #78 on 4 dpi and nasal sample from piglet #79 on 4 dpi were not available for testing in RT-iiPCR assay. All positive RT-iiPCR results were associated with R1 value above 4.5.

samples gave negative results in both FMDV RRT-PCR (no C_T values) and FMDV RT-iiPCR assays (R1 valued ranged between 0.93 and 1.02) (data not shown).

The clinical signs in pigs began to appear on 2 dpi. Both the RT-iiPCR and RRT-PCR assays detected FMDV RNA in the nasal and oral swabs collected from pig #78 on 2 dpi. The laboratory-based RRT-PCR assay was able to detect FMDV in nasal swab sample from pig #78 on 3 dpi, but the same sample was not available for testing by RT-iiPCR assay. The RRT-PCR assay was able to detect FMDV RNA in oral swabs from pigs #78, #79 and #80 on 4 dpi, but failed to detect in nasal swabs. The RT-iiPCR assay was able to detect FMDV RNA in all three samples (oral swab from pig #79, and the oral and nasal swabs from pig #80) tested on 4 dpi. Nasal and oral samples collected from pig #78 and nasal sample from pig #79 on 4 dpi were not available for testing by RT-iiPCR.

Epithelial tissues from freshly ruptured vesicles are one of the preferred tissue samples for FMDV detection. Total RNA was extracted from epithelial tissues collected from freshly ruptured vesicles (within a few hours of rupture) from 2 pigs infected with either the A IRN 1/2005 or SAT1 ZAM 9/2000 strains, and tested by the FMDV RT-iiPCR. Epithelial tissues collected from tongue and soft palate from a healthy piglet were used as negative controls. The RT-iiPCR assay detected FMDV RNA in epithelial tissue samples in all FMDV-infected piglets and showed no cross-reactivity with RNA extracted from epithelial tissue sample collected from uninfected piglets. The results were comparable to that of the FMDV RRT-PCR assay (Table 5A).

Oral fluid represents a convenient and cost-effective tool for disease monitoring and surveillance in commercial pig herds. To determine whether the FMDV RT-iiPCR assay can be used to detect FMDV in oral fluids, samples collected from 4 groups of piglets infected with FMDV O/UKG/11/2001 were tested (Table 5B). The directly inoculated animals developed clinical signs on 2 dpi and the contact pigs 24–48 h later. The FMDV RT-iiPCR assay was able to detect FMDV in oral fluid samples collected as early as 1 dpi from all 4 pens, and the results were comparable to that observed with the FMDV RRT-PCR. The RT-iiPCR assay did not show any cross-reactivity with RNA extracted from oral fluid samples prior to FMDV inoculation (0 dpi).

Table 5. Detection of FMDV by RT-iiPCR in oral fluids (A) and epithelial tissues (B)

(A)

Species/Organ	FMDV Strain	DPI	RT-iiPCR (Result)	RRT-PCR (C_T)
Pig 3/Tongue	A/IRN/1/2005	0	–	No C_T
Pig 3/Soft Palate	″	0	–	No C_T
Pig #77/Coronary band	″	1	+	15.77
Pig #76/Coronary Band	SAT1/ZAM/9/2000	1	+	18.75
Pig #77/Interdigital	A/IRN/1/2005	1	+	15.34
Pig #76/Interdigital	SAT1/ZAM/9/2000	1	+	16.22

(B)

Group	DPI	RT-iiPCR (Result)	RRT-PCR (C_T)
1	0	–	ND
	1	+	33.99
	2	+	22.53
	3	+	21.59
2	0	–	ND
	1	+	24.23
	2	+	21.93
	3	+	19.26
3	0	–	ND
	1	+	23.21
	2	+	24.38
	3	+	22.32
4	0	–	ND
	1	+	27.58
	2	+	22.87
	3	+	21.72

In RRT-PCR assay, a sample was considered positive when the threshold cycle (C_T) value was lower than 36. DPI = days post-infection. ND = not done. All positive RT-iiPCR results were associated with R1 value above 4.5.

Vesicular fluid collected from three pigs infected with FMDV O/UKG/11/2001 and one pig with SAT1/ZAM/9/2008 strain was serially diluted in nuclease-free water (1 : 5, 1 : 10 and 1 : 20) and tested (Table 6). All the dilutions were positively identified by the FMDV RT-iiPCR assay, and the vesicular fluid collected from an SVDV Por 1/2003-infected animal did not show any cross-reactivity.

taco™ mini extraction and POCKIT™ RT-iiPCR detection

Sample types other than vesicular fluid are complex and heterogeneous and therefore require processing (nucleic acid extraction) prior to their use on PCR-based assays. Using a taco™ mini instrument, total nucleic acid was extracted from multiple sample types including epithelial tissues, oral, nasal and lesion swabs collected from FMDV-infected pigs, cattle and sheep. The extracted nucleic acid was tested in the FMDV RT-iiPCR assay and the results

Table 6. Detection of FMDV in unextracted vesicular fluid samples

Strain	Pig #	DPI	Dilution	RT-iiPCR Result (R1)
FMDV O/UKG/11/2001	15	4	1 : 5	+ (5.0)
			1 : 10	+ (5.0)
			1 : 20	+ (4.9)
	4	4	1 : 5	+ (4.9)
			1 : 10	+ (4.9)
			1 : 20	+ (4.9)
	24	7	1 : 5	+ (4.9)
			1 : 10	+ (4.9)
			1 : 20	+ (5.0)
FMDV SAT1/ZAM/9/2008	81	6	1 : 5	+ (2.7)
			1 : 10	+ (4.8)
			1 : 20	+ (5.0)
SVDV POR 1/2003	5	17	1 : 5	– (1.0)
			1 : 10	– (1.0)
			1 : 20	– (1.0)

Vesicular fluid samples were serially diluted in nuclease-free water, and 5 μl of each sample was tested in the FMDV RT-iiPCR assay. DPI = days post-infection.

were compared with that of the FMDV RRT-PCR assay which used nucleic acid extracted from the same samples using MagMAX™-96 Total RNA Isolation Kit (Life Technologies). The taco™ mini/RT-iiPCR assay could detect FMDV RNA in all the samples that were positive with the FMDV RRT-PCR assay using nucleic acid extracted with MagMAX™-96 total RNA isolation kit (Table 7). Additionally, the taco™ mini/RT-iiPCR combination could detect FMDV RNA in the samples that were shown negative with MagMAX™-96/RRT-PCR assay (nasal swab samples were collected from pig #80 on 4 dpi, and the oral swab samples were collected from sheep #566 on 1 and 2 dpi) (Table 7).

Discussion

Rapid, highly sensitive detection, preferably onsite, is critical for FMD control. Towards this goal, a number of portable, immunological and molecular assays have been developed and evaluated. The lateral-flow-based immunological assays are relatively quick (<10 min), cheap and user-friendly; however, they are less sensitive (~80%) compared with molecular assays (Ferris et al., 2009, 2010; Yang et al., 2013, 2015; Morioka et al., 2015).

In contrast, PCR-based assays such as RRT-PCR assays are highly sensitive (>95%) and specific and therefore widely used for routine laboratory-based detection of FMDV. Over the last decade, a number of attempts were made to transfer the laboratory-based RRT-PCR assays to portable PCR equipment that can be operated in the field (Callahan et al., 2002; Hearps et al., 2002; Risatti et al., 2003; Tomlinson et al., 2005; Rasmussen et al., 2008; Takekawa et al., 2010; Madi et al., 2012; Molsa et al., 2012,

Table 7. Detection of FMDV in samples extracted using the taco™ mini portable automated nucleic acid extraction system

Species	Animal #	Sample type	FMDV Strain	DPI	RT-iiPCR (Result)	RRT-PCR (C_T)
Pig	16	10% Epi. Tissue Sus.	O/UKG/11/2001	3	+	ND
	14	"		3	+	ND
	80	Oral Swab	A/IRN/1/2009	4	+	24.53
		Nasal Swab		4	+	No C_T
		Lesion Swab Feet		3	+	ND
Cattle	1	Oral Swab	O/UKG/11/2001	1	+	24.13
				2	+	21.41
				3	+	20.65
	2	Oral Swab	O/UKG/11/2001	1	+	14.50
				2	+	21.79
				3	+	22.38
Sheep	566	Oral Swab	O/UKG/11/2001	1	+	44.62
				2	+	No C_T
				3	+	35.89

In RRT-PCR assay, a sample was considered positive when the threshold cycle (C_T) value was lower than 36. ND = not done. DPI = days post-infection. All positive RT-iiPCR results were associated with R1 value above 4.5.

2015; Liu et al., 2014; Howson et al., 2017). These portable PCR instruments, however, depend on precision thermocycling, are highly sophisticated and therefore expensive and delicate for field use.

Isothermal amplification methods such as loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA) and iiPCR offer alternative means for rapid and sensitive detection of pathogens in clinical samples. The iiPCR and LAMP assays typically provide comparable or better detection compared with conventional PCR and RRT-PCR assays. The instrumentations needed for those assays are relatively inexpensive, less complicated, less delicate and easily decontaminated or disposed of between each use. Recently, a number of RT-LAMP assays have been developed and validated for detection and typing of FMDV (Dukes et al., 2006; Chen et al., 2011a,b; Yamazaki et al., 2013; Ding et al., 2014; Kasanga et al., 2014; Ranjan et al., 2014; Waters et al., 2014; Farooq et al., 2015; Howson et al., 2017). The assays were rapid, highly sensitive and specific, and more resistant to inhibitors than conventional and RRT-PCR assays. A novel real-time reverse transcription RPA (RRT-RPA) assay has been developed for detection of FMDV in the field, and its performance was evaluated during an FMD outbreak (Abd El Wahed et al., 2013). The assay generated results faster than the LAMP assay; however, it showed ~100 times lower sensitivity (1436 FMDV genome copies) compared with that of the RRT-PCR assays. The RRT-RPA assays need relatively long primers (30 nucleotides) and probes (at least 50 nucleotides), and therefore, designing RPA assays is challenging especially for viruses with highly variable genomes such as FMDV.

Here we described evaluation and validation of a portable RT-iiPCR assay for detection of FMDV in clinical samples. The assay which is highly sensitive, specific and reproducible can be carried out in a commercially available simple, relatively inexpensive (compared to thermocyclers), compact portable instrument, the POCKIT™ analyser (Balasuriya et al., 2014; Wilkes et al., 2014, 2015a,b; Ambagala et al., 2017; Lung et al., 2016). The instrument can be operated using AC voltage or car battery and is less delicate and easily decontaminated using a mild detergent between each use. It can process 8 samples at a time with an average run time of 57 min. At the end of each run, the results are displayed on the instrument as '+' or '−' and therefore require no data interpretation by the user. The data are stored onto an SD card for subsequent evaluation if needed. All reagents for RT-iiPCR assays are provided in lyophilized form. The POCKIT™ analyser is available as a component of POCKIT™ Xpress mobile laboratory, a rugged suitcase containing a POCKIT™ analyser, a mini centrifuge (cubee™), two pipettes and a taco™ mini automatic nucleic acid extractor. The taco™ mini automated nucleic acid extractor greatly reduce hands-on time from samples to results.

The FMDV RT-iiPCR assay positively identified all 63 FMDV laboratory isolates tested and showed no cross-reactivity with non-target viruses. The assay was able to detect as low as 9 copies of IVT RNA with 95% confidence. When a dilution series of FMDV genomic RNA belonging to all seven serotypes (extracted using MagMAX™ system) were tested in both FMDV RT-iiPCR and RRT-PCR assays, it was evident that the FMDV RT-iiPCR assay was equally or slightly more sensitive (with some strains) than the FMDV RRT-PCR assay. The variability between serotypes may be attributed to the nucleotide sequence differences and/or the presence of secondary structures in or around the 3D genomic region in different FMDV strains. Based on our

experience on iiPCR development, iiPCR assays are able to tolerate more base pair mismatches than conventional PCR (Ambagala et al., 2017). One hypothesis is that primer annealing in an iiPCR can occur within a temperature gradient which may allow the primers to hybridize with sequences with multiple mismatches and still initiate chain elongation. The clinical sensitivity and specificity of the RT-iiPCR assay were demonstrated using multiple samples types (nasal, oral and lesion swabs, oral fluids, vesicular fluid and epithelial tissues) collected from FMDV-infected cattle, sheep and pigs. The RT-iiPCR was able to detect FMDV RNA in all RRT-PCR-positive clinical samples, and additionally in some (~14%) of the RRT-PCR-negative samples. The higher sensitivity was clearly demonstrated with the preclinical samples collected from sheep experimentally infected with FMDV O/UKG/11/2001, which were positively identified by the FMDV RT-iiPCR assay from 1 dpi but not until 3 dpi by the FMDV RRT-PCR assay. The difference in sensitivity observed could also be due to three different nucleic acid extraction systems used in this experiment (PetNAD™/taco™ mini for samples tested in POCKIT™ analyser and MagMAX™ for the samples processed by Applied Biosystems® 7500 Real-Time PCR System).

Oral fluid can be easily collected from pens of pigs and used for the diagnosis of a number of key endemic and exotic diseases as well as biomarkers of stress or metabolic state (Prickett et al., 2008; Kittawornrat et al., 2010, 2012; Detmer et al., 2011; Ramirez et al., 2012; Goodell et al., 2013; Vosloo et al., 2015; Panyasing et al., 2014). Pen-based oral fluid provides a non-invasive sample type that is easy to collect, resulting in a lower overall cost compared with individual animal sampling. Pen-based oral fluids provide higher herd-level sensitivity and herd-level specificity for detection of pathogens compared with individual pig samples. Currently, the pen-based oral fluid samples are shipped to central veterinary diagnostic laboratories for testing. In this study, we demonstrated that the pen-based oral fluid can be used for rapid onsite detection of FMDV-infected animals using RT-iiPCR. The FMDV RT-iiPCR assay readily detected FMDV RNA in oral fluid samples from all pens as early as 1 dpi.

One of the major bottlenecks of performing molecular assays in the field is the lack of a convenient, effective and safe procedure for nucleic acid isolation. Previously we have shown that diluted serum samples from CSFV-infected pigs can be used directly, without nucleic acid extraction, for detection of CSFV RNA by a CFSV RT-iiPCR assay (Lung et al., 2016). In this study, we demonstrated that the RT-iiPCR assay can detect FMDV RNA in intact vesicular fluid samples without RNA extraction process. The R1 values for all positive samples were 4.9 or 5, except for the 1 : 5 diluted vesicular fluid sample from pig # 81. The R1 value reduction observed (2.7) on this sample could be due to inhibitory effects of proteins in vesicular fluid on the RT-iiPCR assay. Similar observations were noted when undiluted serum samples were tested in the CSFV RT-iiPCR assay (Lung et al., 2015b). For more complex samples, however, nucleic acid extraction is required to ascertain high sensitivity. Most of the nucleic acid extraction methods available to date are too elaborate, labour-intensive and require trained technicians to perform the procedure. Simplified column-based extraction systems are available and they can be used to isolate total nucleic acids from clinical samples in the field (Ambagala et al., 2017), but still require a considerable amount of manual handling of the samples which can lead to potential contaminations and/or degradation of viral RNA. The field-deployable fully automated taco™ mini extraction system we demonstrated here can be used successfully to extract nucleic acid from more complex samples such as tissue suspensions and swab samples in the field. The taco™ mini is a relatively inexpensive, light weight (5 kg), small footprint (30(H) × 26.5(D) × 26(W) cm) magnetic bead-based extraction system that can extract total nucleic acids from 8 samples within 45 min.

In conclusion, in this study we have evaluated an alternative, field-deployable, highly sensitive and specific molecular assay that can be run on a commercially available, user-friendly, relatively inexpensive, field-deployable POCKIT™ instrument for rapid detection of FMDV. The total assay time (sample to results) for vesicular fluid samples is less than 1 h and for other samples types, when taco™ mini extraction system is used, is less than 2 h. The FMDV RT-iiPCR assay reagents are commercially available as a single-tube, lyophilized and ready-to-use commercial kit. Both the POCKIT™ instrument and the taco™ mini extraction system can be operated using a car battery in the field. The assay can be deployed to veterinary offices, slaughter houses or borders in an outbreak to support onsite diagnosis by the veterinarians. FMDV RT-iiPCR assay can be performed by anyone with basic laboratory skills (veterinary technicians, veterinarians, inspectors, etc.) after a few hours (2–3 h) of training. This onsite detection tool will allow the veterinarians to confirm clinical diagnosis quickly and take necessary actions to contain the spread of this highly contagious virus before the laboratory confirmations are available. Each taco™ mini/POCKIT detection system can process only 6 samples plus a negative control and a positive control in a single run, and similar to other pen-side detection tools available, are unlikely to replace the testing done at the centralized laboratories. Considering the commercial availability, rapid turnaround time, ease of use and portability, the FMDV RT-iiPCR assay evaluated here warrants a comprehensive field validation.

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Conflict of Interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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