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ScienceDirect
Veterinary Microbiology 126 (2008) 101–110
veterinary microbiology
www.elsevier.com/locate/vetmic

Detection of foot-and-mouth disease virus by nucleic acid sequence-based amplification (NASBA)

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Received 30 December 2006; received in revised form 9 July 2007; accepted 10 July 2007

Abstract

A study was conducted to evaluate the performance of a nucleic acid sequence-based amplification (NASBA) assay for the detection of foot-and-mouth disease virus (FMDV). Two detection methods: NASBA-electrochemiluminescence (NASBA-ECL) and a newly developed NASBA-enzyme-linked oligonucleotide capture (NASBA-EOC) were evaluated. The diagnostic sensitivity of these assays was compared with other laboratory-based methods using 200 clinical samples collected from different regions of the world. Assay specificity was also assessed using samples ($n = 43$) of other viruses that cause vesicular disease in livestock and genetic relatives of FMDV. Concordant results were generated for 174/200 (87.0%) of suspect FMD samples between NASBA-ECL and real-time RT-PCR. In comparison with the virus isolation (VI) data, the sensitivity of the NASBA-ECL assay was 92.9%, which was almost identical to that of the real-time RT-PCR (92.4%) for the same set of samples. There was broad agreement between the results of the NASBA-ECL and the simpler NASBA-EOC detection method for 97.1% of samples. In conclusion, this study provides further data to support the use of NASBA as a rapid and sensitive diagnostic method for the detection and surveillance of FMDV.

Keywords: Foot-and-mouth disease virus; Diagnosis; NASBA-electrochemiluminescence; NASBA-enzyme-linked oligonucleotide capture; Real-time RT-PCR

1. Introduction

Foot-and-mouth disease (FMD) is a highly infectious disease affecting cloven-hoofed animals, predominantly cattle, sheep, pigs, and goats. It spreads rapidly if the disease is not properly controlled and can

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doi:10.1016/j.vetmic.2007.07.008

pose severe threats to the livestock industry (Burrows, 1966). FMD is caused by a single-stranded RNA virus, foot-and-mouth disease virus (FMDV), which belongs to the family *Picornaviridae*, genus *Aphthovirus* and has a genome of approximately 8 kb in size. Seven serotypes of FMDV are recognized (O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1). The disease is characterized by acute fever, generally acquired after viral particle entry into the pharynx epithelium of the respiratory tract, followed by the development of blisters chiefly in the mouth and on the feet of infected animals.

FMD is endemic in many countries across Africa, South America and Asia. It has a considerable impact on the farming industry leading to significant economic losses particularly in countries that are normally FMD-free, as was demonstrated by the outbreak in the United Kingdom in 2001 caused by the pan-Asia type O virus strain (McLaws et al., 2006). Approximately 6.5 million animals both infected and suspected of carrying the virus were slaughtered to prevent the spread and to ultimately eliminate the disease. A sensitive detection method for rapid identification of FMDV-infected animals is therefore important to minimize the unnecessary slaughter of uninfected herds and to aid FMD surveillance.

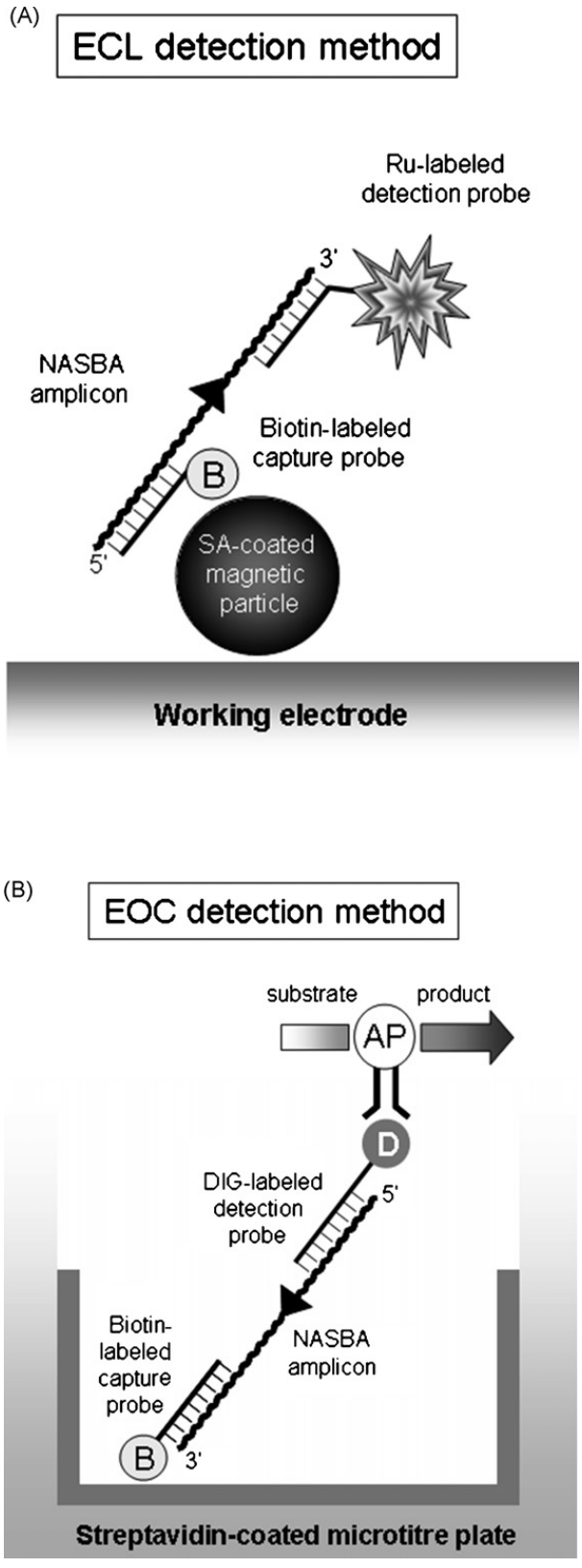
Current identification methods for FMDV include virus isolation (VI) in cell culture, immunoassays to detect antigen, and assays to detect nucleic acid sequences of the FMDV genome. Of the latter, reverse-transcription polymerase chain reaction (RT-PCR) is currently the most commonly applied method for the amplification of viral targets. In recent years, nucleic acid sequence-based amplification (NASBA) has been increasingly used for the detection of viruses, including avian influenza virus (Collins et al., 2002a; Lau et al., 2004), Newcastle disease virus (Cui et al., 2007; Lau et al., 2006), dengue fever virus (Baeumner et al., 2002), and FMDV (Collins et al., 2002b; Lau et al., 2006). Developed in 1991, NASBA technology is a continuous, isothermal and enzyme-based method to amplify single-stranded RNA and is therefore particularly suited to RNA virus detection (Compton, 1991). The technology employs three enzymes: reverse-transcriptase, ribonuclease-H and T7 RNA polymerase, a set of target-specific forward and reverse oligonucleotide primers and two types of detection probes. The forward primer has a 5′ extension containing the promoter sequence for T7 bacteriophage DNA-dependent RNA polymerase, while the reverse primer has a 5′ extension containing a complementary binding sequence for a DNA oligonucleotide detection probe labelled with a ruthenium (Ru)-based electrochemiluminescent (ECL) tag for electromagnetic detection (Fig. 1A). Under optimum conditions, NASBA is more sensitive than RT-PCR of equivalent targets (Keer and Birch, 2003). Moreover, NASBA amplification can occur at a constant temperature (41 °C): therefore the process can be performed in a heat-block or even in a temperature-controlled water bath which circumvents the need for expensive thermocycling equipment.

We previously developed a sensitive NASBA-based assay for FMDV (Collins et al., 2002b). In the current study, we evaluated the performance of the NASBA-ECL method for the detection of FMDV using clinical samples from a variety of sources, which had previously been tested using VI, ELISA and real-time RT-PCR. We also assessed a newly developed NASBA-enzyme-linked oligonucleotide capture (NASBA-EOC) colorimetric method (Fig. 1B). This technique is particularly suitable for use in mobile or portable (field-based) laboratories or in central laboratories with limited access to molecular and conventional diagnostic techniques.

2. Materials and methods

2.1. Test samples

Two hundred samples from the FAO World Reference Laboratory for Foot-and-Mouth Disease (WRL for FMD), Pirbright, were tested (Table 1). These samples were from suspect FMD cases and were collected from around the world (1967–2005) and predominantly comprised ∼10% suspensions of vesicular epithelium (ES) prepared in phosphate buffer (Ferris and Dawson, 1988). Additional samples containing other viruses that can cause vesicular disease in livestock were also tested: swine vesicular disease virus (SVDV; $n = 8$); vesicular stomatitis virus (VSV; $n = 18$) and vesicular exanthema of swine-like viruses (VESV; $n = 10$). Samples of equine rhinitis A virus and bovine rhinitis-3 virus (members of the *Aphthovirus* genus) were also tested by NASBA and real-time RT-PCR.



2.2. Virus isolation

All the samples had been tested upon receipt by VI in primary calf thyroid cell cultures and/or in a permanent line of IB-RS-2 cells. The FMDV specificity of samples showing a cytopathic effect was confirmed by ELISA (Ferris and Dawson, 1988).

2.3. Extraction of RNA viruses for NASBA and real-time RT-PCR

Total nucleic acid was extracted from the sample suspensions by an automated procedure using a MagNA Pure LC (Roche) robot, as previously described (Reid et al., 2003). Nucleic acid was aliquoted and stored at −70 °C until tested by real-time RT-PCR or NASBA.

2.4. NASBA amplification

The primers (Invitrogen, Shanghai, China) used for the NASBA amplification were previously described in Collins et al. (2002b) and are listed in Table 2. For amplification, 5 μl of nucleic acid was added to a mixture of 80 mM Tris pH 8.3, 24 mM $MgCl_2$, 140 mM KCl, 10 mM DTT, 2 mM each dNTP, 4 mM each NTP, 30% (v/v) DMSO, and 0.4 μM of each primer. The reaction mixture was heated to 65 °C for 5 min and then cooled to 41 °C for 5 min. After cooling, 5 μl enzyme mix (6.4 U/μl T7 RNA polymerase, 1.3 U/μl AMV-RT, 0.02 U/μl RNase H and 0.42 μg/μl BSA) was added to make up a final reaction volume of 20 μl which was then incubated at 41 °C for 90 min.

Fig. 1. (A) Schematic illustration of the principle of NASBA-ECL detection. The biotin-labelled capture probe is attached to the magnetic bead through a biotin-streptavidin interaction. Its 3′ end is complementary to a sequence conferred to the product by the forward primer. The ruthenium-labelled detection probe is complementary to a sequence conferred to the product by the reverse primer. Detection is at 620 nm in an ECL reader. (B) Schematic illustration of the principle of NASBA-EOC detection. The biotin-labelled capture probe is attached to the well of a streptavidin-coated microtitre plate (grey). As in NASBA-ECL, its 3′ end is complementary to a sequence on the product. The DIG-labelled detection probe is complementary to a sequence conferred to the product by the reverse primer. After substrate addition and incubation (see Section 2), the reaction was stopped and absorbance at 405 nm measured in a spectrophotometer.

Table 1
Samples from suspect cases of FMD utilised in this study

O	A	C	Asia 1	SAT 1	SAT 2	SAT 3	NVD
AFG 56/2003	BHU 41/2002	AUR 4/73	TAI 1/98[2]	BOT 2/68	BOT 13/2002	RHO 1/74	BHU 42/2002
AFG 17/2003	BHU 6/2003	BEL 1/69	TUR 3/2000	BUN 2/99	BUN 7/99	RHO 2/74	BHU 43/2002
BKF 1/2002	IRN 7/2000	BEL 1/72	PAK 20/2003	GHA 3/69	CAR 5/2000	SAR 4/80	BHU 44/2002
BHU 7/2002	IRN 6/2002	C ARG/84	PAK 69/2003	KEN 11/96	ERI 1/98	SAR 7/80	BHU 47/2002
BHU 17/2004	IRN 5/2003	FRA 2/72		KEN 1/2005	ERI 4/98	ZIM 1/84	BHU 48/2002
HKN 13/99	IRN 26/2003	FRA 3/74		MOZ 11/78	IVC 8/75	ZIM 6/91	BHU 52/2002
HKN 2/2000	IRN 14/2005	HUN 2/72		NIG 11/68	KEN 7/99		BHU 1/2003
HKN 3/2003	IRN 16/2005	ISO 3 bovine		RHO 1/80	KEN 2/2002		BHU 2/2003
HKN 12/2005	IRN 17/2005	ISO 5 NV 19		SAR 6/74	KEN 2/2002		BHU 3/2003
IRN 55/99	IRQ 17/2000	ITL 4/89		SAR 17/80	KEN 7/2004		BHU 4/2003
IRN 8/2001	IRQ 108/2002	IND 16/81		SUD 8/74	LIB 1/2003		BHU 5/2003
IRN 71/2001	MAY 1/2003†	JOR 11/69		SWA 1/80	LIB 7/2003[3]		BHU 10/2003
IRQ 27/2000	MAY 1/2003†	PHI 3/88		TAN 2/96	MAL 4/75		BHU 10/2004
ISR 2/2004[1]	MAY 4/2003	PHI 2/89		TAN 28/99	MAL 7/91		GHA 1/2002
KUW 1/2002	MAY 5/2003	PHI 3/94		TAN 36/99	MOZ 20/78		GHA 3/2002
LAO 20/2003	PAK 3/2003	POR 2/80		TAN 52/99	RHO 3/80		GHA 6/2002
LAO 21/2003	PAK 11/2003†	ROM 1/72		ZAM 28/2004	SAU 4/2000		HKN 5/2003
LAO 25/2003	PAK 11/2003†	TUN 3/67		ZIM 3/85	SAU 13/2000		IRN 13/2000
LAO 27/2003†	PAK 12/2003	UKG 15/72		ZIM 25/89	UGA 20/98		IRN 54/2001
LAO 27/2003†	SYR 5/2002	UKG 70/74			ZIM 19/89		IRN 3/2002
LAO 30/2003	TAI 2/99[2]	USSR 4/72			ZIM 11/91		IRN 4/2002
LAO 32/2003	TAI 4/2003				ZIM 2/97		IRN 6/2005
LEB 3/2002	TAI 9/2003						IRN 11/2005
LEB 1/2003							IRN 15/2005
MAU 19/2000							IRQ 39/2002
MAY 4/2002							IRQ 44/2002
MAY 5/2002							IRQ 45/2002
MAY 2/2003							IRQ 46/2002
MYA 1/2002							IRQ 57/2002
NEP 12/2000							IRQ 58/2002
NGR 1/2001							IRQ 112/2002
PAK 7/2003							PAK 23/2003
PAK 13/2003[1]							PAK 38/2003
PAT 3/2002[1]							PHI 16/2003
PHI 7/2000							SEN 12/2002
PHI 17/2003							SYR 7/2002
PHI 20/2003							TUR 17/2002
PHI 1/2005							UKG 553/01
PHI 2/2005							UKG 642/01
PHI 3/2005							UKG 2243/01
SRL 4/99							UKG 2653/01
SYR 1/2002							UKG 4059/01
TAW 1/99[1]							UKG12632/01
TUR 8/69							UKG13314/01
UAE 496-01							UKG14035/01
UAE 2/2000							UKG14854/01
UAE 1/2003							UKG15006/01
UAE 2/2003							UKG 40/2002
UKG 6922/01							
UKG11509/01							
UKG13305/01							
UKG13309/01							
UKG13715/01							

Table 1 (*Continued*)

O	A	C	Asia 1	SAT 1	SAT 2	SAT 3	NVD
URU 1/2000							
VIT 19/99							
VIT 17/2002							
VIT 18/2002							

All samples (assigned three letter country-codes and year of submission) were clinical material from suspect cases of FMD except four samples denoted by superscript '1' which were derived from cell culture. All clinical samples were vesicular epithelium except 2 samples[2] which were vesicular fluid and a single sample of milk[3]. Two samples (†) comprised a mixture of serotypes O and A. Three samples (underlined) were repeated in the panel. Samples were serotyped using ELISA: NVD (no virus detected by VI and ELISA).

2.5. Real-time RT-PCR amplification

Two-step real-time RT-PCR set up of samples was performed by manual pipetting of reagents as previously described (King et al., 2006). Briefly, cDNA was prepared by adding 6 μl nucleic acid to 9 μl of RT reaction mix. PCR amplification of 7 μl of cDNA in a final reaction volume of 25 μl was performed in an Mx4000 Quantitative PCR System (Stratagene) using primers and a probe that target the conserved 5′ untranslated region (5′UTR) of the FMDV genome as previously described (Reid et al., 2003).

2.6. Detection of NASBA amplification products by the ECL method

Detection of RNA amplicons generated by NASBA reaction was performed using the electrochemiluminescence method as described previously (Collins et al., 2002b). The sequence of the biotinylated capture probe is listed in Table 2. Briefly, 5 μl of amplicon solution was added to 20 μl of 1:1 mixture of the ECL generic and capture probe solutions and incubated at 41 °C for 30 min. Assay buffer (phosphate buffer, pH 7.5, tripropylamine and Trixton-100) (BioMerieux, Boxtel, The Netherlands) (0.3 ml) was added to this mixture and the emitted light intensity at 620 nm was measured by an ECL detector (BioMerieux). The principle of this technique is illustrated in Fig. 1A.

2.7. Detection of NASBA amplification products by the EOC method

This enzyme-based colorimetric detection method is illustrated in Fig. 1B. The sequence of the biotin-labelled capture probe (Invitrogen) was identical to the detection probe used for the ECL assay (Collins et al., 2002b) while the EOC detection probe [labelled with digoxygenin (DIG), Invitrogen] had a sequence identical to the ECL capture probe (Table 2). The biotin-labelled capture probe was linked to a streptavidin-coated microtitre plate during hybridization. For each sample, the probe solution (2 μl, comprising a 1:1 mixture of 26 μM biotinylated capture probe and 26 μM DIG-labelled detection probe) was added to 5 μl of the NASBA amplicon and 93 μl hybridization buffer [20× SSPE, pH 7.4; 50 mM Tris–HCl, pH 8.8; 1% (w/v) BSA], mixed thoroughly and the mixture aliquoted into a 96-well streptavidin-coated microtitre

Table 2
Nucleotide sequence and genomic location of the oligonucleotides used in the NASBA amplification reaction

Name	Sequence (5′–3′)	GenBank accession no.	Nucleotide coordinates	Genome location
U1 (primer)	AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GCT TCT CAG ATC CCG AGT GTC	NC_002527	551–570	5′UTR (IRES)
U2 (primer)	GAT GCA AGG TCG CAT ATG AGT GTT TCG TAG CGG AGC ATG	NC_002527	341–359	5′UTR (IRES)
CP-U (capture probe for ECL method)	Biotin-GAT GCC CTT CAG GTA CCC CGA GGT A	NC_002527	520–544	5′UTR (IRES)
DP-U (detection probe for EOC method)	Digoxygenin-GAT GCC CTT CAG GTA CCC CGA GGT A	NC_002527	520–544	5′UTR (IRES)

UTR, untranslated region; IRES, internal ribosome entry site.

plate (Nalge Nunc International Corp., Naperville, IL, USA) and incubated at 41 °C for 1 h. The supernatant was discarded and wells washed three times with 0.2 ml 1× TBS. Detection solution (0.1 ml, 1:500 monoclonal α-DIG-alkaline phosphatase conjugate, Sigma Chemical Co., St. Louis, MO, USA) was added and incubated at room temperature for 30 min. The supernatant was discarded and the wells were washed three times with 0.2 ml 1× TBS. Substrate solution (0.1 ml, *p*-nitrophenyl phosphate liquid substrate system, Sigma Chemical Co.) was added to each well and the plate was incubated in the dark for 5 min at room temperature. The reaction was terminated by the addition of 0.1 ml 3 M NaOH. The absorbance at 405 nm (with a 630 nm reference filter) was measured in an ultraviolet/visible microtitre plate spectrophotometer (Titertek Multiskan® PLUS MKII).

2.8. Calculation of cut-off values (COV)

Signals higher than the cut-off values were considered "positive" whereas those below the COV were considered "negative". COV for NASBA-ECL and NASBA-EOC were previously defined as 3000 and 0.25, respectively, by testing the systems against a number of known negative samples (data not shown). Alternatively, for the ECL method, the cut-off value can be defined by using the reading obtained for the Instrument Reference Solution by the ECL reader as described previously (Collins et al., 2002b). The COV for real-time RT-PCR corresponded to a C_t value (the cycle number at which the amplification curve crosses a set threshold line) of 40 (Reid et al., 2003).

3. Results

RNA extracted from all 200 suspected FMD samples was tested blindly by NASBA and real-time PCR.

3.1. Comparison of NASBA-ECL with real-time RT-PCR

Fig. 2 shows a comparison between the results generated by NASBA-ECL and real-time RT-PCR. These data [scored for a diagnostic result of positive or in cases where no virus was detected (NVD)] is summarized in Table 3. There was broad agreement between NASBA-ECL and real-time RT-PCR for 174 (87.0%) of the samples. One hundred and thirty eight of these samples were positive on both assays, while FMDV was not detected by either assay in 36 samples. Results for 26 samples were not in agreement: 12 were NASBA-ECL positive and real-time RT-PCR negative, while 14 were real-time RT-PCR positive and NASBA-ECL negative.

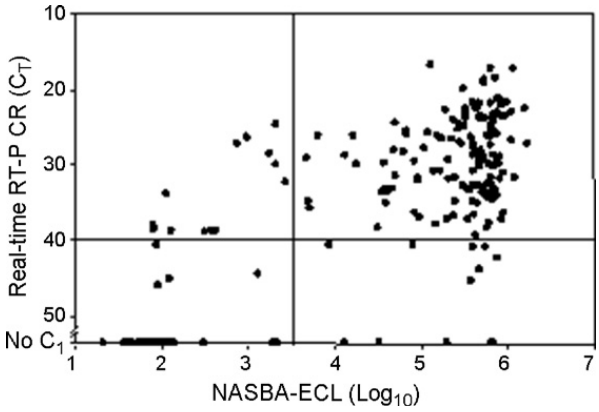


Fig. 2. Plot of real-time RT-PCR results vs. NASBA-ECL results log_{10}. The four quadrants indicate the regions of within and outside the cut-off values. Compatibility is seen in the top right (positive) and bottom left (negative).

3.2. Comparison of NASBA-ECL with virus isolation

VI identified FMDV in 140 cases, NASBA-ECL in 150 cases and real-time RT-PCR in 152 cases, respectively (Tables 4a and 4b). Of the 194 samples where VI data was available, 169 (87.1%) had compatible results (either positive or negative) with NASBA-ECL. Results for 25 samples (12.9%) were not in agreement. Of these, 15 samples were negative by VI but positive by NASBA-ECL. Interestingly,

Table 3
Summary of the results generated by the NASBA-ECL and real-time RT-PCR on the samples from suspect cases of FMD

Real-time RT-PCR	NASBA-ECL	
	Positive (150)	NVD (50)
Positive (152)	138	14
NVD (48)	12	36

NVD, no virus detected.

Table 4a
Summary of the results generated by the NASBA-ECL in retrospective comparison with those samples ($n = 194$) studied by virus isolation

Virus isolation	NASBA-ECL	
	Positive (145)	NVD (49)
Positive (140)	130	10
NVD (54)	15[a]	39

NVD, no virus detected.
[a] 13/15 of these samples were also positive by real-time RT-PCR.

FMDV genome was also detected in 13/15 of these samples by real-time RT-PCR. Conversely, 10 samples positive by VI were negative by NASBA (with ECL values lower than 3000) (Table 4a). Of these, eight (four each of serotypes O and A) were positive by real-time RT-PCR suggestive of false negative results by NASBA, while two (a serotype C isolate and a milk sample containing SAT 2 virus) were also negative by real-time RT-PCR.

Comparing real-time RT-PCR with VI, 164 (84.5%) of the results were in agreement (Table 4b). Of the 30 anomalous results, 18 (9.3%) were positive by RT-PCR where VI was negative. Fourteen of these were also positive by NASBA-ECL suggesting these samples failed to be amplified in cell culture. The remaining four samples were positive by real-time RT-PCR but negative by VI and NASBA. Of the 12 negative real-time RT-PCR results for samples positively identified by VI, eight were in the borderline cut-off range (C_t values between 40.0 and 50.0) in which samples should be retested to confirm results. Only two of these 12 samples were negative by NASBA-ECL.

There were only two cases in which neither molecular method detected the virus when VI was positive.

Table 4b
Summary of the results generated by the real-time RT-PCR in retrospective comparison with those samples ($n = 194$) studied by virus isolation

Virus isolation	Real-time RT-PCR	
	Positive (146)	NVD (48)
Positive (140)	128	12
NVD (54)	18	36

NVD, no virus detected.

3.3. Analytical specificity

The results achieved by NASBA-ECL and NASBA-EOC (where available) on samples containing other vesicular disease viruses (SVDV, VSV and VESV) and genetic relatives to FMDV are shown in Table 5 and demonstrate that both detection methods have high specificity for FMDV.

3.4. Correlation of NASBA-ECL with NASBA-EOC

The results achieved by NASBA-ECL and NASBA-EOC were in agreement for 97.1% of samples tested (Fig. 3). For 6 of the 200 cases NASBA-ECL and NASBA-EOC gave inconsistent results. Four were close to the positive borderline values for low ECL. These four had all been detected as positive by VI and NASBA-ECL, indicating a lower sensitivity of NASBA-EOC at low positive values. Neither of the remaining samples, which also had low NASBA-ECL values close to the borderline cut-off range, had been detected by real-time RT-PCR. One was also negative by VI. The complete absence of false positives demonstrates high specificity of the primers for FMDV.

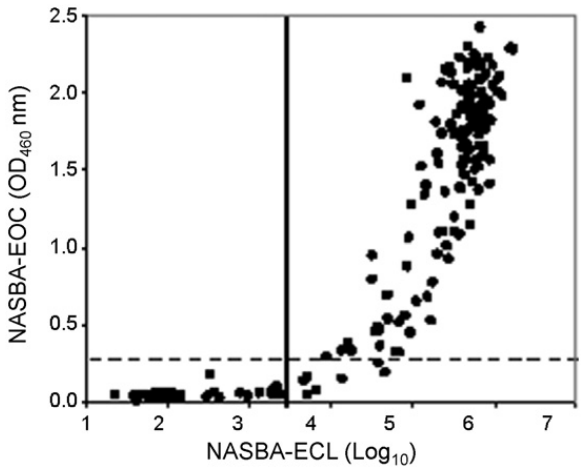


Fig. 3. Plot demonstrating primer specificity for NASBA, comparing the distribution of results from NASBA-EOC compared with that from NASBA-ECL (given as $\log_{10}$) from results given in Table 4a. The four quadrants indicate the regions of within and outside the cut-off values. No false positives were detected. This logarithmic plot clearly indicates the high degree of correlation between the two methods and the high specificity of the primers for FMDV, there being no false positive results for either NASBA-ECL or NASBA-EOC.

Table 5
Specificity of reaction of NASBA on samples of other vesicular disease viruses and on genetic relatives of FMDV

Virus	Isolate	NASBA-ECL	NASBA-EOC (405 nm)
SVDV[1]	HKN 1/80	78	0.060
	HKN 1/82	37	0.040
	FRA 1/73	98	0.030
	HKN 1/80	97	0.026
	SWI 1/74	26	0.050
	AUT 1/73	61	0.050
	AUT 1/73	121	0.026
	HKN 1/76	117	0.060
	FRA 1/73	94	0.044
	SWI 1/74	55	0.027
	POR 2/2004	110	0.038
	HKN 1/82	106	0.041
VSV[2] New Jersey	RV31 15/88 CP211634	124	0.033
	VSV-NJ COL 1/93	120	0.029
	VSV-NJ El Salvador	142	0.028
	VSV-NJ Colorado-2	119	0.034
	VSV-NJ Nicaragua 71	2098	0.055
	VSV-NJ Costa Rica 66	117	0.039
VSV Indiana	VSV Ind 16677 Columbia	212	0.044
	SV Ind 15156 Columbia	81	0.033
	COL 4/93	92	0.034
	El Salvador 71	118	0.001
	Costa Rica 79	150	0.043
	El Salvador 78	142	0.002
	Maraba Brazil 83	109	0.018
	Maipu Argentina	102	0.044
	VSV Ind-2	101	0.034
	Rancharia Brazil 66	98	0.026
	Agulhas Negras B	172	0.016
	Agulhas Negras B	114	0.072
SMSV[3]-1	SMSV-1	81	0.035
SMSV-6	SMSV-6	85	0.049
SMSV-7	SMSV-7	53	0.033
SMSV-9	SMSV-9	84	0.044
SMSV-11	SMSV-11	99	0.046
PPRV[4]	IRQ 57/2000	78	0.030
VESV[5]-J56	VESV-J56	90	0.084
Primate CV[6]	Gorilla CV SDZ 022	110	0.067
Reptile CV	Rattlesnake CV 780010	97	0.043
Bovine CV	Bovine Tillamook CV	79	0.034
Skunk CV	SKUNK CV	127	0.005
ERAV[7]	ERAV	136	0.029
BRV-3[8]	BRV-3	78	0.028

Viruses tested were swine vesicular disease virus[1], vesicular stomatitis virus[2], San Miguel sea lion virus[3], Peste-des-Petits Ruminants virus[4], vesicular exanthema of swine virus[5], calicivirus[6], equine rhinitis A virus[7], bovine rhinovirus[8].

4. Discussion

The high suitability and sensitivity of NASBA for the detection of FMDV was demonstrated in this study. A newly developed NASBA-EOC was also evaluated in conjunction with the traditional NASBA-ECL and showed a similar performance to it. The sensitivity of NASBA for the detection of FMDV was

assessed by testing samples submitted from suspect cases of FMD. RNA isolated from these samples was tested in parallel by real-time RT-PCR and the results were also compared retrospectively with the original VI data that had been obtained for these samples. These results indicated that NASBA has equivalent sensitivity to real-time RT-PCR. Only 10/140 cases were positively identified by VI and not by NASBA (8 of these were also scored positive by real-time RT-PCR) while 12/140 of these VI positive cases failed to be detected by real-time RT-PCR. In contrast, NASBA was able to detect FMDV in 15 additional samples that were designated NVD by VI. Of these, 13 were also positive by real-time RT-PCR suggesting that these results reflect an increased sensitivity of molecular assays for FMDV detection and are not false positive results. A higher sensitivity of real-time RT-PCR compared with VI has been demonstrated in previous studies (Shaw et al., 2004).

Failure of both NASBA and real-time RT-PCR to detect individual isolates may be caused by nucleotide mismatches in the primer and/or probe regions. The real-time RT-PCR assay used in this study targets the 5′UTR of the FMDV genome. The findings from this study were in broad agreement with previous work evaluating the diagnostic sensitivity of this assay (King et al., 2006). This assay has been shown to perform less well against FMD viruses of the three SAT serotypes, which may explain the negative real-time RT-PCR results obtained for eight SAT virus samples in this study. Similar heterogenicity in the regions targeted by the NASBA assay may explain the results for the 14 samples that were positive by real-time RT-PCR but negative by NASBA.

The high specificity of the NASBA assay was confirmed by testing RNA from other vesicular viruses (SVDV, VSV and VESV) and genetic relatives of FMDV. Suspensions of negative epithelia tested by NASBA also yielded negative results (data not shown).

In this study, the COV for the NASBA-ECL and NASBA-EOC assays were 3000 and 0.25, respectively. These COV were determined previously by testing a number of known negative samples. The mean value of all signals generated ($\bar{X}$) was calculated plus a multiple of standard deviations (nS.D., where n is an integer) and a constant (K), i.e., $\mathrm{COV} = \bar{X} + n \times \mathrm{S.D.} + K$. The K value was used to increase the COV beyond the highest value that was observed from testing known negative samples (this value may change due to different sample types, laboratory conditions, etc.). These COVs achieved high sensitivity and specificity in both detection systems in comparison with VI and real-time PCR. For routine use, individual laboratories can establish their own COV to achieve best performance of the assay.

NASBA and real-time RT-PCR are both effective molecular methods for the detection of FMDV. Both methods can reduce diagnosis time, providing results faster than VI. The total assay times are similar for NASBA-ECL and real-time RT-PCR but NASBA-EOC takes an extra 2 h to complete. However, this relatively minor increase in running-time could be offset by the reduced cost of NASBA-EOC which does not require expensive and specialized equipment such as an ECL detector or real-time PCR machine. The ease of use and low cost of NASBA-EOC offers hope to regions where diagnostic laboratories are not well equipped and with staff of minimal training. The NASBA-EOC technique is generally a more affordable option in that it can be performed simply using a temperature-controlled water bath and a 96-well microplate spectrophotometer. Alternative isothermal formats such as RT-LAMP (Dukes et al., 2006) that may provide rapid and simple diagnosis of FMD are also being explored. Therefore, the early and rapid detection of FMDV in developing, as well as developed nations is now a reality and these methods can be used to minimize the loss of livestock that is devastating to an economy and thus far, particularly unavoidable in the developing world.

Acknowledgements

We thank Sarwat Fatima from Hai Kang Life Corporation Limited, for her assistance in preparation of this article and Nick Knowles (IAH Pirbright) for providing some of the viruses tested in this study. This work was supported in part by Defra project SE1121.

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