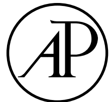
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A method to detect major serotypes of foot-and-mouth disease virus

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Abstract

Nucleic acid sequence-based amplification (NASBA) is an isothermal technique that allows the rapid amplification of specific regions of nucleic acid obtained from a diverse range of sources. It is especially suitable for amplifying RNA sequences. A rapid and specific NASBA technique was developed, allowing the detection of foot-and-mouth disease virus genetic material in a range of sample material, including preserved skin biopsy material from infected animals, vaccines prepared from denatured cell-free material, and cell-free antigen-based detection kits. A single pair of DNA oligonucleotide primers was able to amplify examples of all major FMD virus subtypes. The amplified viral RNA was detected by electrochemiluminescence. The method was at least as sensitive as existing cell-free antigen detection methods. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Nucleic acid sequence-based amplification; Foot-and-mouth disease; Electrochemiluminescence; Internal ribosome entry site

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed ruminants, including cattle, pigs, sheep, goat, and antelope among others. The causative agent, foot-and-mouth disease virus (FMDV), is a member of the *Picornaviridae* family and exists as seven antigenically and genetically distinct serotypes, i.e., A, C, O, Asia-1, SAT-1, SAT-2, and SAT-3. The virus genome consists of a single-stranded positive-sense RNA molecule of approximately 8.4 kb [1]. Establishment of a persistent yet asymptomatic infection (carrier status without clinical signs) in unvaccinated as well as vaccinated animals is well documented in ruminants, especially cattle [2,3]. Animals that recover from the disease can remain carriers for several months or years after infection [3–5], and may intermittently shed the virus and cause FMD outbreaks [6]. One of the major problems in controlling outbreaks of FMD is the difficulty of rapidly analysing livestock for the presence of the virus so that appropriate control measures can be initiated. Nucleic acid testing is a technique that is able to provide such rapid diagnosis. Nucleic acid testing for FMDV has principally been confined to research uses involving a variety of PCR and RT-PCR techniques [7–12]. PCR methods are often slow and cumbersome to perform due to the need for gel electrophoresis analysis or other manipulations of the amplified product prior to detection. Quantitative TaqMan PCR methods have been developed but are not practical in monitoring large outbreaks rapidly [13].

Nucleic acid sequence-based amplification (NASBA) is a continuous, isothermal, enzyme-based method for the amplification of nucleic acid and offers an attractive alternative to PCR-based methods [14]. The technique employs a mixture of reverse transcriptase, ribonuclease-H, RNA polymerase, and two specially designed DNA oligonucleotide primers. The forward primer has a 5′ extension containing the promoter sequence for bacteriophage T7 DNA-dependent RNA polymerase. The reverse primer has a 5′ extension containing a complementary binding sequence for a DNA oligonucleotide detection probe labelled with a ruthenium-based electrochemiluminescent (ECL) tag. During the amplification process, the 5′ primer extensions are fully incorporated into the amplified sequence, allowing both highly efficient production of complementary RNA template (directed by the RNA polymerase) and specific detection by the ECL-tagged probe during the detection stage. The technique is particularly suited for the

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amplification of single-stranded RNA and has been successfully used in the detection of numerous different RNA and DNA viruses, bacteria, fungi, parasites and cytokines. For example, NASBA protocols have been described for avian influenza virus [15], human, simian, and feline immunodeficiency viruses [14,16,17], dengue virus [18], cytomegalovirus [19], hepatitis C virus [20], Epstein–Barr virus [21], human rotavirus [22], *Escherichia coli* [23], *Salmonella enterica* [24], *Chlamydia trachomatis* [25], *Candida* spp. [26], *Aspergillus* spp. [27], *Plasmodium falciparum* [28], macrophage-derived chemokine mRNA [29], tissue factor mRNA [30], and human TNF-α mRNA [31], among many others. Other advantages of NASBA include the fact that amplification levels can reach 10^9–10^{12} after 2 h. In addition, the amplification process is isothermal (41 °C) and does not require a thermal cycler or other specialised amplification equipment. DNA contaminants do not cause artefacts, as there is no thermal denaturation step to allow primer annealing. The error rate during NASBA amplification is lower than *Taq* polymerase, improving the fidelity of transcripts, a factor that may be important in the phylogenetic analysis of isolates [32].

It is the purpose of this study to demonstrate the utility of the NASBA detection system in amplifying and detecting nucleic acid derived from FMDV isolates of the seven major subtypes. This preliminary study will act as the foundation for further studies into the potential application of this technique for rapidly monitoring FMD outbreaks around the world. This is the first description of a convenient, rapid, accurate and sensitive NASBA-based assay able to detect all major subtypes of FMDV using a single pair of primers.

Materials and methods

Viruses and control antigens. Dr. Wilna Vosloo, ARC-Onderstepoort Veterinary Institute, South Africa, provided freeze-dried RNA from FMDV SAT-1 (KNP 196/91/1), SAT-2 (KNP 19/89/2), and SAT-3 (KNP 10/90/3). Dr. Yehuda Stram, Kimron Veterinary Institute, Israel, provided RNA from FMDV-O1(G) Geshune/Israel. A FMDV/SVDV antigen detection ELISA kit containing denatured cell-free extracts of subtypes A (isolates A5, A22, and A24 combined), C (C3 Resende), O (O1 Manisa), SAT-1 (BOT 1/68), SAT-2 (ZIM 5/81), SAT-3 (ZIM 4/81), Asia-1 (CAM 9/80), and swine vesicular disease virus (UKG 27/72) was purchased from the Institute for Animal Health, Pirbright, UK. Newcastle disease virus RNA was provided by the Agriculture, Fisheries and Conservation Department of the Hong Kong SAR.

Sequence alignment and primer selection. Nucleotide sequences of the internal ribosome entry site (IRES) located in the 5′-untranslated region (5′-UTR) from about 50 FMDV isolates representing all major subtypes obtained from GenBank were aligned using the BioEdit software program [33]. Conserved regions were used to design NASBA amplification primers and the NASBA capture probe. After the primer and probe sequences were selected, they were compared with sequences submitted to the GenBank nucleotide database using a standard nucleotide–nucleotide comparison tool (BLASTN version 2.2.1; [34]) using default search parameters to search for potentially cross-reacting sequences. In addition, primer and probe sequences were aligned with specific sequences using a pairwise comparison tool (BLAST 2 sequences; [35]) using default comparison parameters.

Nucleic acid isolation. Briefly, 1 vol of sample (resuspended swab) was added to nine volumes of lysis buffer. The sample was mixed gently by vortex mixing. This inactivated infectious virus and stabilised the nucleic acids by denaturing nucleases. Acid-treated silica (50 μl, 1 mg/ml) was added to the lysate. The sample was kept at room temperature for 10 min and vortexed vigorously every 2 min. The liberated influenza virus RNA segments bound to the silica and collected in the solid phase. The silica and nucleic acid complex was pelleted by centrifugation for 30 s at 10,000*g* and washed repeatedly (twice with 5.25 M guanidine isothiocyanate, 50 mM Tris, pH 6.4, 20 mM EDTA; twice with 70% ethanol and once with acetone). The acetone was evaporated from the silica pellet by warming the sample in a 56 °C water bath for 10 min. DEPC-treated water (50 μl) was added to the dry pellet and incubated in a 56 °C water bath for 10 min. The tube was centrifuged for 1 min at 10,000*g* to separate the silica from the water containing the eluted nucleic acid.

NASBA primers. The forward and reverse primers used for amplifying all major subtypes were designated "universal" U1 and U2, respectively (Table 1). Primers were obtained from Gibco-BRL, Life Technologies (Tseun Wan, Hong Kong). The forward primer was based on the IRES sequence of FMDV-O/Taiwan/97 (Chu Pei) (GenBank Accession No.: NC_002527.1, nucleotides 550–571) and contained a 5′ extension of 25 nucleotides complementary to the bacteriophage T7 DNA-dependent RNA polymerase promoter linked to the FMD sequence by a six nucleotide purine bridge to enhance polymerase extension of GC-rich sequences. The reverse primer was derived from the same source (nucleotides 341–359) and contained a 5′ extension of 20 nucleotides complementary to a ruthenium-labelled oligonucleotide detection probe.

Amplification by NASBA. To 5 μl nucleic acid extract, 10 μl mixture containing 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% DMSO, and 0.4 μM each primer was added. This mixture was heated to 65 °C for 5 min in a water bath and then cooled to 41 °C for 5 min. Once cooled, 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 and the reaction was incubated at 41 °C for 90 min in a water bath. The final volume was 20 μl.

Capture probe. The amplified products of the NASBA reaction (amplicons) were immobilised by binding to paramagnetic silica beads to which had been attached a capture probe specific for the universal amplicons. The sequence of the capture probe was determined after selection of the amplification primers by aligning the nucleotide sequences of the putative amplification products. The universal capture probe, CP-U, was derived from FMDV-O/Taiwan/97 (Chu Pei), nucleotides 520–544 and was labelled at the 5′ end with biotin (Table 1). The capture probe was obtained from Gibco-BRL.

Detection of NASBA amplification products. The NASBA reaction amplicons were detected by hybridisation analysis using an electrochemiluminescence (ECL)-based detection system following manufacturer's instructions. Briefly, the amplicons were immobilised by hybridisation to a capture probe that had been attached to paramagnetic silica beads through a streptavidin:biotin interaction. The amplicon solution (5 μl) was added to 20 μl of a 1:1 mixture of the ECL generic probe solution and capture probe solution and incubated at 41 °C for 30 min. Assay buffer (0.3 ml) was added to this mixture and the emitted light intensity at 620 nm was measured by the detector (NucliSens ECL Reader, Organon Teknika, Boxtel, The Netherlands).

Reverse transcription-polymerase chain reaction. RT-PCR was used to generate a DNA copy of the RNA amplicons to facilitate sequencing. The reaction mixture contained 5 μM random hexamer (Gibco-BRL) and 1 μg RNA from the nucleic acid extraction procedure in RNase-free water. The mixture was incubated at 70 °C for 10 min. Then, 1 U/μl RNasin inhibitor (Promega, WI, USA), 1× first-strand buffer (Gibco-BRL), 10 mM DTT (Gibco-BRL), and 1 mM dNTPs (Pharmacia Biotech, Sweden) were added and the mixture was kept at room temperature for 10 min, followed by incubation at 42 °C

Table 1
Primers and probes used in this study

Name	Sequence (5′–3′)	GenBank Accession No.	Nucleotide coordinates	Genome location	GC content (%)	Product size
U1	**AAT TCT AAT ACG ACT CAC TAT AGG G**AG AAG GCT TCT CAG ATC CCG AGT GTC	NC_002527	551–570	5′-UTR (IRES)	23/51 (45%)	279 (U1+U2)
U2	GAT GCA AGG TCG CAT ATG AGT GTT TCG TAG CGG AGC ATG	NC_002527	341–359	5′-UTR (IRES)	20/39 (51%)	
CP-U	Biotin-GAT GCC CTT CAG GTA CCC CGA GGT A	NC_002527	520–544	5′-UTR (IRES)	15/25 (60%)	
SP-FOR	CTT CTC AGA TCC CGA GTG TC	NC_002527	551–570	5′-UTR (IRES)	11/20 (55%)	
SP-REV	TGT TTC GTA GCG GAG CAT G	NC_002527	341–359	5′-UTR (IRES)	10/19 (53%)	

for 2 min. Finally, 10 U/μl SUPERSCRIPT II RNase H-Reverse Transcriptase (Gibco-BRL) was added into the reaction mixture and incubated at 42 °C for 50 min, followed by 70 °C for 15 min to stop the reaction. After the RT reaction, 0.15 U/μl ribonuclease-H (Gibco-BRL) was added to the reaction mixture and incubated for 20 min at 37 °C to degrade the RNA in the DNA–RNA hybrid.

PCR was performed in a thermocycler (Robocycler, Stratagene, Amsterdam, Holland). The reaction mixture contained 1× PCR buffer (Gibco-BRL), 1.5 mM $MgCl_2$, 200 μM dNTPs (Pharmacia Biotech, Sweden), forward and reverse sequencing primers (SP-FOR and SP-REV, Table 1), and 0.04 U/μl *Taq* DNA polymerase (Gibco-BRL). The PCR cycle began with 2 min at 94 °C, followed by 40 amplification cycles: 94 °C for 1 min, 55 °C for 40 s, and 72 °C for 1 min. Further extension at 72 °C for an additional 10 min completed the reaction. PCR products were analysed by electrophoresis on 2% agarose gels. The PCR products were subsequently sequenced to confirm their identity (data not shown).

DNA sequencing and analysis. The products of the amplification reaction were sequenced to ensure that the amplicons corresponded to the intended target sequence (data not shown). DNA sequencing was performed with an AutoRead 100 Sequencing Kit (Pharmacia Biotech, Sweden) using an automated fluorescent sequencer (Pharmacia LKB. A.L.F. DNA Sequencer, Pharmacia Biotech, Sweden). In brief, 8 μg template DNA was denatured with 400 mM NaOH and incubated at room temperature for 10 min. The DNA was precipitated with 100% ethanol and 3 M potassium acetate (pH 4.8) and then washed with 70% ethanol. The DNA pellet was dried and resuspended in distilled water (10 μl). Annealing primers were added to the DNA template to 0.15 nM. The mixture was heated at 65 °C for 5 min and immediately incubated at 37 °C for another 10 min. They were incubated at room temperature for at least 10 min following which extension buffer, DMSO and T7 DNA polymerase were added. The reaction mixture was divided equally into four different tubes containing adenine-, cytosine-, guanine-, and thymine-dideoxynucleotides, respectively. The reaction mixture was further incubated at 37 °C for 5 min, followed by the addition of stop solution. They were heated at 90 °C for 3 min before being loaded into a sequencing gel. DNA sequences were analysed by sequence similarity searches with EMBL and GenBank DNA databases.

Results

Use of electrochemiluminescence

The ECL signal obtained during the experiments was normalised relative to an internal reference standard (IRS) solution supplied by the manufacturer of the ECL reader. The IRS solution produces an ECL signal of about 30–40,000 arbitrary luminescence units. While ECL emission is proportional to the amount of amplified nucleic acid products, in the absence of standardisation with appropriate internal amplification controls, no information on the initial amount of infectious agent, target RNA or degree of amplification can be obtained. The cut-off value for differentiating positive and negative samples is critical to the appropriate interpretation of data. In this study, cut-off values are defined as 0.025 × IRS. Other users of NASBA systems have reported similar methods for calculating the cut-off value, including 0.01–0.025 × IRS [25,36,37] and 200 ECL units [38]. The use of appropriate internal controls for quantifying HIV viral load using the NASBA technique has been described [14] and the technique is applicable to other targets. Analysing the same sample multiple times assessed reproducibility. Replicate analyses of the same sample conducted over a period of several months by different operators produced the same qualitative result as the original determination (data not shown).

Sequence alignment and primer specificity

The primers and capture probe used in this assay system were designed after a comprehensive analysis of sequence data submitted to publicly available sequence databases. The universal primers were capable of amplifying examples of all seven subtypes of FMDV provided as positive controls in a commercially available ELISA-based detection kit in addition to FMDV samples isolated from the field (Table 2). There was no cross-reactivity of the primer pair or capture probe with other single-stranded RNA viruses (e.g., swine vesicular disease virus, avian influenza virus, and Newcastle disease virus), double-stranded DNA viruses (e.g., hepatitis B virus), or endogenous porcine, bovine, ovine or avian DNA. Comparison of the primer and capture probe

Table 2
Specificity of U1, U2, and CP-U in amplifying and detecting various samples

Sample	ECL signal	Sensitivity (ECL/cut-off limit)
FMDV-A5/A22/A24 combined	707,564	512
FMDV-C3 Resende	172,925	125
FMDV-O1 Manisa	194,805	141
FMDV-SAT-1 BOT 1/68	297,440	215
FMDV-SAT-2 ZIM 5/81	488,586	354
FMDV-SAT-3 ZIM 4/81	815,723	591
FMDV-Asia-1 CAM 9/80	277,513	201
FMDV-SAT-1 KNP 196/91/1	562,580	407
FMDV-SAT-2 KNP 19/89/2	602,159	436
FNDV-SAT-3 KNP 10/90/3	242,541	183
FMDV-O (Hong Kong 2/2002)	14,743	10.8
FMDV-O1/G/Geshune/Israel	103,695	75.9
SVDV UKG 27/72	458	0.332
Influenza A/chicken/Hong Kong/1725-1/97 (H5N1)	332	0.240
Newcastle disease virus	404	0.293
Hepatitis B virus DNA	225	0.163
Porcine muscle (fresh) DNA	890	0.644
Bovine muscle (fresh) DNA	105	0.099
Ovine muscle (fresh) DNA	108	0.102
Chicken muscle (fresh) DNA	371	0.269
Instrument reference standard (IRS)	55,255	NA
Cut-off value (0.025× IRS)	1381	NA
Negative control	129 ± 19	NA

All reactions performed with 90 mM KCl. NA, not applicable.

sequences with the complete genome sequences of important veterinary pathogens such as swine vesicular disease virus, vesicular stomatitis virus, vesicular exanthema virus, and distantly related single-stranded RNA viruses, such as enterovirus and coxsackie virus using sequence comparison software (BLAST 2 sequences) revealed no significant sequence similarity (data not shown).

Sequencing

The amplified target RNA molecules were sequenced to confirm their identity. No differences between the expected sequence and that obtained were observed (data not shown).

Effect of KCl

The efficiency of amplification was examined at various potassium chloride (KCl) concentrations using the universal primers and RNA was prepared from FMDV-A obtained from a commercially available cell-free antigen ELISA kit. The results are shown in Table 3. As the amplified RNA is extracted from the amplification medium prior to detection, there is no carry-over of KCl into the detection stage. Thus, the ECL signal generated reflects the effect of KCl on nucleic acid amplification and not on other components of the detection process. Within the range 70 ± 20 mM KCl, the ECL signal did not vary by more than $\pm 12\%$. Increasing the KCl concentration above 90 mM profoundly affected amplification efficiency and increased the sensitivity of detection, measured as the ratio of the ECL signal to the cut-off limit, to over 300 compared with 200 at 70 mM (Table 3). Maximum amplification occurred at a KCl concentration of 110 mM, where the ECL signal increased by more than 50% compared with that observed at 70 mM (Table 3). This demonstrates the robustness of the amplification reaction. During these studies, experiments were performed between 70 and 90 mM KCl to reduce the possible deleterious effects of excessive salt on other components of the reaction.

Limit of detection

The limit of detection of the NASBA/ECL method was examined by determining the ability of the universal primers to amplify a serially diluted RNA template derived from all major FMDV subtypes obtained from a commercially available cell-free antigen ELISA kit. The amount of nucleic acid in the initial RNA preparation could not be correlated with the virus concentration used to generate the cell-free antigen preparation. Thus, the results of the serial dilution assay can only be used to give a general estimation of the minimum number of virus particles necessary to yield a positive signal. The RNA template was serially diluted in nuclease-free water, prior to amplification. The ECL signal for each dilution of template is shown in Table 4. Dilution of the template by 10^3–10^4-fold yielded positive ECL signals for all seven major subtypes. Thus, the NASBA/ECL assay for FMDV is extremely sensitive and able to detect viral RNA from very small samples. The effect of varying KCl concentration on the efficiency of amplification of FMDV-O was also examined (Table 4). The benefits of higher KCl concentration were lost as the sample was diluted, as the sensitivity (ECL/cut-off limit) declined markedly compared with the absolute ECL signal. This emphasises the importance of optimising the assay for particular uses.

Discussion

FMDV poses a serious economic threat, especially to nations with high-density intensive livestock-rearing industries. Rapid, accurate and sensitive assays for the presence of FMDV are required if future outbreaks are to be contained [39]. Nucleic acid sequence-based amplification coupled with electrochemiluminescent detection (NASBA/ECL) is an appropriate technology on which to base a rapid assay. The technique has been demonstrated to provide the necessary speed, accuracy and sensitivity when applied to other single-stranded

Table 3
Effect of KCl on FMDV-A amplification efficiency

[KCl] mM	ECL signal (mean ± range, $n = 2$)	Amplification[a] (wrt 70 mM KCl, %)	Sensitivity (ECL/cut-off limit)
50	201,642 ± 19,511	87.9	176
60	201,787 ± 496	88.0	176
70	229,277 ± 61,974	100	200
80	216,827 ± 10,523	94.6	189
90	257,459 ± 15,571	112	225
100	314,867 ± 74,293	137	275
110	350,827 ± 39,293	153	306
Negative control (70 mM KCl)	121 ± 26	NA	NA
Instrument reference standard	45,786	NA	NA
Cut-off limit (0.025× IRS)	1145	NA	NA

NA, not applicable.
[a] FMDV-A RNA template was extracted from a commercially available cell-free antigen detection ELISA Kit (IAH, UK) and amplified with primers U1 and U2.

Table 4
Limit of detection and sensitivity of NASBA/ECL using universal primers and probes

Sample	ECL signal[a] (sensitivity[b])							
	Asia-1	C	SAT-1	SAT-2	SAT-3	A	O	O[c]
Undiluted	140,616 (120)	153,174 (131)	164,772 (137)	209,132 (174)	352,570 (262)	147,664 (130)	ND	ND
10^{-1} Dilution	175,257 (149)	222,669 (190)	37,103 (31)	223,013 (186)	62,721 (46.6)	182,813 (161)	246,175 (167)	251,740 (171)
10^{-2} Dilution	140,805 (120)	24,848 (21)	10,324 (8.61)	52,975 (44.2)	11,434 (8.49)	226,072 (199)	290,196 (197)	366,338 (248)
10^{-3} Dilution	103,805 (88.5)	19,898 (17)	3,473 (2.9)	76,580 (63.9)	2,147 (1.6)	183,570 (161)	15,654 (10.6)	2,767 (1.9)
10^{-4} Dilution	ND	ND	168 (0.14)	443 (0.37)	1989 (1.48)	ND	22,255 (15.1)	6,488 (4.4)
Negative control	220		177		226	221	275	226
IRS[d]	46,918		47,978		53,820	45,566	59,059	
Cut-off limit (0.025 × IRS)	1173		1199		1346	1139	1476	

ND, not done.
[a] FMDV RNA template was extracted from a commercially available cell-free antigen detection ELISA kit (IAH, UK) and amplified as described in Materials and methods in the presence of 70 mM KCl.
[b] Defined as ECL signal/cut-off limit.
[c] Amplified as described in Materials and methods in the presence of 90 mM KCl.
[d] Instrument reference solution.

RNA viruses of veterinary and economic importance, i.e., avian influenza virus subtype H5 [15].

The NASBA/ECL system consists of three elements: nucleic acid isolation, amplification, and detection. Nucleic acid can be isolated from a wide variety of sample matrices using a guanidinium thiocyanate/Triton X-100 lysis buffer and silica bead method [40]. Oropharyngeal swabs, blood, faeces, and skin lesions may all be used as a source of viral nucleic acid for the NASBA/ECL process. In addition, animal bedding, pen swabs, and other environmental samples may also be used as a source of viral material, rapid examination of which provides a convenient means of assessing the efficiency of decontamination procedures following an outbreak. Nucleic acid amplification involves alternate rounds of cDNA and RNA synthesis using avian myeloblastosis virus reverse transcriptase, ribonuclease-H, and bacteriophage T7 DNA-dependent RNA polymerase. The RNA polymerase generates 100–1000 copies of target sequence per round of amplification, each of which can be used as the substrate for the next round. As a result, the degree of amplification with NASBA is significantly higher than that obtained with traditional nucleic acid amplification techniques, such as the polymerase chain reaction (PCR). This adds greatly to the sensitivity of the detection method. The amplified target is immobilised by hybridisation to a biotin-labelled oligonucleotide capture probe attached to a streptavidin-

coated paramagnetic particle. Contaminants are removed by extensive washing, after which a ruthenium-labelled DNA oligonucleotide detection probe is added. The application of a current to the assay mixture induces the ruthenium label to emit light at 620 nm, which can be detected by a photo-multiplier tube incorporated into the ECL reader. The use of a labelled capture probe immediately presents the opportunity of using a standard 96-well microtitre ELISA plate as an alternative mechanism for isolating and detecting the amplified nucleic acid, although sensitivity compared with the ECL method may be reduced.

The universal primers were designed against the highly conserved IRES sequence located in the 5′-UTR of the FMDV genome. Using isolates of known provenance, the ability of the universal primers and capture probe to amplify and detect examples of all seven major subtypes was demonstrated (Table 2). From an examination of publicly available sequence databases, it is likely that a significant number of FMDV isolates can be amplified with this pair of primers. The NASBA technique can tolerate 1–2 mismatches, provided they are not located at the termini, thus, extending the coverage of subtypes detected.

FMDV is a member of the *Picornaviridae*, a family that contains other single-stranded RNA viruses of clinical, veterinary, and economic importance, such as swine vesicular disease virus (SVDV), coxsackie virus, enteroviruses, and poliovirus. FMD is clinically indistinguishable from infection with SVDV, vesicular stomatitis virus, and vesicular exanthema virus and it is important for control measures that these diseases can rapidly be differentiated. Due to phylogenetic similarity, it is possible that primers designed for FMDV may cross-react with SVDV. The antigen detection ELISA kit obtained from the institute for Animal Health (Pirbright, UK) contains the SVDV strain UKG/27/72 as a negative control. The primers U1 and U2 failed to amplify the SVDV sample, indicating that the NASBA/ECL system described can adequately discriminate between SVDV and FMDV. In addition, sequence comparison of the primers and capture probes with SVDV, vesicular stomatitis virus, and vesicular exanthema virus demonstrated the absence of significant sequence similarity. Comparison of primer and capture probe sequences with the genomic sequences of distantly related viruses, such as swine and bovine enteroviruses, coxsackie virus, and poliovirus, indicated no significant sequence similarity was present. Thus, it is unlikely that the primers described here will amplify nucleic acid from viruses other than FMDV.

The effect of potassium chloride (KCl) on the efficiency of nucleic acid amplification is well known [41]. Generally, primer pairs with long amplification products work more efficiently at low salt concentrations, whereas primer pairs with short amplification products work more efficiently at high salt concentrations, where longer products become harder to denature [41]. Standard PCR buffers contain about 50 mM KCl, but this can vary depending upon the application. The optimum KCl concentration may help overcome non-optimal parameters such as product size and GC content. The products of the NASBA reaction described in this work are about 300 nucleotides in length and so are expected to be amplified more efficiently at relatively high KCl concentrations. This was demonstrated for the universal primers, which produced their maximum amplification at 110 mM (Table 3). High concentrations of salt are not the only factor affecting amplification efficiency. Most data relating to the effects of salt concentration have been derived from standard PCR and RT-PCR applications. Few data are available for NASBA applications, which contain two additional enzymes. For this reason, it was decided to limit the KCl concentration used in these studies to 70–90 mM.

Limit of detection studies was performed using the universal primers and nucleic acid prepared from cell-free antigen of all major FMDV subtypes. The RNA template was serially diluted and amplified. Even after 10^3–10^4-fold dilution, a detectable ECL signal greater than the cut-off limit was obtained for all subtypes tested. The effect of varying the KCl concentration on the efficiency of the NASBA reaction was also recorded for FMDV-O. Using 90 mM KCl enhanced the ECL signal at low template dilutions. However, this advantage was lost when the template was diluted further (Table 4). Thus, it is important to optimise the NASBA/ECL reaction for the particular purpose required. The limit of detection using a standard antigen detection ELISA method is about 1/625 dilution of sample. The NASBA/ECL method is able to detect all serotypes at 10^3–10^4 dilutions, a 1.6–16-fold increase in sensitivity. The absolute ECL signal in terms of luminescence units and the sensitivity (ECL signal/cut-off limit) increased significantly for most of the serotypes when the sample was diluted 10-fold from the original undiluted RNA template preparation. The degree of signal enhancement due to limited dilution ranged from 6.6% (SAT-2) to 45.4% (C). This enhancement may be due to decreased inhibition of amplification caused by the presence of unidentified inhibitory substances in the RNA template preparation.

The rapid identification of FMD is a critical factor in ensuring that the disease does not spread [39]. Rapid implementation of quarantine and eradication measures is required following confirmation of FMD outbreaks. The presence of infected but asymptomatic carriers is a major problem in the control of FMD. Identification of viral genetic material in such animals offers a convenient and accurate method of confirming their disease status. Similarly, seroconversion of animals following infection may be incomplete or absent, further confusing attempts

to control the spread of disease. The mass culling of livestock that followed the 2001 FMD outbreak in the United Kingdom put a great strain on the ability of the authorities to dispose of carcasses efficiently. Contamination of air and watercourses was a major concern as a consequence of the disposal activities [42]. This was a result of the adoption of a stamping out policy that required all animals within a defined containment area to be culled, irrespective of their infection status. The sensitivity of the NASBA/ECL technique afforded by the extremely high degree of nucleic acid amplification allows multiple samples to be pooled to increase testing throughput. Mass screening could be used to identify uninfected animals so that these animals could be set aside and culling deferred until the appropriate disposal of confirmed positive cases. NASBA/ECL might even be used to spare expensive livestock from slaughter as the absence of the infectious agent can be demonstrated. In contrast, antibody-negative animals are not necessarily free of disease as the animals may be asymptomatic carriers or have been tested prior to seroconversion, a process that takes 1–4 weeks [43]. Antibody-based detection methods lack sensitivity and are relatively time-consuming to perform. Other techniques, such as RT-PCR, are prone to contamination, especially when used to analyse atypical samples, such as faeces. NASBA/ECL offers a rapid, specific and sensitive method for the detection of FMDV and is a highly appropriate technique to confirm the presence of virus.

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