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Development of a Lightcycler-based reverse transcription polymerase chain reaction for the detection of foot-and-mouth disease virus

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One step TaqMan real-time reverse transcription polymerase chain reaction (R/T RT-PCR) using a set of primers/probes was developed for the detection of foot-and-mouth disease (FMD) virus. The gene-specific probes labeled fluorogen for the internal ribosomal entry site, Leader sequence and 2B regions were used to detect FMD virus (FMDV). This assay specifically detected FMDV both in cell culture preparations and clinical samples, and was capable of distinguishing FMD from other viral diseases similar to clinical signs (swine vesicular disease, vesicular stomatitis and bovine viral diarrhea). This assay was shown to be 1000-fold more sensitive than the conventional RT-PCR method. The detection limits of this assay was 1 $TCID_{50}$/ml of the FMDV RNA concentration. Quantification was obtained by a standard curves plotting threshold cycle values versus known infectivity titer. The assay was sensitive, specific and rapid enough to detect FMDV RNA genome in probang samples. As such, the described method is reliable and provides faster disease diagnostics than the conventional RT-PCR procedure to detect FMDV.

Key words: FMDV, quantification, TaqMan R/T RT-PCR

Introduction

Foot and mouth disease virus (FMDV) is the causative agent of an economically important viral animal disease reported all over the world. Although mortality associated with foot and mouth disease (FMD) is usually low, the disease decreases livestock productivity and for the affected country, severe restrictions are placed on international trade of animals and animal products [2,3].

There were 16 FMD cases reported in Korea on 2002, most of which (except for one case) had occurred in pig farms [6]. Thirteen of 16 outbreak farms were sacrificed within 24 hours after diagnosis, which was an important factor in reducing the spread of the disease [13]. The laboratory confirmation of FMD outbreak was carried out according to FMD diagnostic methods [7]. Upon the submission of samples from the infected premises, FMD was diagnosed by the detection of virus antigen in clinical samples using a combination of enzyme-linked immunosorbent assay (referred to as Ag-ELISA hereinafter) and specific gene of FMDV by reverse transcription polymerase chain reaction (RT-PCR). The virus isolation in cell culture was performed only for the first several outbreak cases because this assay takes at least 4 days for the interpretation of the result. For this reason, in the majority of the cases subsequent to the initial outbreaks, samples were tested using conventional RT-PCR. However, conventional RT-PCR has been regarded as not being sufficiently preferable to replace virus isolation for the diagnosis of FMD due to higher risk of cross-contamination compared to virus isolation [4,10]. Moreover, the final products amplified by the conventional RT-PCR are usually analyzed by gel electrophoresis that is laborious, insensitive and time-consuming leaving its interpretation subjective [10]. For this reason, the conventional RT-PCR has been performed only as a part of a diagnostic strategy where it is used to supplement the other test procedures.

To minimize this risk of cross-contamination and inconvenience of these methods, we have developed a fluorogenic real-time RT-PCR(R/T RT-PCR) method for the detection and quantification of FMDV using the FMDV-specific probes on the highly conserved region with a set of primers on its both sides. This assay didn't show any nonspecific reaction with other animal diseases that show similar clinical signs such as vesicular stomatitis, bovine viral diarrhea and swine vesicular disease diseases.

Materials and Methods

Design of FMDV-specific primers and probes

FMDV nucleotide sequences were retrieved from GenBank

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Table 1. Description of FMDV strains

Serotype	Virus strain	Genebank accession numbers
O	TAW/2/99/BOV	AJ539137
O	Tibet/CHA/99	AJ539138
O	UKG/35/2001	AJ539141
O	OM III	AJ359854
O	O1 Campos	AJ320488
O	TAW/2/99TC	AJ539136
O	O/SKR/2002	AY312588
O	O/SKR/2000	AJ539139
SAT2	KEN/3/57	NC003992
Asia 1	IND/63/72	AY304994
C	C3 Ind iso19	AY593806
A	A24/Cruzeiro/Brazil/55	AJ251476

(Table 1) and aligned using the Dnastar software (Dnastar, USA). The target region was analyzed for the 12 strains. The sequence for A24/cruzeiro/brazil/55 strain, however, covering from Leader to partial 2B region was reflected only for the designing of leader primer/probe set. Specific primers and a fluorogenic probes were designed to target the conserved regions of FMDV gene using the Lightcycler (LC) probe design software (Roche, USA), according to the manufacture's guidelines for the design of PCR primers and TaqMan probes. The PCR primers and TaqMan probes used in this experiment are listed in Table 2. A blast search analysis confirmed that the primer and probe sequences had no homology with those of other viral sequences and were sufficiently FMDV specific.

In TaqMan R/T RT-PCR, amplification is monitored by the fluorescence gain associated with the Taq polymerase-mediated hydrolysis of a specifically hybridizing TaqMan probe.

Preparation of viruses and samples

To assess the sensitivity of the assay, serial dilution (1 $TCID_{50}$~10^5 $TCID_{50}$/ml) of cell culture supernatants from the two Korean isolates of FMDV (O/SKR/2000 and O/SKR/2002) grown in monolayers of BHK-21 cells was performed. Virus infectivity was titrated in 96 well plates by standard method.

In addition, to evaluate the specificity of the assay, the cell culture supernatants infected by swine vesicular disease virus (SVDV) (10^5 $TCID_{50}$/ml), vesicular stomatitis virus (VSV) ($10^{5.5}$ $TCID_{50}$/ml) and bovine viral diarrhea virus (BVDV) (10^5 $TCID_{50}$/ml) were prepared in a similar manner to the samples infected by FMDV, ascertaining that this assay did not co-amplify the target gene of these three viruses. The RNA samples from cell culture supernatants infected by O/SKR/2000 at the titer of 10^5 $TCID_{50}$/ml and uninfected epithelial suspension were routinely included as positive and negative controls, respectively.

Vesicular fluids and epithelial tissues collected from 14 pig farms during the FMD outbreak in 2002 were prepared. The Probang samples collected between 0 DPI and 7 DPI were collected from animals (cows and pigs) experimentally infected with O/SKR/2000 virus to test the presence of virus or virus genome. All samples of 10% concentration in phosphate-buffered saline (PBS) were distributed in 1.5 ml tube and then kept at −70°C until use.

RNA extraction

All viral RNAs were extracted using QIAamp Viral RNA Mini Kit (Qiagen, Germany), following manufacturer's instructions. In brief, the 560μl of lysis buffer (Buffer AVL-containing Carrier RNA; Qiagen, Germany) was mixed with 140 μl of clinical sample or cell culture supernatant and incubated at room temperature for 10-minutes. Microcentrifuge tube was short-centrifuged to remove drops from inside of the lid, after which 560 μl of absolute ethanol was added to the sample and mixed. Microcentrifuge tube was again short centrifuged to remove drops from inside of the lid and 630 μl of the solution was applied to the QIAamp spin column (Qiagen, Germany) and centrifuged at 8000 rpm for 1 minute. Viral RNA was washed with 500 μl of washing buffer 1 (Buffer AW1; Qiagen, Germany) and washing buffer 2 (Buffer AW2; Qiagen, Germany) by short centrifugation, and extracted by adding 60 μl of elution buffer (Buffer AVE; Qiagen, Germany).

Table 2. Design of FMDV-specific primers and probes

Region	Name	Sequence	Position (size of amplicon)
IRES	Forward	5'-TGTGTGCAACCCCAGCAC-3'	845
	Reverse	5'-CGAGTGTCGCRTGTTACC-3'	972
	Probe	5'-mACAGGCTAAGGATGCCCTTCAGGTACC xp-3'	(128 bp)
Leader	Forward	5'-AACACGCYGTSTTYGCSTG-3'	1521
	Reverse	5'-GCGTCCAKGGGTARAAGTC-3'	1595
	Probe	5'-ACCTCCAACGGGTGGTACGCGAT-3'	(75 bp)
2B	Forward	5'-AGATGCAGGARGACATGTCAA-3'	4000
	Reverse	5'-TTGTACCAGGGYTTGGCYT-3'	4125
	Probe	5'-mAAACACGGACCCGACTTTAACCGxp-3'	(126 bp)

*RT-PCR primers and probes for all targets were designed using available GenBank data.
The 5' end of probes was labeled with 6-FMA whereas the 3' end was labeled with TAMRA.

TaqMan R/T RT-PCR

One-step R/T RT-PCR reaction was conducted using QuantiTaq probe RT-PCR (Qiagen, Germany) in a single capillary tube according to the manufacturer's guidelines for individual component concentrations. The reaction was performed in a final volume of 20 µl containing 1 µM each primer, 0.2 µM probe, 10 µl 2X Quantitech probe RT-PCR Master Mix, Quantitech probe RT Mix 2U/reaction, 3.6 µl RNase-free water and 5 µl template RNA.

Samples were amplified by using a program that included a reverse transcription procedure consisting of one cycle of an incubation at 50°C for 20 min and 94°C for 15 min, followed by 55 cycles of denaturation step at 94°C for 1 sec and annealing/extension step at 60°C for 1 min with the ramp of 20°C/sec for each cycle.

Amplification and product detection were performed under the LC system (Roche, Germany). During PCR, the probe hybridizes to its complementary single-strand DNA sequence within the PCR target. As amplification occurs, the probe is degraded due to the exonuclease activity of Taq DNA polymerase, thereby separating the quencher from reporter dye during extension. During the entire amplification cycles, the light emission increases exponentially.

A positive result was determined by identifying the threshold cycle (C_T) value at which reporter dye emission appeared above background. If the fluorescence signal was not detected within 55 cycles, the sample was considered negative.

Comparison of the detection limit of R/T RT-PCR and other tests

To compare the results obtained by R/T RT-PCR with those by other diagnostic tools, the conventional RT-PCR and Ag-ELISA were performed with the cell culture supernatants of different $TCID_{50}$ concentrations. The conventional RT-PCR procedures were undertaken with a primer set targeting the 3ABC region of FMDV [12]. The Ag-ELISA was carried out using the test kit produced by Pirbright Lab, Institute for Animal Health [9]. The sample was considered positive if the net OD value was ≥0.1.

Results

R/T RT-PCR of the standard dilution series and selection of adequate primer/probe sets

Standard curves for FMDV were obtained using serial dilution from 10^5 $TCID_{50}$/ml to 1 $TCID_{50}$/ml with three different primer/probe sets. The absolute negative for any test sample or negative control corresponded to a C_T value of 35. The C_T values obtained from the standard dilution in assays with three different primer and probe sets were plotted. The respective C_T values were determined and a linear relationship was established between C_T values and the logarithm of the standard dilution of FMDV RNA concentration of infectivity equivalent. All sets of primer/probe had similar sensitivity but the standard curve generated using 2B primer/probe set was more sensitive and specific for FMDV detection than the others (Fig. 1).

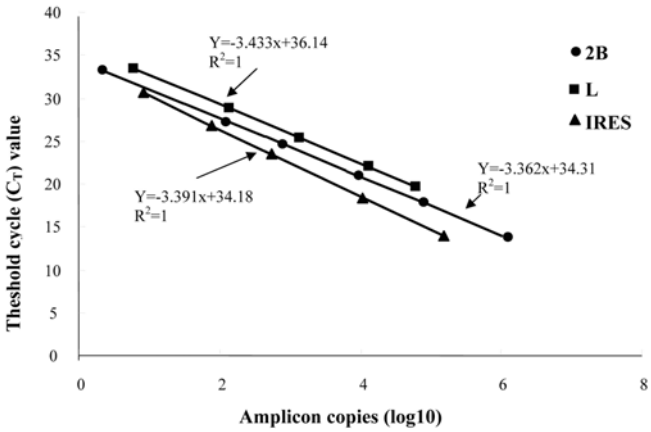

Fig. 1. Standard curves for FMDV O/SKR/2002 obtained using serial dilutions with three different primer/probe sets. The quantitative RT-PCRs with TaqMan probes were performed on a serial dilution of 10^5 $TCID_{50}$/ml to 10^0 $TCID_{50}$/ml of FMDV standard RNA to evaluate its detection limits. With increasing amounts of standard RNA templates, the respective C_T values were determined and a linear relationship was established between the C_T values and the logarithm of initial template amounts. R/T RT-PCR efficiencies were calculated according to the equation $E = 10^{-1/slope}$. The coefficient of reliability (R^2) of the regression was 1.

Sensitivity of R/T RT-PCR in comparison with those of conventional RT-PCR and Ag-ELISA

To evaluate the sensitivity of the R/T RT-PCR, cell culture supernatants of various $TCID_{50}$ concentrations were prepared. Analytical sensitivity was determined by testing sequential 10-fold dilutions in D-MEM (Gibco, USA). The sensitivity of the R/T RT-PCR was compared with those of other diagnostic tests such as conventional RT-PCR and Ag-ELISA. Table 3 shows that the samples of at least 1 $TCID_{50}$/ml viral RNA concentration were positive in the R/T RT-PCR. The detection limit of the conventional RT-PCR was the viral RNA concentration of 10^3 $TCID_{50}$/ml, whereas no antigen detection was observed when tested by Ag-ELISA. The R/T RT-PCR assay had a 1000 fold higher sensitivity than conventional RT-PCR assay.

Specificity

The analytical specificity of the R/T RT-PCR was determined by testing different non-FMDV isolates related to vesicular diseases or causing diseases with similar symptoms (SVDV, VSV and BVDV). None of the SVDV, VSV and BVDV isolates tested were positive by R/T RT-PCR (Fig. 2). Also, whether the result of the R/T RT-PCR depended on the type of tissue, we determined by testing different tissues collected from a non-infected cow. All tissues tested did not react by R/T RT-PCR.

Table 3. Comparisons of diagnostic methods with different virus titers of O/SKR/2002

FMD virus concentration	2B R/T RT-PCR ($C_T \pm SD$)	Conventional RT-PCR*	Ag-ELISA‡
10^5 $TCID_{50}$/ml	13.76 ± 0.589	+	-(0.02)
10^4 $TCID_{50}$/ml	17.84 ± 0.550	+	-(0.00)
10^3 $TCID_{50}$/ml	20.89 ± 2.273	+	-(0.01)
10^2 $TCID_{50}$/ml	24.51 ± 1.692	-	-(0.01)
10^1 $TCID_{50}$/ml	27.29 ± 1.022	-	-(0.01)
10^0 $TCID_{50}$/ml	31.94 ± 0.671	-	-(0.01)
Negative	- §	-	-(0.01)

*+; positive -; negative.
†Mean values and standard deviations were based on three different experiments.
‡If the net OD value in Ag-ELISA is above 0.1, the sample is considered positive.
§Threshold cycle (C_T) value ≥35: negative.

Analysis of R/T RT-PCR results using clinical and experimental samples

To determine whether the preliminary experiment with the viruses grown in cell culture using O/SKR/2002 would generate the same results for the diagnosis of FMDV in clinical samples, further experiment had been conducted against a panel of epithelial suspensions and vesicular fluids. Of the 17 samples with a positive result by R/T RT-PCR, twelve were positive by conventional RT-PCR (Table 4). The samples negative in R/T RT-PCR were also negative by conventional RT-PCR, indicating identical specificities for both RT-PCR systems (data not shown).

For the detection of FMDV RNA in Probang samples, R/T RT-PCR and conventional RT-PCR were performed, and the results were compared (Table 5). Both live virus and viral RNA could be detected by R/T RT-PCR in Probang samples taken from cattle between 1 DPI and 6 DPI, and pigs were positive for viral RNA between 1 DPI and 3.DPI. However, both live virus and viral RNA could be detected by conventional RT-PCR between 1 DPI and 2 DPI.

Discussion

A number of previously published studies on R/T RT-PCR methods have targeted the different sequences from either internal ribosomal entry site (IRES) [4,5,11] or 3D region [1,8] in an effort to introduce a rapid diagnosis of FMD. In this study, we have developed optimal TaqMan primers and probes using the LC probe design software to allow the detection of all serotypes of FMDV by way of targeting a highly conserved region within the 2B gene. The ability of these primers to specifically amplify FMDV RNAs was verified by obtaining the fluorescent gain only in the FMDV samples when applied to the cell culture supernatants infected by SVDV, VSV, BVDV and FMDV.

The cell culture supernatants infected with FMDV were tested by the R/T and conventional RT-PCR in parallel with Ag-ELISA. The R/T RT-PCR was more sensitive than the other diagnostic methods for the detection of FMDV. Both RT-PCR methods has successfully detected specific gene of FMDV even though the sensitivity of conventional RT-PCR failed to identify the sample of less than 10^2 $TCID_{50}$/ml titer when compared to the R/T RT-PCR which could detect the viral RNA concentration as low as 10$TCID_{50}$/ml. Whereas the viral RNAs of serial dilutions (titers; 1 $TCID_{50}$ ~ 10^5 $TCID_{50}$/ml) using the Korean isolate O/SKR/2002 had a C_T value in the range of 13.76 ± 0.589 to 31.94 ± 0.671, the Ag-ELISA did not detect any antigen in these samples. The clinical samples positive by conventional RT-PCR have also been diagnosed as positive under the fluorogenic R/T RT-PCR system. Moreover, with this automated 5'-nuclease probe-based RT-PCR procedure (using LC system) described

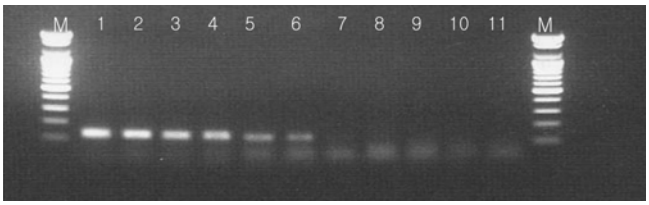

Fig. 2. Specificity of R/T RT-PCR for O/SKR/2002 using 2B primer/probe set. BVDV, VSV (Indiana and New Jersey strain) and SVD of the titer of 10^5 $TCID_{50}$/ml was used to assess the specificity of the R/T RT-PCR. M: 100 bp ladder; 1: 10^5 $TCID_{50}$/ml; 2: 10^4 $TCID_{50}$/ml; 3: 10^3 $TCID_{50}$/ml; 4: 10^2 $TCID_{50}$/ml; 5: 10^1 $TCID_{50}$/ml; 6: 10^0 $TCID_{50}$/ml; 7: Negative sample; 8: BVDV; 9: VSV Indiana; 10: VSV New Jersey; 11: SVDV.

Table 4. Diagnostic comparison between the conventional and R/T RT-PCR on the epithelial suspensions and vesicular fluids from animals infected with O/SKR/2002 FMDV

Sample description	Type of sample*	Conventional RT-PCR†	Ct values by R/T RT-PCR
JC1	ES	-	23
BY1	CC	-	25
YI1	VF	+	15
YI6	ES	-	18
PT1	VF	+	16
YI2	VF	+	16
JC2	ES	-	23
PT2	VF	+	15
AS4	VF	+	18
AS9	VF	+	18
AS7	ES	+	22
BY2	VF	+	26
YI7	ES	-	24
AS1	VF	+	15
YI3	ES	+	21
Y14	ES	+	25
YI5	ES	+	16

*ES: epithelial suspension; CC, cell culture supernatant fluid; VF, vesicular fluid.
†+: positive , -: negative.

Table 5. Comparison between R/T RT-PCR (R) and conventional RT-PCR (C) for the detection FMDV in probang samples from animals infected experimentally

Animals	Animal no.		Days post inoculation (DPI)							
			0	1	2	3	4	5	6	7
Bovine	4	R	-	+	+	+	NT	+	+	-
		C	-	+	-	-	NT	-	-	-
	78	R	-	+	-	-	+	+	+	-
		C	-	-	-	-	-	-	-	-
Swine	1134	R	-	+	+	+	-	-	-	-
		C	-	-	+	-	-	-	-	-
	1135	R	-	+	+	+	-	-	-	-
		C	-	+	+	-	-	-	-	-

-: FMDV negative; +: FMDV positive. NT: not tested.

above, the test results could be made within 2 hours after the submission of the clinical samples. This would greatly contribute to the implementation of effective control measures in the face of an FMD outbreak, especially in pigs.

All these findings demonstrate that the automated R/T RT-PCR assay is suitable for the rapid, accurate and reliable detection of FMD virus in clinical samples. In particular, this assay may have the advantage over the conventional procedure of virus isolation in cell cultures for the diagnosis of samples containing low concentration of virus, which is neither detected by the ELISA nor produce a cytopathic effect in cell culture [10]. In this study, this procedure was found to be more convenient to use than the conventional RT-PCR and produced objective results and saved test-time.

As described elsewhere, we have used a conserved 2B sequences in designing a set of primer/probe which, in principle, could enable the detection all serotypes of FMDV. The standard curve generated using 2B primer/probe set offered a highly sensitive, high throughput and rapid method for FMDV detection. However, this assay has been applied only to the samples of FMDV Korean strain type O due to the lack of other FMDV strains. For this reason, further experiment will need to be performed in order to clarify whether this assay can detect the FMDV irrespective of its serotypes or not.

Vesicular viral diseases consist of large portion of the principal animal health problems in pigs and an effective method of distinction is required among these viruses (FMDV, SVDV and VSV). Therefore, the next step will be to develop a diagnostic scheme to differentiate the FMDV infection from those of these diseases with similar clinical signs. This work will lead us to take advantage of this new technology, which will allow the rapid diagnosis of economically devastating animal diseases that need to be promptly controlled.

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