Comparison of stabilisers for development of a lyophilised multiplex reverse-transcription PCR mixture for rapid detection of foot and mouth disease virus serotypes

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Summary

Multiplex reverse-transcription polymerase chain reaction (mRT-PCR) assay is a sensitive and rapid method for the detection and serotyping of foot and mouth disease virus (FMDV). However, the method has not been used to its full potential, because of factors such as cost, a lack of infrastructure and the complexity of the reaction mixture. This study was undertaken to optimise and validate a thermostable, lyophilised, ready-to-use mRT-PCR kit for the rapid detection of FMDV in field laboratories in India. Trehalose, PEG-8000 and glycerol were evaluated for stabilisation of the PCR mixture at ambient temperatures. The lyophilised mRT-PCR kit was validated and found robust enough for use in field-level laboratories. The PCR reaction mixture in the ready-to-use kit has low complexity, so chances of cross-contamination during the preparation of the mixture are limited, but may easily be monitored by using lyophilised internal positive and negative controls. In addition, the requirement to

maintain live FMDV isolates as internal positive controls at field-level regional laboratories is eliminated.

Keywords

Foot and mouth disease – India – Lyophilised RT-PCR reagent – Ready-to-use RT-PCR kit.

Introduction

Foot and mouth disease (FMD) is a highly contagious viral disease with transboundary importance. Cattle, buffalo, sheep, goats, pigs, elephants and many other species of livestock and wild animals are susceptible. The causative agent, FMD virus (FMDV), belongs to the genus *Aphthovirus* in the family *Picornaviridae* (1) and occurs as seven genetically and antigenically distinct serotypes (O, A, C, Asia-1 and Southern African Territories [SAT] 1, 2 and 3) and multiple subtypes (2). In India, three of the seven serotypes (O, A, Asia-1) are reported throughout the year in many parts of the country (3). The control of FMD in India is achieved through a systematic, biannual, preventive vaccination programme and by strict monitoring of the disease by a network of field-level regional FMD diagnostic laboratories, located in different parts of the country and working under the All-India Co-ordinated Research Project (AICRP) on FMD (4). A vaccination-based FMD control programme was launched in 2003 in 54 selected districts in eight states of India. Since the 2006–2007 programme (all programmes run for a 12-month period from April to March of the following year), numbers of cases/outbreaks have dropped significantly in the regions covered by the control programme. Only a few sporadic cases due to free animal movement from the neighbouring unvaccinated districts/states have been recorded (4). After the initial success, an additional 167 districts were included in the programme in 2010–2011 (4).

Early diagnosis of FMD at field-level regional laboratories contributes to the rapid implementation of preventive measures to check any further spread of the disease in the region. Suspected FMDV materials from outbreaks are tested for serotype determination in an in-house,

antigen-capture, sandwich enzyme-linked immunosorbent assay (ELISA), with 100% specificity for heterologous FMDV and 80% sensitivity for detection in clinical samples (5). The samples collected by the regional laboratories are then referred to the central FMD laboratory at Mutkeswar for detailed examination, including serotype confirmation and virus isolation and characterisation. Clinical samples that test negative in the sandwich ELISA are retested in a more sensitive, in-house, multiplex reverse-transcription polymerase chain reaction (mRT-PCR) assay that has a diagnostic sensitivity of 96.5% (6) and has been used for serotype identification for six years. India is mainly a subtropical country and maintenance of the cold chain for transporting clinical samples is sometimes difficult to achieve; this occasionally results in decomposition of the samples, leading to false-negative results. Among the samples received at the central laboratory in Mukteswar from suspected FMD cases, the virus serotype could not be determined in 43.4% of the samples collected in 2007–2008; 41% of those collected in 2008–2009; 34% of those collected in 2009–2010; 39% of those collected in 2010–2011; and 37% of those collected in 2011–2012. The explanation could be that the samples had decomposed during transportation or that they were truly negative for FMDV. In addition, shipping samples to the central laboratory increases the time required to confirm the diagnosis.

For early diagnosis in field laboratories, highly sensitive and specific assays, such as a ready-to-use PCR assay, are required. In addition to the antigen-capture sandwich ELISA, use of the in-house mRT-PCR for the simultaneous detection and serotype determination of FMDV at the central laboratory increased the rate of diagnosis by 8% in 2007–2008; by 5.7% in 2008–2009; by 3.3% in 2009–2010; by 5.5% in 2010–2011; and by 7.5% in 2011–2012. Thus, an assay with a user-friendly, ready-to-use format that can be easily transported for use in field-level laboratories is required. In the present study the stabilisers trehalose, PEG8000 and glycerol were evaluated for stabilisation of the mRT-PCR master mixture for lyophilisation. The lyophilised master mixtures were evaluated and compared with freshly prepared master mixture for the detection and serotype determination of FMDV in clinical samples.

Materials and methods

Foot and mouth disease viral RNA template

Isolates of FMDV serotypes O (IND R2/1975), A (IND 40/2000) and Asia-1 (IND 93/2008) were obtained from the national FMDV repository (Project Directorate on FMD, Mukteswar) and expanded in healthy BHK-21 cells. Infected cell culture supernatants were collected after incubation for 18–20 h, when cytopathic effects were complete. Total RNA was extracted from the supernatants using a QiAmp viral RNA extraction kit (Qiagen) and quantified in a nanodrop spectrophotometer (Thermo Scientific).

One-step reverse-transcription and PCR mixture

Complementary DNA was synthesised from the extracted FMDV RNA in an *in vitro* amplification process using a one-step mRT-PCR mix (Qiagen). For freeze drying of 50 µl reaction volumes, the mRT-PCR mixture was prepared in nuclease-free vials by adding 10 µl of 5XQiagen One-Step RT-PCR buffer, 400 µM of each deoxyribonucleotide triphosphate (dNTP), 10 pmols of FMDV-specific pNK61 reverse primer (7) targeting the 2B region (Table I), 10 pmol each of the forward primers DHP13, DHP15 and DHP9 (specific for serotypes O, A, and Asia-1, respectively) (Table I), and 2.0 µl of Qiagen One Step RT-PCR enzyme mixture. The total volume was adjusted to 45 µl with RNase-free water.

Positive and negative multiplex PCR controls

Equimolar ratios of RNA extracted from the reference strains of serotypes O, A and Asia-1 were pooled and freeze dried in 50 ng quantities in nuclease-free glass vials to serve as an internal RNA-positive control. Similarly, 50 ng of RNA extracted from healthy BHK-21 cells was freeze dried to serve as an internal viral RNA-negative control.

Lyophilisation of mRT-PCR mixture, internal positive and negative RNA controls

The addition of chemicals as stabilisers in the mRT-PCR mixture may have detrimental effects on PCR efficiency, so stabilisers and their optimal concentrations should be chosen with care for stabilisation during and after lyophilisation, without any significant effect on the efficiency of the PCR. The three stabilisers were tested individually for stabilisation of the mRT-PCR mixture components during and after lyophilisation. To determine the optimum concentration of each stabiliser that would have a minimal effect on the analytical sensitivity of the PCR, differing concentrations of the stabilisers were mixed with mRT-PCR master mixtures (Table II) containing RNA in decreasing concentrations. This was followed by reverse-transcription and *in vitro* amplification. Freshly prepared mRT-PCR mixtures were compared with and without stabiliser.

Three sets of mRT-PCR mixtures and positive and negative internal RNA controls containing optimal concentrations of the individual stabilisers were prepared in 200 µl volumes and lyophilised in nuclease-free glass vials. The PCR reagents were lyophilised for 24 h in a freeze drier (Lyodryer, LSI India) under a range of conditions (Table III). One set of mRT-PCR reagents without stabiliser was also lyophilised to serve as a negative control. After lyophilisation, the vials were sealed under vacuum and stored at –86ºC until further use.

Reverse-transcription polymerase chain reaction

The lyophilised mRT-PCR mixtures were reconstituted with 180 µl of RNase-free water. The internal RNA positive and negative controls were reconstituted with 50 µl of RNase-free water and then 10 µl of each control was added to 90 µl of the mRT-PCR mixture. The reaction mixtures were placed in a thermal cycler and amplified at 50ºC for 30 min for reverse transcription, followed by one cycle of initial denaturation at 95ºC for 15 min and 35 cycles of denaturation at 95ºC for 30 s. Annealing took place at 60ºC for 30 s and extension at 72ºC for 45 s, as described by the authors (6). Final extension was at 72ºC for 10 min. The amplified products and a 100 bp DNA reference

ladder (Fermentas) were resolved in 2% agarose gel electrophoresis and visualised by ethidium bromide staining under a UV light illuminator.

Determination of the thermal stability of the lyophilised reagents

The lyophilised mRT-PCR mixtures and the internal positive and negative RNA controls, together with the individual stabilisers, were incubated at –86ºC, –20ºC, 4ºC, room temperature and 37ºC. Three sets of vials from each of the incubation temperatures were reconstituted with 200 µl of nuclease-free water. Reaction mixtures were assembled for amplification of the lyophilised internal positive and negative RNA controls on days 1, 2, 3, 5, 7, 14, 30, 60, 90, 120, 180 and 365. In parallel, reaction mixtures were assembled for amplification of freshly extracted RNA from cell-culture-derived FMDV serotypes O, A, and Asia-1 (1×10^{-1} 50% tissue culture infective dose [$TCID_{50}$]) to cross-check the stability of the lyophilised RNA controls.

Analytical sensitivity

Lyophilised reagents incubated at –20ºC for three months were used to estimate analytical sensitivity. Isolates of serotypes O, A and Asia-1 were grown in cell culture and titrated, using the method of Reed and Muench (8). Serial two-fold dilutions of the three serotypes were prepared in maintenance medium to obtain virus at titres of 1×10^{3} to 1×10^{-2} $TCID_{50}$/ml. Viral RNA was extracted from the diluted material and amplified in mRT-PCR with both freshly prepared and lyophilised mRT-PCR mixtures. The procedure was repeated three times to check repeatability.

Validation of lyophilised reagents in mRT-PCR for serotype determination

The lyophilised reagent-based mRT-PCR for serotype determination was validated by testing FMDV-suspected clinical samples of tongue epithelium and foot lesions. Tissue material ($n = 105$) from suspected

cases, collected in field outbreaks during 2009–2011 and stored in phosphate buffered saline (PBS) with 50% glycerol, were obtained from the national FMDV repository for testing. All the clinical samples were triturated to extract viral RNA, using QiAmp viral RNA extraction kits (Qiagen). The extracted RNA was tested with both lyophilised and freshly prepared mRT-PCR reagents in separate reactions and the results compared. Although variations in results among technical staff should be minimised with the use of the mRT-PCR pre-mixture, variation was nonetheless evaluated by testing a set of 12 clinical samples (six positive, six negative), performed by three different staff on three different days, and the results compared. Lyophilised reagent-based kits were shipped without a cold chain to three field-level regional laboratories, more than 1,000 km from the central Indian laboratory. A total of 57 tissue samples collected from animals suspected of having FMD during field outbreaks were tested. A set of lyophilised reagents was then brought back to the central laboratory, incubated at –20ºC and tested quarterly for up to one year to evaluate the stability of the kits in relation to thermal fluctuations during transportation.

Results

The maximum concentrations of the stabilisers at which no significant drop in PCR efficiency was observed were 100 mM for trehalose, 0.1% for PEG-8000 and 2% for glycerol. Accordingly, the mRT-PCR mixtures were lyophilised with individual stabilisers at these concentrations. In addition, gradient freeze-drying conditions were required to obtain stable mixtures: these were obtained by a gradual decrease in temperature followed by a gradual increase in vacuum (Table III).

On day zero, the lyophilised mRT-PCR mixtures, stabilised with the three stabilisers, were used to amplify the FMDV RNA controls to sizes 249 bp, 376 bp and 537 bp, specific for serotypes O, A, and Asia-1, respectively. The positive RNA controls could not be amplified by lyophilised mRT-PCR mixture without stabiliser. No amplification was observed in any of the negative RNA controls. The

analytical sensitivity of the lyophilised mRT-PCR mixture was estimated by amplifying RNA extracted from serial two-fold dilutions (1×10^3 to 1×10^{-2} $TCID_{50}$/ml) of serotype A isolates (Fig. 1). The minimum detection limit of the lyophilised mRT-PCR was estimated as 1×10^{-1} $TCID_{50}$/ml of serotype A, which was equal to that of the mRT-PCR with freshly prepared reagents, as described by the authors (6). Similar results were obtained for serotypes O and Asia-1.

The thermostability of the lyophilised mRT-PCR mixtures was evaluated by incubating vials at different combinations of time and temperature. Extracted RNA from virus at 1×10^{-1} $TCID_{50}$/ml was used to evaluate the lyophilised mRT-PCR mixture and the criterion for success was defined as amplification of the RNA in all three replicates of the lyophilised reagents. Although amplification in the glycerol-stabilised mRT-PCR mixture was successful on day 1 (the day immediately after freeze-drying), it failed afterwards at all temperatures. In contrast, amplification was successful up to day 3 with mRT-PCR mixtures stabilised with PEG-8000 and trehalose at all incubation temperatures. The thermostabilities of the stabilised mRT-PCR mixtures are shown in Table IV. Amplification was successful up to day 7 with the mRT-PCR mixture stabilised with trehalose and incubated at 37ºC; however, at the same temperature, amplification was observed only up to day 4 with PEG-8000 (Table IV). For lyophilised mRT-PCR mixture stabilised with PEG-8000 and trehalose, and stored at 4ºC, amplification could be achieved up to day 15 and day 30, respectively. Similarly, with mRT-PCR mixtures stabilised with PEG-8000 and trehalose and stored at –20ºC, amplification was successful up to day 120 and day 180, respectively; at –86ºC it was successful up to one year.

On the basis of these thermostability experiments, reagents stabilised with 100 mM trehalose were chosen for further use. Comparison of lyophilised and freshly prepared mRT-PCR mixtures for the detection of FMDV in suspected tissue samples ($n = 105$) showed 100% concordance. Among these samples, 47 tested positive for serotype O, 11 for serotype Asia-1 and three for serotype A. Using the antigen-capture sandwich ELISA, only 54 samples could be serotyped, of

which 42 were serotype O, 10 were Asia-1 and two were A. Results with the lyophilised kit were highly repeatable: there were no differences when three different technicians tested a set of 12 samples on three different occasions.

The robustness of the kit was confirmed by shipping without any cold chain to field-level laboratories. Tissue samples from animals (n = 57) suspected of FMDV infection were tested with ready-to-use, lyophilised mRT-PCR reagents in the field: among the samples, 18 were reported as serotype O, one sample was serotype A and five samples were serotype Asia-1. These results were verified by testing the same samples at the central FMD diagnostic laboratory. Similarly, the performance of the kit was satisfactory when three sets of kit (in triplicate) were transported, without any cold chain, to two different regional laboratories and brought back to the central laboratory and tested periodically for up to one year.

Discussion

Early detection of FMDV is the foremost requirement in preventing the further spread of the disease. Together with conventional diagnostic methods, such as the antigen-capture sandwich ELISA and virus isolation, molecular diagnostic methods such as PCR and real-time PCR are now routinely used to diagnose FMD in India (3). In addition to their high sensitivity, molecular methods generate results much faster than other methods. In particular, the mRT-PCR assay for the detection and identification of FMDV has been used extensively in India over recent years. However, its use has been limited to a few field-level FMD diagnostic laboratories because of such factors as the cost of testing, a lack of infrastructure and the complexity of the mRT-PCR mixture. Samples from FMD-suspected animals are referred to the central laboratory for FMDV detection by mRT-PCR, considerably increasing the time required for a confirmatory diagnosis. In addition, possible sample deterioration due to a break in the cold chain during shipping can lead to an erroneous diagnosis. In view of these factors, a thermostable, ready-to-use mRT-PCR kit was optimised. Three different stabilisers were evaluated for stabilisation

of the PCR mixture. The stabilisation of reverse transcriptase and polymerase enzymes is vital during lyophilisation, as enzymes are prone to lose their structural conformation and functional activity. Many stabilisers can stabilise the mRT-PCR components during and after the lyophilisation procedure; however, PCR efficiency may be reduced and thus optimisation of stabiliser concentration in the mRT-PCR mixture was required.

In the present study, trehalose, PEG-8000 and glycerol were evaluated individually and in combination for the thermostabilisation of the mRT-PCR master mixtures. All three stabilisers protected the enzymes during lyophilisation. However, reagents preserved with glycerol at a range of working concentrations worked only on day 1, but not on successive days at any storage temperature, therefore the investigation of glycerol-stabilised mRT-PCR reagents was discontinued. Trehalose at a concentration of 100 mM was found to be more effective than PEG-8000 for preserving the reagents for a longer duration. The use of trehalose 5% (w/v) for stabilising RT-PCR reaction components at ambient temperature has been described for the detection of *Vibrio cholerae*, *Leptospira* spp., *Mycobacteria* spp. and *Salmonella* spp. in clinical samples (9, 10, 11, 12), and stabilised the PCR reagents for up to one year at –20ºC. Qu *et al.* (13) developed lyophilised thermostable real-time PCR reagents for the detection of *Yersinia* spp. that remained effective for 79 days at 37ºC. The use of carbohydrate mixtures (13) may further prolong the stability of lyophilised RT-PCR reagents at ambient temperatures. Nevertheless, the trehalose-stabilised mRT-PCR reagents in the present study were stable for up to one year when stored at –20ºC with thermal fluctuations, which is adequate for shipping the kit to field-level diagnostic laboratories.

Some freeze-dried PCR mixtures are also available commercially, although for diagnostic use they require the addition of FMDV-specific primers for individual serotypes, whereas the present kit is optimised for the diagnosis of FMD in India and requires only reconstitution with nuclease-free water and the addition of viral RNA extracted from clinical samples.

Lyophilised internal positive and negative FMDV RNA controls are also provided in the kit, to aid in quality control and eliminate the requirement for maintaining live FMDV at field-level laboratories. Use of the kit also significantly reduces the chances of cross-contamination during the assembly of reaction mixtures, as components are provided in individual vials. Production of the kit takes place at the central laboratory, with the further advantage that quality control is uniform. It was observed that personnel at the field-level laboratories can easily perform the mRT-PCR assay with the new kit and little training.

Since the implementation of systematic vaccination as part of the FMD control programme in India, occurrences of the disease have been reduced. However, the vaccine only prevents clinical manifestations and does not protect animals from infection with the virus. Hence, effective monitoring is essential for controlling the spread of disease. The ready-to-use, lyophilised mRT-PCR mixture is robust enough for use across the Indian subcontinent and will help in FMD diagnosis at field-level laboratories. The lyophilised reagent is very user-friendly and the preparation of the reaction mixture is simple (9, 12). The ability of field-level laboratories to test tissue samples by mRT-PCR assay will help to generate results immediately, enabling the rapid adoption of control measures at field level. The kit is sufficiently stable to withstand fluctuations of temperature during shipping to field-level laboratories and so, with little assistance, specific and early diagnosis of FMD is now possible, augmenting the FMD surveillance and control programme in India. Following the success of the lyophilised mRT-PCR kit, the production of lyophilised lineage-differentiating PCR and real-time PCR assay, which were previously developed in India (14, 15), are now being developed for use in field-level laboratories and will support FMDV molecular epidemiological studies in India.

Conclusions

This study leads to the following conclusions:

– the ready-to-use, lyophilised mRT-PCR kit is user-friendly and thermostable for use in Indian conditions

– the kit is now in use for FMDV detection and serotype determination at field-level regional FMD diagnostic laboratories

– the requirement for a cold chain for transportation of the mRT-PCR reagents will be abolished

– in comparison with the earlier system of referring samples to the central FMD laboratory for diagnosis, suspected clinical samples can now be tested at field-level regional diagnostic laboratories, considerably reducing the time required to make a confirmatory diagnosis

– the provision of internal positive and negative PCR controls eliminates the requirement to maintain live FMDV at field-level regional diagnostic laboratories.

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Table I

Specific serotype primers and amplified products of type O, A and Asia-1 in mRT-PCR

Primer designation	Serotype	Primer sequence 5'-3'	Location/gene segment	Product length (bp)	Reference
DHP13	O	GTGACTGAACTGCTTTACCGCAT	1D	249	6
DHP 15	A	CAACGGGACGARCAAGTACTC	1D	367	6
DHP 9	Asia-1	GACCTGGAGGTYGCGCTTGT	1D	537	6
NK 61	–	GACATGTCCTCCTGCATCTG	2B	universal primer	7

mRT-PCR: multiplex reverse-transcription PCR

Table II

Concentrations of trehalose, PEG-8000 and glycerol used for stabilisation of the mRT-PCR master mixture during and after lyophilisation

Trehalose	PEG-8000	Glycerol
10 mM	0.05%	1%
50 mM	0.10%	2%
100 mM	0.20%	4%
200 mM	0.30%	5%

mRT-PCR: multiplex reverse-transcription PCR

Table III

Procedure for lyophilisation of mRT-PCR mixture in freeze drier

Cycles	**Freeze**				**Primary lyophilisation**												**Secondary lyophilisation**
Temperature (ºC)	–20	–25	–30	–40	–25	–15	–10	–5	–5	0	5	15	20	20	25	25	25
Time (min)	90	60	60	60	120	150	120	90	120	150	60	90	60	90	30	30	30
Vacuum (millitorr)	n/a	n/a	n/a	n/a	500	450	450	450	400	350	350	300	300	200	200	100	100

mRT-PCR: multiplex reverse-transcription PCR

n/a: not applicable

Table IV
Stability of lyophilised mRT-PCR mixtures with added stabilisers and exposure to a range of temperatures
Numbers indicate stability of mRT-PCR mixtures in days

Stabiliser concentrations	Temperature				
	–86ºC	–20ºC	4ºC	25ºC	37ºC
Trehalose					
10 mM	90	30	7	14	1
50 mM	90	120	14	21	4
100 mM	365	180	30	21	7
PEG-8000					
0.05%	90	30	1	1	1
0.10%	180	30	4	4	1
0.20%	365	120	14	14	4
Glycerol					
1%	1	1	0	0	0
2%	1	1	0	0	0
Without stabiliser	0	0	0	0	0

mRT-PCR: multiplex reverse-transcription PCR

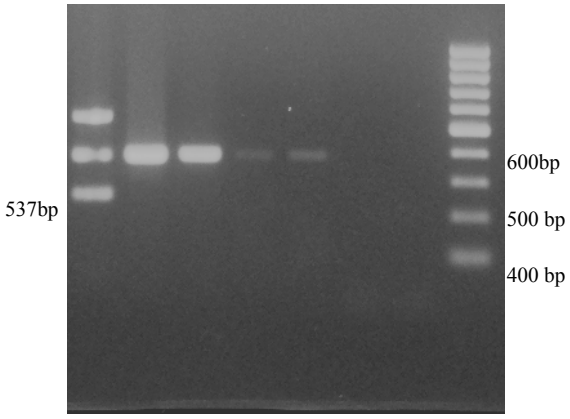

Fig. 1
Amplification of RNA from serial dilutions of serotype A foot and mouth disease virus in multiplex reverse-transcription PCR

Lane 1: mRT-PCR internal positive control

Lane 2: RNA from serotype A (1×10^{3} $TCID_{50}$/ml)

Lane 3: RNA from serotype A (1×10^{2} $TCID_{50}$/ml)

Lane 4: RNA from serotype A (1×10^{1} $TCID_{50}$/ml)

Lane 5: RNA from serotype A (1×10^{-1} $TCID_{50}$/ml)

Lane 6: RNA from serotype A (1×10^{-2} $TCID_{50}$/ml)

Lane 7: mRT-PCR internal negative control

Lane 8: 100bp DNA ladder