DETECTION OF FMDV SEROTYPES O, A AND ASIA 1 BY REAL-TIME RT-PCR

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ABSTRACT

In countries like Iran where foot-and-mouth disease (FMD) is endemic, identification of the serotypes of the causative virus strains is important for vaccine selection and for tracing the source of the outbreaks. Real-time reverse transcription polymerase chain reaction (rRT-PCR) assays for the specific detection of FMD virus (FMDV) serotypes O, A and Asia 1 were evaluated using primers/probe sets designed from the VP1 region of the FMDV genome.

RNA extracted from suspensions of vesicular epithelia of representative strains of serotypes O, A and Asia 1 was tested by new one-step rRT-PCR assays run in parallel with pan-serotype specific assays (5’ UTR and 3D). These new assays incorporated primers/probe sets intended for specific detection of serotype O, A and Asia 1 virus sequences, respectively. Strains were chosen from sub-groups of each serotype recently circulating in the Middle East. Serotype O strains belonged exclusively to the PanAsia-2 lineage, type A strains were from the Iran-05 lineage while the Asia 1 viruses were selected from three relevant lineages.

The assays with the universal primers and probes were useful in confirming the presence of FMDV in each sample. All three serotype-specific primers/probe sets were strongly positive against RNA from homotypic viruses and no cross-reactivity was observed with heterotypic viruses except with one serotype O virus which also gave a weaker reaction with the serotype A-specific primers/probe set. The cause of this apparent non-specificity is being investigated but analysis of the sequence did not reveal a significant similarity with those of the type A-specific primers and probe. The results illustrate the potential of using rRT-PCR for the detection and discrimination of FMDVs belonging to geographically distinct sub-groups of serotypes O, A and Asia 1 from the Middle East. A similar approach could be used to develop typing assays for other geographical regions of the world.

2. INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease of wild and domestic cloven-hoofed animals, particularly cattle, sheep, pigs and goats. It is the most economically important viral disease of domesticated livestock throughout the world today, being endemic in many countries of Africa, Asia and South America. However, recent outbreaks have occurred in countries that are normally free of FMD including Japan and Korea (2000), France and The Netherlands (2001) and the United Kingdom (UK) (2001 and 2007). The causative agent, FMDV virus (FMDV), is a single-stranded positive sense RNA virus of around 8.4 kilobases in length, belonging to the genus Aphthovirus within the family Picornaviridae (Belsham, 1993). There are seven immunologically distinct serotypes: O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1 and a diverse antigenic spectrum of virus strains within each serotype. The seven serotypes are not distributed equally around the world (Knowles and Samuel, 2003). Serotypes O and A are widely disseminated but type C viruses are rare; having recently only been found only in Brazil, Kenya and Ethiopia while Asia 1 is mainly confined to the Asian continent. The SAT (Southern African Territories) serotypes are normally, but not exclusively, restricted to sub-Saharan Africa. In countries normally free of FMD, rapid confirmation of FMDV as the causative virus in material from suspect cases is the primary goal of field and laboratory investigations. A number of pan-serotype specific real-time reverse transcription polymerase chain reaction (RT-PCR) assays have been developed to target highly conserved regions of the RNA genome of FMDV (Reid et al., 2002;
Callahan et al., 2002; Moniwa et al., 2007) to detect all seven serotypes in clinical samples. These assays have undergone extensive evaluation: parallel testing of samples has shown the sensitivity of these molecular assays to be at least equal to that of the current gold standard method of in-vitro virus isolation in cell culture (Reid et al., 2003; Shaw et al., 2004; King et al., 2006). Further optimisation including the use of automated robotic equipment to prepare template RNA has simplified and speeded up the assays for routine use (Shaw et al., 2007; Reid et al., 2008, manuscript submitted for publication). A limitation of RT-PCR procedures targeting conserved sequences in non-structural and untranslated regions of the genome is that they cannot determine the serotype identity of the causative FMDV. Therefore, it is not currently possible to serotype samples that are RT-PCR positive but VI/ELISA negative. Serotyping such samples may only be accomplished by use of serotype-specific primers and probes and/or through nucleotide sequencing.

In countries such as Iran where the disease is endemic, identification of the serotypes of the causative virus strains is important for vaccine selection, disease containment and for tracing the source of the outbreaks. Previous attempts have shown that it is difficult to achieve maximum sensitivity with serotype-specific primers by conventional RT-PCR to cover the genetic diversity within all FMDV serotypes (Reid et al., 1999; Reid et al., 2001). While these procedures could be used in conjunction with antigen-detection ELISA and VI to provide additional information, they were insufficiently sensitive to replace them for primary diagnosis of FMD (Reid et al., 1999). Alternative RT-PCR assays reported by Suryanarayana et al. (1999) and Alexandersen et al. (2000) for serotype-specific diagnosis were extremely cumbersome and unsuitable for routine use in an epidemic. Giridharan et al. (2005) describe a conventional RT-PCR procedure for differentiation of FMDV serotypes native to India using multiple primers based mostly on nucleotide sequences of viruses circulating in that geographical area. Whilst able to differentiate Indian FMDV serotypes O, A, C and Asia 1; these primers would be too restrictive for detection of viruses belonging to other lineages or sub-groupings within these serotypes. However, this work demonstrated the potential to develop tailored molecular tools for detection of serotypes of FMDV.

Real-time RT-PCR (rRT-PCR) assays for the specific detection of FMDV serotypes O, A and Asia 1 recently circulating in the Middle East were evaluated in this study using primers/probe sets designed from the VP1 region on clinical samples submitted to the FAO World Reference Laboratory for Foot-and-Mouth Disease, Pirbright (WRLFMD) for virological investigation and on samples held at the Central Veterinary Laboratory in Tehran. To our knowledge, this is the first reported use of serotype-specific primers/probe sets for the detection of FMDV in clinical samples by rRT-PCR.

3. MATERIALS AND METHODS

Specific primers/probe sets were designed at the WRLFMD using PrimerExpress (Applied Biosystems) from VP1 sequence alignments of FMDV serotype O strains belonging exclusively to the PanAsia-2 lineage, from serotype A viruses of the Iran-05 lineage and from Asia 1 viruses from three relevant lineages. For serotypes O and A, two primer sets with the same probe for each (O1, O2 and A1, A2 respectively) were designed while four forward primers, two reverse primers and four probes were designed for detection of the Asia 1 strains (Table 1).

Table 1; Sequences and serotype-specificity of the primers/probes sets

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>FMDV serotype- specificity</th>
<th>Sequence (5′ – 3′)</th>
<th>Name of set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward 1</td>
<td>O</td>
<td>CCGAGACAGCGTTGGATAACA</td>
<td>O1a</td>
</tr>
<tr>
<td>Reverse 1</td>
<td>O</td>
<td>CCATACTTGCAGTTCCCGTTT</td>
<td>O2b</td>
</tr>
<tr>
<td>Forward 2</td>
<td>O</td>
<td>CCGAGACAGCGTTGGAYAAYA</td>
<td>O1/O2</td>
</tr>
<tr>
<td>Reverse 2</td>
<td>O</td>
<td>CCATACTTGCAGTTCCCGTTT</td>
<td></td>
</tr>
<tr>
<td>Panacea probe</td>
<td>O</td>
<td>CCGACTTGCACTGCCTTACACGGC</td>
<td></td>
</tr>
<tr>
<td>Forward 1</td>
<td>A</td>
<td>CACGACCATCCACGAGCTT</td>
<td>A1c</td>
</tr>
<tr>
<td>Reverse 1</td>
<td>A</td>
<td>GCAGAGGCGCTGGGACAGTAG</td>
<td>A2d</td>
</tr>
<tr>
<td>Forward 2</td>
<td>A</td>
<td>ACAGCCATCCACGACGTTC</td>
<td></td>
</tr>
<tr>
<td>Reverse 2</td>
<td>A</td>
<td>RCAGAGGCGCTGGGACAGTAG</td>
<td></td>
</tr>
<tr>
<td>A Iran-05 probe</td>
<td>A</td>
<td>CGTGCCGATGAAACGTGCCG</td>
<td>A1/A2</td>
</tr>
<tr>
<td>Forward 1</td>
<td>Asia 1</td>
<td>GCTGTAAGGGCTGAAACCATCAC</td>
<td></td>
</tr>
<tr>
<td>Forward 2</td>
<td>Asia 1</td>
<td>GCAGTWAAAGGCYGAGACATYAC</td>
<td></td>
</tr>
</tbody>
</table>
Forward 3  Asia 1  GCAGTWAAGGCGAGASCATYAC
Forward 4  Asia 1  GCAGTWAAGGCGAGASCATYAC
Reverse 1  Asia 1  GCAAAGGCCTAGGGCAGTATG
Reverse 2  Asia 1  GCAAGGCCTAGGGCAGTATG
Probe 1  Asia 1  TTGATTTCGACATGAAACGTCGGAG
Probe 2  Asia 1  AGCTTTTGATTCGCATGAAACGCGG
Probe 3  Asia 1  AGCTGTTGATTCGCATGAAACGCGG
Probe 4  Asia 1  AGCTTGTTGATTCGCATGAAACGCGG

aForward primer 1/Reverse primer 1/PanAsia O probe.
bForward primer 2/Reverse primer 2/PanAsia O probe.
cForward primer 1/Reverse primer 1/A Iran-05 probe.
dForward primer 2/Reverse primer 2/A Iran-05 probe.

Total nucleic acid was extracted from suspensions of vesicular epithelia of serotype O PanAsia-2, serotype A Iran-05 and Asia 1 viruses from the three lineages by an automated procedure as previously described (Shaw et al., 2004). The best performing primers/probe sets (O1, A2 and the Asia 1 set: Forward 3/Reverse 2/Probe 4) were then selected following evaluation of the new primers/probes on templates of the serotype O, A and Asia 1 viruses with the one-step rRT-PCR protocol described by Shaw et al. (2007).

All templates were tested by these selected one-step rRT-PCR assays (incorporating the primers/probe sets intended for specific detection of serotype O, A and Asia 1 virus sequences, respectively) run in parallel with pan-serotype specific assays (5’ UTR and 3D) using the protocol of Shaw et al. (2007). To check the specificity of the primers/probe sets, total nucleic acids were similarly extracted from FMDV serotype C, SAT 1 and SAT 2 and from swine vesicular disease virus (SVDV) isolates and each were tested by the new assays.

The O1, O2, A1 and A2 primers/probe sets were also used to test homo- and heterotypic FMDV O and A viruses at the Central Veterinary Laboratory in Tehran using a locally-employed rRT-PCR procedure.

4. RESULTS

The assays with the universal primers and probes were useful in confirming the presence of FMDV in each sample. All three serotype-specific primers/probe sets were strongly positive against total nucleic acid from homotypic viruses and no cross-reactivity was observed with heterotypic viruses except with one serotype O virus which also gave a weaker reaction with the serotype A-specific primers/probe set (Table 2). Analysis of the sequence of the type O virus did not reveal a significant similarity with those of the type A-specific primers and probe but this result will be investigated further by repeat testing. No cross-reactivity was observed when the serotype-specific primers/probe sets were tested against the other FMDV serotypes of C, SAT 1 and SAT 2 or with SVDV isolates.

Table 2: Summary of the results obtained at the WRLFMD using parallel one-step rRT-PCR assays with pan-serotypic (5’UTR and 3D) or serotype-specific primers/probe sets

<table>
<thead>
<tr>
<th>FMDV serotype or virus tested</th>
<th>Lineage</th>
<th>Ratio of number of samples positive to total number tested by rRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5’UTR 3D O^a A^b Asia 1^c</td>
</tr>
<tr>
<td>O    PanAsia-2</td>
<td>19/21</td>
<td>21/21 21/21 1/21 0/21</td>
</tr>
<tr>
<td>A    Iran-05</td>
<td>18/18</td>
<td>18/18 0/18 18/18 0/18</td>
</tr>
<tr>
<td>Asia 1 3 lineages</td>
<td>12/12</td>
<td>11/12 0/12 0/12 12/12</td>
</tr>
<tr>
<td>C, SAT 1, SAT 2 Various</td>
<td>9/10</td>
<td>10/10 0/10 0/10 0/10</td>
</tr>
<tr>
<td>SVDV Various</td>
<td>0/3</td>
<td>0/3 0/3 0/3 0/3</td>
</tr>
</tbody>
</table>

^aO1 primers/probe set.
^bA2 primers/probes set.
^cAsia 1-specific primers/probe set (Forward 3/Reverse 2/Probe 4 – see Table 1).
Evaluation of serotype-specific primers/probes in Tehran indicated that the sets A1 and A2 performed better than sets O1 and O2. When four serotype O viruses were tested, three were positive with the O1 and O2 sets but weaker positive with the A1 and A2 sets – the other virus being negative with the O1 and O2 sets but positive with sets A1 and A2. Two serotype O/A viruses were positive with the A1 and A2 sets but negative with the O1 and O2 sets and 5 out of 6 serotype A viruses were positive with sets A1 and A2 (the other being positive with set A1 only and all 6 negative with sets O1 and O2).

5. DISCUSSION

While pan-serotypic rRT-PCR assays for FMDV have been extensively validated for routine use, several challenges face the design of serotype-specific primers/probes for rRT-PCR. In-silico analysis can be used to attempt to find conserved sequences for primer/probe binding sites that allow the specific recognition of all viruses within a particular serotype, but do not cross-react with viruses of other serotypes. This work is made difficult by the high variability of the FMDV genome and lack of consistent sequences that are conserved within, and restricted to a particular serotype. For these reasons, efforts have concentrated on detecting viruses of particular serotypes from distinct geographic regions or lineages rather than all strains within a serotype. The results from this study have demonstrated the potential of using rRT-PCR for the detection and discrimination of FMDVs belonging to geographically distinct sub-groups of serotypes O, A and Asia 1 currently circulating in the Middle East. This is the first step in developing a suite of molecular tools for different countries and regions. Typing assays could similarly be developed for other geographical regions of the world. For this study, a simple approach was employed whereby specific primers/probe sets were designed from sequence alignments using basic PrimerExpress software. To complete this study, more strains (particularly reference strains) from the desired lineages need to be tested to further evaluate the diagnostic sensitivity of each primers/probe set and the analytical sensitivity of the assays remains to be determined.

6 AUTHOR’S CONCLUSIONS

The results have demonstrated the potential of using rRT-PCR for the detection and discrimination of FMDV strains belonging to geographically distinct sub-groups of serotypes O, A and Asia 1 currently circulating in the Middle East. To our knowledge, this is the first reported use of serotype-specific primers/probe sets for the detection and discrimination of FMDV in clinical samples by rRT-PCR. The study is the first step towards the development of a suite of molecular tools for different countries and regions.

7 AUTHOR’S RECOMMENDATIONS

Molecular typing assays should similarly be developed by reference laboratories for other geographical regions of the world.

8. ACKNOWLEDGEMENTS

The authors thank Dr Nigel Ferris and Geoffrey Hutchings from the WRLFMD, Pirbright for characterisation and supply of viruses and colleagues from the Central Veterinary Laboratory, Tehran for their co-operation. This work was funded by Defra, UK (Project number: SE1124).

9. REFERENCES
