DETECTION OF FOOT-AND-MOUTH DISEASE VIRUS IN BIOLOGICAL SAMPLES - RECENT DIAGNOSTIC POSSIBILITIES

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Abstract

Primary bovine thyroid cell cultures and IB-RS-2 continuous cell line were used for foot-and-mouth disease virus (FMDV) isolation. In both cell culture systems, all tested samples gave positive results and the specificity of isolated virus was confirmed by the Ag-ELISA. Results of virus isolation test agreed with those obtained by RT-PCR and rRT-PCR, which enabled detection of the genetic material of FMDV. This indicates a high and comparable sensitivity of the applied diagnostic assays, which permit a reliable detection of FMDV in biological material.

Key words: foot-and-mouth disease virus, isolation, Ag-ELISA, RT-PCR, rRT-PCR.

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease that affects domestic and wild ruminants and swine. The disease has been still considered one of the most important viral diseases causing massive losses and hindering animal trade on the world wide scale. At present, in spite of a beneficial epizootic situation in several European countries, including Poland, the disease has been frequently imposing a threat such as the latest epidemics in the Western Europe in 2001 and Turkish Thrace in 2005/2006 (10, 11, 13). The causative agent of the disease is foot-and-mouth disease virus (FMDV, family Picornaviridae, genus Aphthovirus) existing in seven serotypes: O, A, Asia 1, SAT 1, SAT 2, and SAT 3 and numerous variants. The virus genome is a single-stranded RNA with a positive polarity. The pathogen can propagate in vitro in sensitive cell culture systems, including the primary cultures of bovine thyroid and swine, calf, and lamb kidney cells, and continuous IB-RS-2 and BHK-21 cell lines (5, 10, 14).

FMD is clinically undistinguishable from swine vesicular disease (SVD) and vesicular stomatitis (VS). Thus, a rapid and reliable laboratory diagnosis is highly needed to recognize FMD; moreover, this diagnosis may form the basis for an immediate implementation of several measures to prevent the dissemination of the disease and limit its economic losses (13). Recently, the Office International des Epizooties (OIE) has recommended a virus isolation, antigen enzyme-linked immunosorbet (Ag-ELISA), reverse transcription-PCR (RT-PCR), and real - time RT-PCR (rRT-PCR) assays for virus identification (14).

The aim of this study was to compare the methods recommended by the OIE for FMDV detection in biological samples and to evaluate their usefulness.

Material and Methods

Samples. The biological samples supplied by the World Reference Laboratory for FMD in Pirbright (U.K.) as a part of FMD/SVD Ring Trials (FMD/08/1, FMD/09/1, FMD/09/2, epithelium from affected animals) and from own collection (FMD/64, FMD/75, FMD/80, cell culture suspension) were used. A positive control was a laboratory FMDV strain, serotype O, propagated on the continuous IB-RS-2 cell line culture, whereas a sample of the epithelium from a healthy and not affected animal was used as a negative control.

Cells. The studies included the primary culture of bovine thyroid cells prepared according to the method described previously (17) and the culture of continuous IB-RS-2 cell line provided by the Instituto Zooprofilattico Sperimentale della Lombardia e dell’ Emilia in Brescia (Italy) and passaged according to generally accepted methods.

Virus isolation. A confluent monolayer in 24-well plates was inoculated with 200 µl of the sample per well and incubated for 1 h at 37°C and 5% of CO₂. Following the addition of 2 ml of Eagle MEM without serum, the plates were incubated for 48 h at 37°C and
5% of CO₂. The infected cultures were daily examined microscopically to check the appearance of cytopathic effects. In the case of lack of the effects, the cell cultures were frozen and then thawed out and used to inoculate fresh cultures (second passage). The specificity of the virus was checked by the detection of the viral antigen using the Ag-ELISA (6, 9).

**Viral RNA extraction.** The total RNA was isolated using RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer’s protocol.

**Reverse transcription-PCR (RT-PCR).** The reaction was carried out using OneStep RT-PCR (QIAGEN, Germany) Kit. Two oligonucleotides P1-5’-CCTAACCTCTTCAACTACGG3’ and P2-5’GAAGGGCCAGGGTTGGACTC3’ were used as the primers to amplify the fragment of 216 bp length (13). The reaction mixture at a volume of 40 µl comprised: 10 µl of 5 x buffer RT-PCR, 2 µl of dNTPs, 0.5 µl of each primer: P1 and P2, 2 µl of enzyme mix, and 25 µl of RNase free water. The mixture was supplemented with 10 µl of RNA (total volume of 50 µl). The reaction was performed in an Eppendorf thermocycler (Mastercycler). The amplification was carried out as follows: reverse transcription – 30 min, 50°C, initial denaturation – 15 min, 94°C, amplification during 40 cycles: denaturation – 1 min, 94°C, primer binding – 1 min, 50°C, elongation – 2 min, 72°C, and final synthesis – 10 min, 72°C.

The amplification products were separated electrophoretically in a 1.5% agarose gel in the presence of TBE buffer at a constant voltage of 70 V. After electrophoresis, the gel was stained with ethidium bromide and photographed in UV light. The presence of PCR products was determined by comparison of their mobility, relative to the DNA ladder, Promega, USA. The result was considered positive when the DNA stripe was seen in the gel at the value expected for the pair of primers.

**Real-time RT-PCR (rRT-PCR).** One TaqMan primer/probe set from internal ribosomal entry site (IRES) sequences (within the FMDV RNA 5’ untranslated region - UTR) was used (14, 21). The primer sequence was as follows: SA-IR-219-246F (forward primer): (5’-CACCTYAAAGRTGACAYTGRTACTGGTAC-3’) and SA-IR-315-293R (reverse primer): (5’-CAGATYCCRAGTGWCICITGTTA-3’), FMDV IRES probe sequence: SAmulti2-P-IR-292-269R (TaqMan probe): (5’-CCTCGGGGTACCTGA YTGRTACTGGTAC-3’). The rRT-PCR was performed in MicroAmp optical 96-well reaction plate (Applied Biosystems) in one-step reaction using the QuantiTect Probe PCR Kit (QIAGEN, Germany). The reaction mixture at the volume of 19 µl contained: 12.5 µl of 2x QuantiTect Probe RT-PCR Master Mix, 1 µl (20 pmol) of each of the primers: SA-IR-219-246F and SA-IR-315-293R, 0.5 µl (5 pmol) of probe SAmulti2-P-IR-292-269R, 1.25 µl of MgSO₄ (25 mM), 0.1 µl of RNasin, 0.2 µl of QuantiTect RT Mix, and 3.45 µl of RNase free water. Six microlitres of extracted RNA was added to the reaction mix (total volume 25 µl) and the reaction was capped using optical caps (Applied Biosystems). The plate was transferred to the thermal cycler (7300 Real Time PCR System, Applied Biosystems) and amplification was carried out using the following programme: 55°C for 30 min, one cycle (reverse transcription), 95°C for 15 min (one cycle) to activate the DNA polymerases and inactivate the reverse transcriptases, and 45 cycles at 95°C for 15 s and at 60°C for 1 min. Fluorescence was measured at the end of the 60°C annealing/extension step. Cycle threshold (Cₜ) value (the point on the x-axis showing the number of cycles of replication where the fluorescence breached a threshold fluorescence line) was assigned to all PCR reactions after the amplification.

**Results**

The results of the isolation of the FMDV in cell cultures are shown in Table 1, and Figs 1 and 2. The presence of infectious FMDV was found in all examined samples. FMD/09/1 and FMD/09/2 samples produced a visible cytopathic effect (CPE) in the first passage after 20 h post inoculation (pi), both in primary bovine thyroid cell and continuous IB-RS-2 cell line cultures. The remaining samples caused CPE in the second passage: FMD/08/1 sample after 19 h pi in the two cultures, FMD/75 and FMD/80 after 24 h pi in primary bovine thyroid cell cultures and after 30 h and 24 h pi in continuous cell line cultures, FMD/64 after 30 h pi in the primary bovine thyroid cell culture, whereas in continuous IB-RS-2 cell line cultures after 48 h pi. The identification of the serotype of the virus responsible for the CPE was performed by Ag-ELISA (Table 1).

Results of the detection of FMDV by the RT-PCR showed the presence of genetic material of the virus in all tested samples (Table 1, Fig. 3). The length of the amplified products was 216 bp. No amplification was found in negative controls.

The results of the rRT-PCR assay were evaluated by the Cₜ value. All FMDV isolates tested by rRT-PCR were recognised as positive (Cₜ <40.0) (Table 1, Fig. 4). Positive control (K+) had Cₜ value of 29.23 and tested FMDV isolates (FMD/09/2, FMD/09/1, FMD/08/1, FMD/64, FMD/75, and FMD/80) had Cₜ values of 26.19, 27.06, 27.21, 34.09, 31.22, and 31.26, respectively. FMDV RNA was undetectable in the epithelium collected from healthy uninfected calf (K - negative control).

The possibility of using rapid, sensitive, and specific assays for FMD recognition is a prerequisite for prevention, control, and eradication of the disease.

The detection of FMDV in biological material involves both routine methods, which have still retained the diagnostic utility (virus isolation, Ag-ELISA) and new ones, whose usefulness has been confirmed (RT-PCR, rRT-PCR). To isolate the FMD virus, both the primary bovine thyroid cell and IB-RS-2 continuous cell line cultures were applied. The cell culture systems used in the studies gave positive results towards all examined samples; FMD/09/1 and FMD/09/2 in the first passage whereas FMD/08/1, FMD/64, FMD/75, and FMD/80 in the second one.
Table 1
Detection of foot-and-mouth disease virus in biological samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Virus isolation in cell culture/Ag-ELISA</th>
<th>RT-PCR</th>
<th>rRT-PCR C\textsubscript{1} value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMD/08/1</td>
<td>CPE II p/19 h serotype A</td>
<td>+</td>
<td>27.21</td>
</tr>
<tr>
<td>BTY</td>
<td>CPE II p/19 h serotype A</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IB-RS-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FMD/09/1</td>
<td>CPE I p/20 h serotype O</td>
<td>+</td>
<td>27.06</td>
</tr>
<tr>
<td></td>
<td>CPE I p/20 h serotype O</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FMD/09/2</td>
<td>CPE I p/20 h serotype O</td>
<td>+</td>
<td>26.19</td>
</tr>
<tr>
<td></td>
<td>CPE I p/20 h serotype O</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FMD/64</td>
<td>CPE II p/30 h serotype C</td>
<td>+</td>
<td>34.09</td>
</tr>
<tr>
<td></td>
<td>CPE II p/48 h serotype C</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FMD/75</td>
<td>CPE II p/24 h serotype A</td>
<td>+</td>
<td>31.22</td>
</tr>
<tr>
<td></td>
<td>CPE II p/30 h serotype A</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FMD/80</td>
<td>CPE II p/24 h serotype O</td>
<td>+</td>
<td>31.26</td>
</tr>
<tr>
<td></td>
<td>CPE II p/24 h serotype O</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

+ positive, p-passage

Fig. 1. Culture of bovine thyroid cells (control).

Fig. 2. Culture of bovine thyroid cells infected with FMDV (sample FMD/08/1).
It was interesting that in the case of FMD/64 and FMD/75, the CPE was seen earlier in primary bovine thyroid cell cultures than in IB-RS-2 cell line. The identification of the virus causing the CPE was performed by Ag-ELISA. The A, O, O, C, A, and O serotypes were found in FMD/08/1, FMD/09/1, FMD/09/2, FMD/64, FMD/75, and FMD/80 samples, respectively (Table 1).

Discussion

Primary bovine thyroid cell culture is the most sensitive system for FMDV detection. This view was confirmed by several authors and our previous and recent studies (2, 7, 17, 25). A continuous supply of these cells for diagnostic purposes is not easy because it needs both: technical and professional preparation.
However, when the supply is inadequate, the cells demonstrating comparable, or similar sensitivities may be utilised. The IB-RS-2 cells used in our experiment demonstrated advantageous properties because the cells may be rapidly provided, passaged, kept frozen, and rapidly resuscitated in a laboratory. The virus isolation and ELISA are the gold standard tests for diagnosis of FMD (16).

An intense development of research techniques involving molecular biology enabled the use of RT-PCR (4, 12, 15, 19, 22) and rRT-PCR (8, 18, 20, 23, 26) assays for the detection of FMDV genetic materials in various biological samples.

For the purpose of conventional RT-PCR, the primers targeting the highly conservative region of 1D and 2AB genome and permitting the detection of seven serotypes of FMDV were selected (1). The viral RNA was detected in all tested samples, apart from the negative controls (Table 1, Fig. 3). The use of RT-PCR assay enabled obtaining results in a shorter time in comparison to those obtained by the virus isolation and Ag-ELISA combined methods, and enhanced laboratory potential for FMD detection.

The electrophoresis of RT-PCR products limits drastically the use of this assay on a large scale because it is quite laborious. It is especially important for FMD, drastically the use of this assay on a large scale because it is quite laborious. It is especially important for FMD, whereas the virus isolation and Ag-ELISA combined methods, and enhanced laboratory potential for FMD detection.

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To utilise this method for our studies, the primers and fluorescence probe selected from the IRES region of FMDV genome, specific for detection of the seven FMDV serotypes, were used (14, 21). By this method it was possible to detect MDV RNA in all tested samples. Similar results were obtained using conventional RT-PCR procedure (Table 1, Fig. 4).

All used techniques gave positive results. This indicates a high and comparable sensitivity and usefulness of these methods. However, several authors reported a higher sensitivity of the rRT-PCR assay in comparison to that of the virus isolation and Ag-ELISA combined (24).

The data from the literature have highlighted that the PCR assays are more frequently used for FMD diagnosis, whereas the virus isolation is becoming rarely applied (2). However, as it was mentioned previously, FMD belongs to the most contagious infectious diseases with regard to its epizootic and economic impact. Thus, diagnostic examinations should not be limited to one method. It seems reasonable to use PCR and virus isolation assays to increase the effectiveness of recognition and to check mutually the results. Moreover, the isolation of the virus from the disease outbreak enables obtaining the material for a further characteristic of isolates and the disease immunoprophylaxis. The presented results show that the used methods permit an effective detection of FMDV in biological materials.

### References

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