FMD – MODERN LABORATORY DIAGNOSIS AS A WAY OF PROVIDING BIO-SECURITY

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Viral, contagious animal diseases, such as FMD are huge threat for health of susceptible animals species. They affect biosecurity of animal breeding and also influence badly the economy of societies.

In Europe, FMD was eliminated in most of the countries in the last decade of 20th century. Present, positive situation is a result of long standing administrative and zoosanitary actions, prophylactic vaccinations, epidemic surveillance, quick and reliable methods of recognizing the disease. FMD diagnostic laboratories have played main part in it through active involvement in every stage of trying to eliminate the disease.

According to the Council Directive 90/423///EWG prophylactic vaccinations against FMD are no longer in practice all around Europe. As a result of it, the population of susceptible animals has become sensitive to contagion of FMD virus. Epidemiologists say it might be an encouragement to some organized actions to make an outbreak of the disease and disturbing European economy.

The examples of FMD epidemics in Great Britain in 2001 and 2007 confirm the risk of probable outbreak and rapid spreading of the plague around the territory of a country and even the whole continent.

Very dynamic spreading of the disease and its negative economical consequences cause that in every single probable appearing of the disease, it is highly important to take diagnostic research in different directions to confirm or eliminate the disease agent.

At present the fastest and the most precise method of recognizing the contagious agent is real-time RT-PCR which enables to detect RNA of the virus.

The causative agent, foot-and-mouth disease virus (FMDV; family *Picornaviridae*, genus *Aphtovirus*) has a single-stranded, positive sense genome of approximately 8.4 Kb (16). There are seven serotypes of FMDV with a large number of variants spread over several regions in the world (4). At present, FMD is widely distributed in different African, South-American, and Asian countries, with serotype O having the highest prevalence, followed by serotype A (6). Outbreaks of FMD result in sanitary barriers that prevent export of bovine and swine products. Furthermore, FMD causes enormous losses to the animal industry due to costs associated with control and eradication measures, including massive vaccination and/or destruction of infected herds, as well as decreases in milk and beef production as the result of clinical disease (1).

The early detection of FMDV is critical to minimising disease spread and the significant economic implications resulting from the introduction of FMDV into a country previously free from the disease. For the laboratory identification of FMDV the Office International des Epizooties (OIE) recommends virus isolation, antigen enzyme-linked immunosorbent assay (Ag-ELISA), and reverse transcription PCR (RT-PCR) (10). In recent years, some conventional RT-PCR methods have been described for the detection and identification of FMDV (13, 15, 17, 20). Whilst this procedure successfully detects small amounts of FMDV genome in biological samples, it is labour intensive, insensitive, non-quantitative, and subjective, so limits the number of samples that can be tested in a day. The nested RT-PCR systems have also a very high risk of contamination. Therefore, several laboratories have recently been developed a fluorogenic PCR-based method (TaqMan-PCR) for the diagnosis of FMDV (2, 12, 14).

The aim of this study was to determine the diagnostic value of real-time RT-PCR (rRT-PCR) for the rapid detection and quantitation of FMDV in biological samples of archival FMDV isolates.

Material and Methods.

Sample origin. The archival FMDV isolates: serotype O (O_1 /BFS 1860/UK/67, O_1 /Manisa/TUR/69), serotype A (A_5/Allier/FRA/60, A_{22}/IRQ/24/64, A_{24}/Cruzeiro/BRA/55), serotype C (C_3/Resende/BRA/55, C/PHI/ 87), serotype SAT 1(SAT 1/BOT/68), serotype SAT 2 (SAT 2/ZIM/81, SAT 2/Eritrea/04), serotype SAT 3 (SAT 3 /ZIM/81), and serotype Asia 1 (Asia 1/PAK/98, Asia 1/Shamir/97) provided by the World Reference Laboratory for FMD (WRL FMD), Pirbright, UK, were used. Additionally, two panels of FMDV isolates supplied for the purposes of ring trial for FMD/SVD 2008 and 2009 were tested. A sample of RNA extracted from FMDV, serotype O_1 /Kaufbeuren/67, kindly provided by Dr O. Marquardt from the Federal Research Centre for Virus Diseases of Animals in Tьbingen (Germany) was used as a positive control. Moreover, an archive swine vesicular disease virus (SVDV) isolate SVDV POL/73 and bluetongue virus (BTV) serotype 8 (BTV-8/GER/2008) collected from BT seropositive cattle imported from Germany were used for checking the specificity of the applied rRT-PCR.

Nucleic acid extraction. The total RNA was isolated using the RNeasy Mini Kit (QIAGEN, cat. No. 74104) as described previously (9).

Oligonucleotide primers and fluorogenic probe. One TaqMan primer/probe set from internal ribosomal entry site (IRES) sequences (within the FMDV RNA 5' untranslated region -UTR) was used according to Reid *et al*. (14). The primers sequence was as follows: SA-IR-219-246F (forward primer): (5'-CACYTYAAGRTGACAYTGRTACTGGTAC-3') and SA-IR-315-293R (reverse primer): (5'-CAGATYCCRAGTGWCICITGTTA-3'), FMDV IRES probe sequence: SAmulti2-P-IR-292-269R (TaqMan probe): (5'-CCTCGGGG-TACCTGAAGGGCATCC-3').

Real-time RT-PCR conditions. 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, cat no. 204443). 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, SA-IR-315-293R, 0.5 µl (5 pmol) of probe SAmulti2-P-IR-292-269R, 1.25 µl of $MgSO_4$ (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 were added to the reaction mix (total volume 25 µl) and the reaction 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 (C_T) 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.

Analytical sensitivity and specificity. In order to establish the analytical sensitivity of the rRT-PCR, 10 serial dilutions (10-fold) of FMDV serotype O (O_1 /Manisa/TUR/69) were prepared. RNA was isolated from 140 µl of each dilution for rRT-PCR analysis using the

RNeasy Mini Kit, Qiagen method. The analytical specificity of the assay was evaluated by testing RNA from SVDV and BTV agents causing vesicular disease and symptomatic look-alike disease, respectively.

Results. The negative C_T value for any test and control sample corresponded to C_T of ≥40.0 and was selected as the positive/negative cut-off C_T values obtained as a consequence of examining the biological samples by three rRT-PCR assays. An example of rRT-PCR results are presented in Fig. 1.

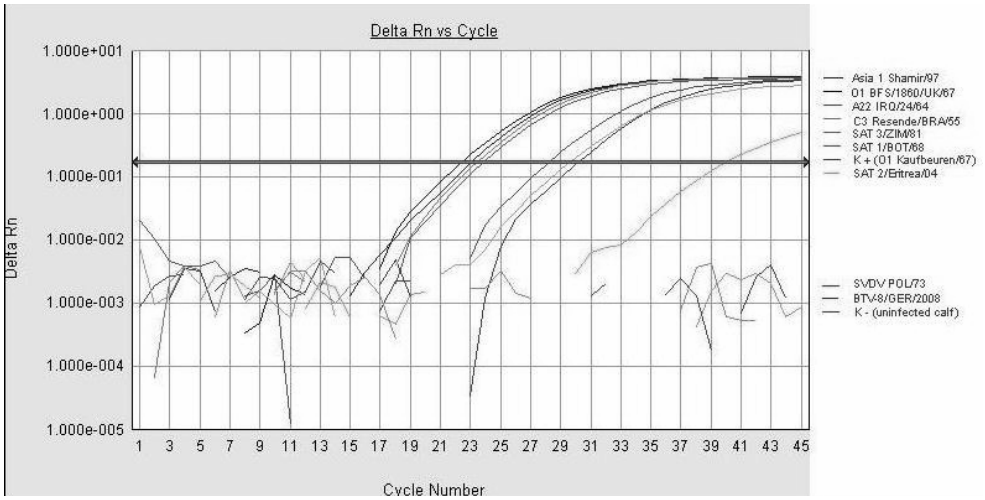

Fig. 1 Logarithmic fluorescence plots versus cycle number resulting from the determination of FMDV RNA in samples of archival FMDV isolates.

All FMDV isolates tested were recognised as positive (C_T <40.0). C_T values of positive control serotype and all serotypes examined are shown in Table 1. SVDV and BTV RNA as well as RNA isolated from the epithelium taken from uninfected calf (negative control) were undetectable in the assay applied (Table 1).

Table 1 – C_T values of control and test FMDV samples obtained by rRT-PCR assay

Virus isolate	*Country of origin*	*C_T value*
K+ (O_1 /Kaufbeuren/67)	Germany	30.14
K- (uninfected calf)	Poland	No C_T
O1 /BFS 1860/UK/67	United Kingdom	23.04
O1 /Manisa/TUR/69	Turkey	13.42
A5/Allier/FRA/60	France	31.22
A22/IRQ/24/64	Iraq	23.40
A_{24}/Cruzeiro/BRA/55	Brazil	25.06
C3/Resende/BRA/55	Brazil	23.81
C/PHI/ 87	Philippines	19.51
SAT 1/BOT/68	Botswana	29.56
SAT 2/ZIM/81	Zimbabwe	35.79
SAT 2/Eritrea/04	Eritrea	39.72
SAT 3 /ZIM/81	Zimbabwe	28.19
Asia 1/PAK/98	Pakistan	20.51
Asia 1/Shamir/97	Israel	22.41
SVDV POL/73	Poland	No C_T
BTV-8/GER/2008	Germany	No C_T

A 10-fold dilution series (100,000 – 0.1 $TCID_{50}$) of O_1 /Manisa/TUR/69 cell culture grown virus was used for the determination of the sensitivity of applied rRT-PCR. The detection limit of viral RNA by rRT-PCR was 1 $TCID_{50}$. Six subsequent dilutions up to 1 $TCID_{50}$ were scored positive by fluorogenic RT-PCR. C_T values of particular dilutions are presented in Table 2. Only 0.1 $TCID_{50}$ dilution was scored negative in the applied assay.

Table 2 – Sensitivity of rRT-PCR assay

O_1 /Manisa/TUR/69 dilutions ($TCID_{50}$)	*C_T value*
100,000	13.36
10,000	17.65
1,000	21.34
100	25.72
10	29.48
1	35.21
0.1	No C_T

Discussion. Rapid and accurate diagnosis plays an important role for the implementation of effective measures to control the spread of disease. Although recently, the European Union (EU) countries are FMD free, the global commercial trade and increasing animal and human transport keep free countries at the constant risk of introduction of the disease into their territory. Therefore, all FMD national reference laboratories and others acting as a FMD diagnostic laboratories must establish a level of preparedness that shall enable them to perform the fast and reliable detection of this pathogen. At the National Reference Laboratory for FMD in Zdunska Wola, the diagnosis of FMD is carried out by the detection of virus antigen in biological materials using a combination of ELISA and virus isolation in cell culture. ELISA is very rapid and easy to perform but the concentration of virus in the sample may be lower than the ELISA detection limit. In contrast, virus isolation in cell culture is very sensitive, although it is labour-intensive, time consuming (taking up to 4 days) and requires property equipped facilities.

In recent years various real-time PCR methods have been implemented and applied to the diagnosis of human and animal diseases (8). Real-time RT-PCR (rRT-PCR) offers certain advantages over conventional RT-PCR. It avoids the use of agarose gel electrophoresis, so decreases the risk of contamination, and is suitable for large scale testing and automation. The target amplicon is usually smaller, reducing the potential problems caused by target degradation. Detection of specific gene sequences by rRT-PCR involves monitoring of the fluorescence generated by cleavage of a target specific oligonucleotide probe during amplification. This eliminates the need to open the reaction tube post-amplification, for neither a nested step nor final agarose gel analysis of the cDNA products, greatly reducing the risk of cross-contamination.

This study describes a highly sensitive and specific rRT-PCR for viral RNA detection in samples of available archival FMDV isolates. The primers and probe for PCR reaction were selected from the IRES region of FMDV genome, the totally conserved and specific to FMD viruses; making this genomic region highly suitable for the detection of all seven serotypes of FMDV. By using these primers/probe set with a commercially available one-master mix we were able to detect viral RNA in samples of all archive FMDV isolates (Fig. 1, Table 1). It was found, that FMDV serotypes O, A, C, and Asia 1 generated high C_T values and only SAT 2 isolates generated borderline C_T values in 5'UTR assay (Fig. 1, Table 1). Our results and similar results obtained by the others (7, 14) may have been due to the nucleotide mismatches with the probe and primers and fact that more sequence information is available for the types O, A, C and, Asia 1 of FMDV for the primer/probe design.

The detection limit of rRT-PCR was quantified for 1 $TCID_{50}$ (Table 2). The C_T of 40 chosen as a positive to negative cut-off equal around 1-10 TCID/mL (approximately 1,000-100,000 molecules of RNA/mL) was adopted as C_T value by others workers (7, 11). Shaw *et al*. (18) found that rRT-PCR method had superior sensitivity to establish diagnostic methods for FMDV was detected in 18% more samples compared with virus isolation and Ag-ELISA combined.

As FMD cannot be clinically distinguished from other vesicular diseases such as SVD, vesicular stomatitis (VS), or symptomatic look-alike disease such as BT, each suspicion of the disease should be considered as FMD and the precise differential diagnosis must be performed. We found that these pathogens can be differentiated in rRT-PCR assay with FMDV specific primers (Fig. 1, Table 1). These results were in agreement with the previous observations (2, 3, 5, 19).

In summary, the current study indicates that rRT-PCR introduced recently in our laboratory is a powerful technique for reliable detection of FMDV in biological samples. The method is more sensitive and much faster to perform (approximately 5 h) than the conventional RT-PCR because it does not require agarose gel electrophoresis. In addition, the used thermal cycler allows the use of 96-well plate formats, which further increases the capacity and speed of the analysis. Therefore it is seen as a valuable tool to complement the routine diagnostic procedure for FMDV diagnosis.

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СУЧАСНА ЛАБОРАТОРНА ДІАГНОСТИКА ЯЩУРА ЯК ШЛЯХ ЗАБЕЗПЕЧЕННЯ БІОЗАХИСТУ

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На теперішній час найбільш швидкий та точний метод визначення збудника ящура – це полімеразно ланцюгова реакція реального часу, яка дає можливість виявлення РНК вірусу.

Метою цієї роботи є визначення діагностичної цінності ПЛР реального часу RT-PCR (rRT-PCR) для швидкого виявлення та кількісного визначення вірусу ящура в біологічних зразках.