

DIAGNOSTIC STRATEGY FOR FOOD-AND-MOUTH DISEASE – THE BULGARIAN APPROACH

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Introduction:

Bulgaria is FMD free country without vaccination since 1996. We use a National surveillance system, based on regular sampling of sentinel animals to prove the FMD free status. In the past only liquid phase blocking ELISA (LPBE) was used for serological investigation. In this case more time is needed to test every sample for each serotype. From 2002 NSP based ELISA tests replaced LPBE as a basic tool for sero monitoring, because they are faster and not type dependent. It is more convenient for countries like Bulgaria applying a non vaccination policy.

Nowadays we use mainly NSP ELISA for serology control combine with LPBE for different serotypes of FMDV, but it is not quite enough related to the continuously increased economic growth and vehicle and people traffic between the countries on the Balkan Peninsula region, Turkey, Middle and Far East.

For definitive diagnosis and detection of FMD virus in test samples we use antigen capture ELISA as a basic method and virus isolation on cell culture which is a “gold standard” but it requires long time to get the final result. In 2006 we implemented high efficiency molecular biological methods for detection of viral genome as real time RT-PCR to improve the laboratory diagnosis of food-and-mouth disease. Also using of pen side tests on the spot is an element of early detection, early warning and prevention system in Bulgaria.

Materials and methods:

1. Virological tests:

1.1. Chromatographic tests:

There are different modifications of chromatographic tests for FMDV detection (pen side tests) as Clearview™, Unipath Ltd., UK; Svanodip, SVANOVA and etc.

We used SVANODIP FMDV – Ag (SVANOVA) under laboratory conditions for direct detection of viral antigen in serum samples and cell culture supernatants.

Only 10 samples were tested on SVANODIP FMDV – Ag devices in 5 times dilutions (from 1:5 to 1:625) and the results were compared with the Antigen FMDV and SVDV ELISA (IAH). We used 3 reference strains of FMDV (O1 Lousana, A 5 Alier and Asia 1 PAK) as positive controls. SVDV antigen was used also to assess the test specificity.

1.2. Indirect sandwich ELISA for FMDV and SVDV antigen detection.

Indirect sandwich ELISA [Ferris, N et al., 1988, Roeder, P. L. et al., 1987] was used routinely for detection and serotyping of FMD virus in test samples. The assay was performed using the standard operating procedure. The results were compared with these from real time RT-PCR assay.

1.3. Molecular – biological tests:

Real time RT-PCR assay:

One step TaqMan real time RT PCR protocol was performed using two different probe/primer sets as follow:

Probe/primer sets	Sequences	Genome location	Research team
5' UTR real time RT-PCR			
Forward Primer:	5' - CAC YTY AAG RTG ACA YTG RTA CTG GTA C - 3'	IRES	
Reverse Primer:	5' - CAG ATY CCR AGT GWC ICI TGT TA - 3'	IRES	Reid, S et al., 2002
Probe:	5' - CCT CGG GGT ACC TGA AGG GCA TCC - 3'	IRES	
3 D real time RT-PCR			
Forward Primer:	5' - ACT GGG TTT TAC AAA CCT GTG A - 3'	3D	
Reverse Primer:	5' - GCG AGT CCT GCC ACG GA - 3'	3D	Callahan J. D et al., 2002
Probe:	5' - TCC TTT GCA CGC CGT GGG AC - 3'	3D	

Total RNA was extracted using manual extraction with QIAamp Viral RNA Mini kit, (QIAGEN) and semi automated extraction with MagaBio total RNA purification kit (Bioer technology) based on magnetic beads. RNA was subjected to PCR amplification.

The reaction mix contains forward primer (10 pmol), reverse primer (10 pmol), and probe (6 pmol) in (2x) TaqMan Master Mix and (40x) RNase + Multiscribe (TaqMan one step RT-PCR, Applied Biosystems) in 25 µl volume. We use the following temperature parameters: 48° C for 30 min. (RT step), 95° C for 10 min (enzyme activation) and 40° cycles at 95° C for 15 sec. and 60° C for 1 min. (c DNA amplification).

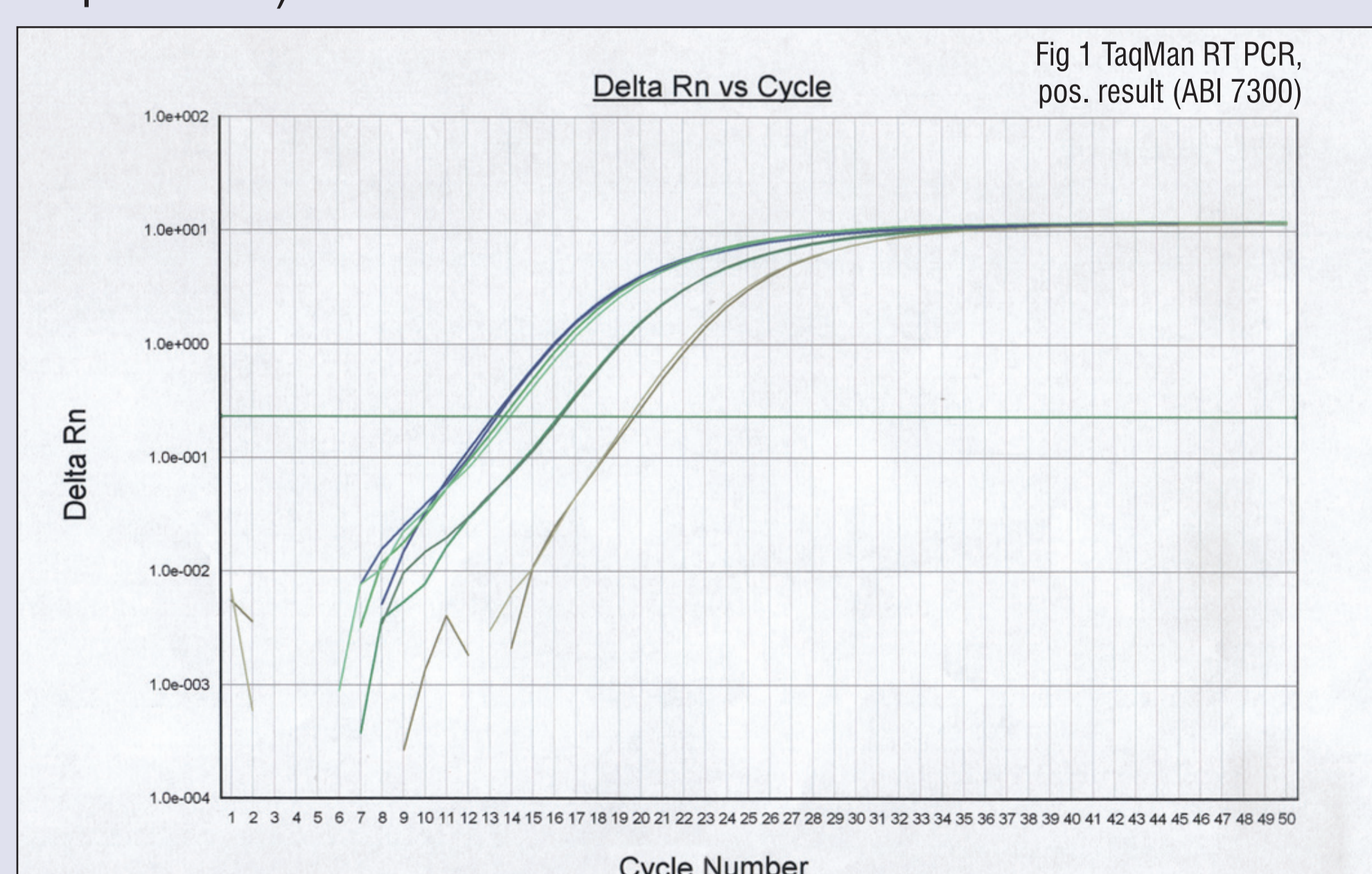


Fig 1 TaqMan RT-PCR, pos. result (ABI 7300)

PCR test panel included 40 samples (cell culture supernatants and positive serum samples). It was used 3 reference FMDV strains (O1 Lousana, A5 Alier and Asia 1 PAK) in 10 times dilutions. All samples were tested in duplicates to assess the intra-assay reproducibility. As a positive were classified all samples with Ct value less than 40.

2. Serological tests:

2.1. NSP ELISA

We used 3 different commercial NSP ELISA tests - Ceditest FMDV-NS (Cedi Diagnostics, The Netherlands), SVANOVIR™ FMDV 3ABC-Ab ELISA (Svanova Biotech AB, Sweden) and CHEKIT-FMD-3ABC (IDEXX laboratory, USA).

2.2. LPBE (Liquid phase blocking ELISA)

Liquid-phase blocking ELISA (LPBE) developed by Hamblin et al. we use as a confirmatory test for NSP positive samples according standard operating procedure using a cut off - 50% PI.

Results and discussion:

There was high correlation between SVANODIP FMDV – Ag devices and Indirect Sandwich ELISA results. 1:125 dilutions of FMDV positive controls were still detectable (weak positive) on chromatographic devices. There was no false positive reaction with SVDV samples.

SVANODIP FMDV – Ag pen side test is easy to perform, enough specific and sensitive for direct detection of FMDV antigen on a field in case of FMD suspicion. It is a tool for preliminary diagnosis and can support the laboratory confirmation by choosing of appropriate samples for laboratory analysis. SVANODIP FMDV – Ag devices are a key element of early detection and early warning system in Bulgaria.

The definitive diagnosis of food-and mouth disease should be based on complex analysis. Indirect sandwich ELISA and VNT tests are recommended by OIE for identification of viral agent in field samples.

Indirect sandwich ELISA (IAH) we use for many years as a basic method for identification and sero typing of FMD virus. Sometimes faster laboratory confirmation is needed and real time RT-PCR assay is the most suitable to supplement the routine diagnostic tests.

From 2006 we started with two step TaqMan real time PCR using probe/primer set designed from highly conserved region of FMD viral genome called IRES (internal ribosomal entry site) for detection of all seven serotypes of FMD virus. (Reid, S et al., 2002)

We used a panel of 40 samples (cell culture supernatants and serum samples) in ten times dilutions to verify the test performance. All positive samples were detected in high dilutions at about 10⁻⁷ due to high sensitivity of the assay. As a strong positive were classified samples with Ct value less than 25 – 28.

Real time PCR still can not replace the conventional methods of virus isolation on cell culture “gold standard” but supplement the conventional virological tests because it is faster, more sensitive and enough specific for detection of viral genome. It improves laboratory testing reducing the risk of carry over contamination and allowing detection of very low quantity of viral agent, below antigen ELISA detection limit.

One year ago we transfer to one step real time RT PCR trying to reduce the time needed for test performing.

Using a single buffer system in one step reaction reduce the possibility of cross contamination during the analysis and allows getting the result in 5–6 hours.

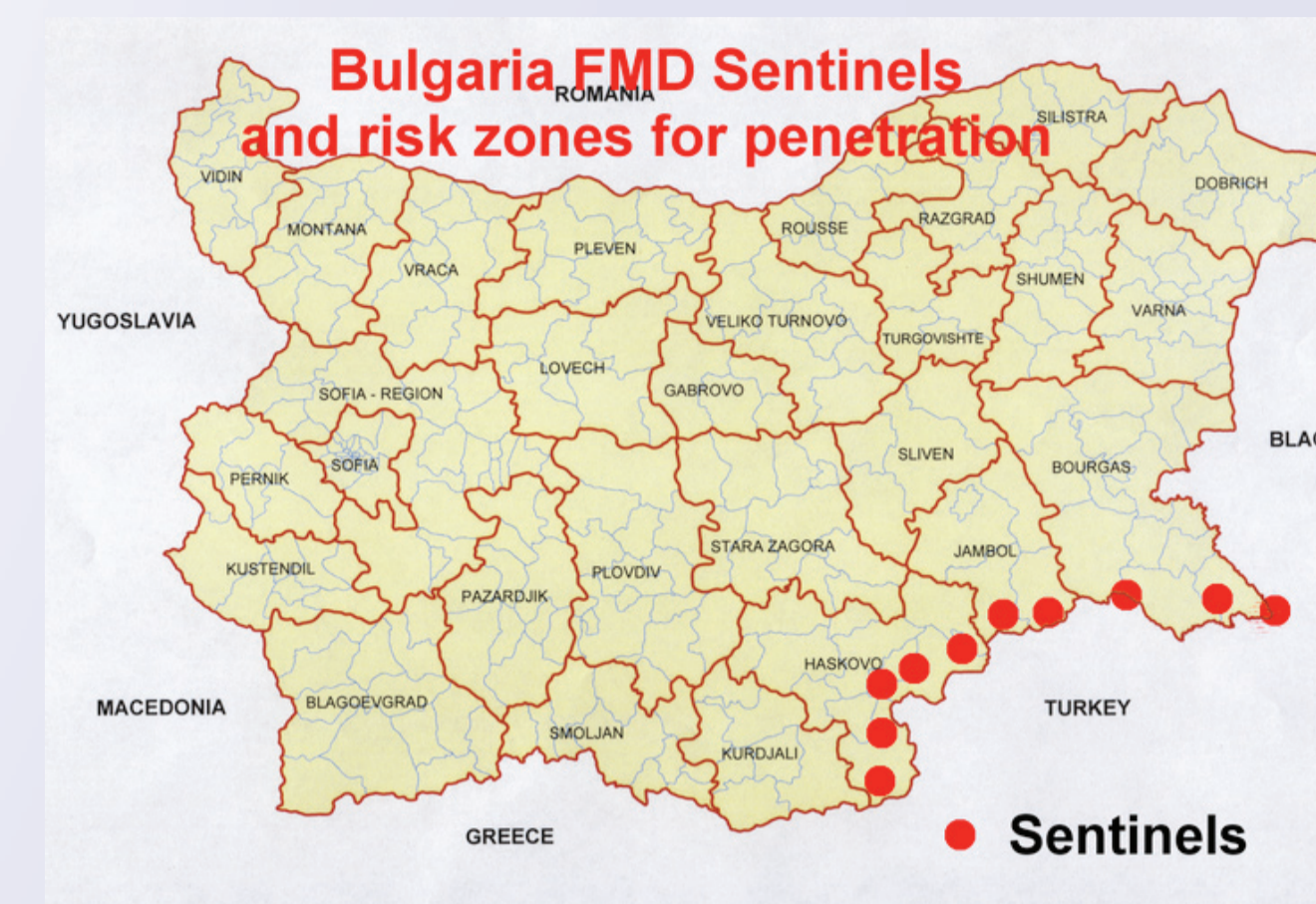
Real time PCR is also an attractive alternative to conventional PCR for viral quantification because of lower intra assay variability and equal or even greater analytical sensitivity and specificity. (Locatelli, G et al., 2000)

Using magnetic beads based procedure for total RNA extraction (MagaBio total RNA purification kit, MagMAX Viral RNA isolation kit) can eliminate the disadvantages of filter-based RNA isolation methods such as clogging and inconsistent yields of RNA.

Taking into consideration the current epidemiological situation and the potential risk of trans boundary spread of food-and-mouth disease we performed annual serological surveillance, since 1996 to prove FMD free status. It is based on regular sampling of sentinel animals located in a high risk area (10 km border zone with system Republic of Turkey).

In the past only LPBE was used for sample analysis. (Ivanov Y. et

all., 1999) From 2002 NSP ELISA replaced LPBE as a basic tool for serological monitoring. (G. Georgiev et al., 2004) This method is enough sensitive for detection not only of infected animals but also “carriers” (28 – 100 dpi). (E. Brocchi et al., 2006) and it is suitable for serological surveillance in countries like Bulgaria that don't apply a prophylactic vaccination against FMD.



Nowadays different commercial NSP ELISA tests and LPBE are in use in our laboratory to monitor the sentinel animals according to National Surveillance programs for food-

and-mouth disease. For positive results confirmation we used the scheme Cedi – Cedi retest followed by SVANOVIR NSP ELISA and LPBE. (David J. Paton et al., 2006) The practical aspect is that LPBE is not suitable for individual diagnosis and export/import purposes, because of false-positive results. VNT test should be used as a confirmatory test especially for defining of individual animal status.

To optimize the diagnostic scheme for food-and-mouth disease it is important not only the sensitivity and specificity of the method in use but also the time needed to get the final result and the ability of the laboratory test to define the actual status of individual sample.

The diagnostic tests used routinely in our lab for detection of FMD virus or for serological testing can be classified as follow:

Virological methods:	Time:	Remarks:
indirect sandwich ELISA for detection of FMDV and SVDV	24 h – 48 h	work with inactivated virus sero typing of FMD virus differentiation from SVDV
real time RT-PCR assay	8 – 16 h	high sensitivity and specificity more expensive
Virus isolation on permanent cell lines (BHK-21 and IB-RS) or primary cell cultures (pfa and lamb kidney)	5 - 10 days	require strict bio security measures (live virus)
Serological methods:	Time:	Remarks:
NSP ELISA	5 – 24 h	more convenient for surveillance purposes and herd diagnosis
LPBE	24 – 48 h	cross reaction between the FMDV serotypes

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