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IMPROVED DIAGNOSTIC STRATEGY FOR FOOT-AND-MOUTH DISEASE IN BULGARIA

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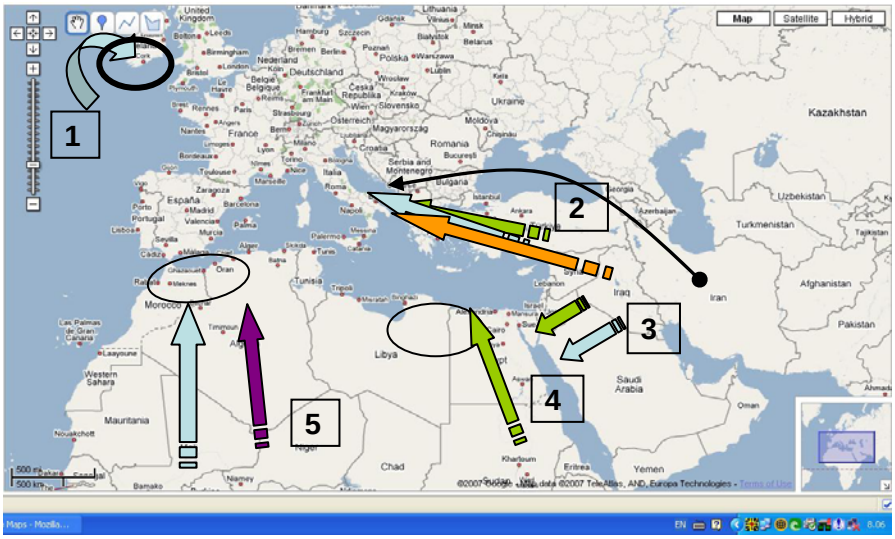
Abstract: Foot – and – mouth - disease is severe, highly contagious disease of cloven – hoofed animals that affects large animal livestock species and various wildlife species. Different countries has a different FMD status which require a disparate approach defining the diagnostic and control strategy. A variety of new diagnostic tests and procedures was developed to improve FMD laboratory diagnosis. The aim of this study is to evaluate the contemporary diagnostic tools and the ability of our laboratory to detect FMD virus or viral genome in field samples and cell culture fluids using an Ag ELISA, TaqMan real-time RT-PCR and Virus isolation combined with chromatographic - LFD (lateral flow devises) tests.

Key words: Foot – and – mouth – disease, molecular diagnostics, ELISA, Real time RT-PCR, FMD viruses, viral genome

Introduction

Foot – and – mouth - disease is severe, highly contagious disease of cloven – hoofed animals that affects large animal livestock species and various wildlifes. Different countries has a different FMD status which require a disparate approach defining the diagnostic and control strategy. The risk of disease penetration in Bulgaria is “moderate” to “high”, because of geographic position of our country (EuroAsian path), the common border with Republic of Turkey, which is FMD endemic and the invasion of new viral stains (A/IRN/05, O PanAsia) from Middle East to Turkey in last 5 years. (Figure 1).



serotype O (blue), A (green), Asia-1 (orange), SAT2 (purple)

Figure 1. Main roads for FMDV penetration in Europe in last several years (*Sumption K., 2008*)

Main priority for the national veterinary authorities in FMD free countries like Bulgaria is early disease identification and implementation of control measures. Essential for effective foot-and-mouth disease control is establishment of early detection and reporting system supplemented by fast and accurate laboratory diagnosis.

The aim of this study is to evaluate the contemporary diagnostic tools and the ability of our laboratory to detect FMD virus or viral genome in field samples and cell culture fluids using an Ag ELISA, TaqMan real-time RT-PCR *(Reid et al., 2002)* and Virus isolation *(Snowdon, 1966; Brehm et al.2008)* combined with chromatographic - LFD (lateral flow devises) tests *(Ferris, et al., 2009)*.

Key element of FMD control is identifying of primary case as soon as possible so appropriate measures to be taken to prevent further spread of the disease. It is important also to have a reliable tool for secondary cases identification.

Materials and Methods

1. Chromatographic tests (LFD, “pen side tests”)

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

Twenty samples were tested in 5 times dilutions (from1:5 to 1:625) and the results were compared with the Antigen FMDV/SVDV ELISA.

We used cell culture supernatants inoculated with 3 different strains of FMDV (O1 Losana, A 5 Alier and Asia 1 PAK) as a test samples and FMDV A 5 Alier - as a positive control. SVDV antigen was used also to asses the test specificity.

2. Indirect sandwich ELISA for FMDV/SVDV antigen detection

Indirect sandwich ELISA *(Roeder 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 (IAH).

3. Real time RT-PCR assay:

We use routinely two step TaqMan r RT-PCR. The probe/primer set are designed from highly conserved regions of FMD viral genome - IRES (internal ribosomal entry site) and 3D ensure detection of all seven serotypes of FMD virus *(Callahan et al., 2002; Ferris et al.2009).*

In 2008 we adapted one step protocols to reduce time for test performing and to minimize the possibility for cross contamination during the analysis.

One step real time RT-PCR for FMDV detection was performed using the same probe/primer set, described in Table 1.

Table 1. Primers/probe sequences and genome location *(Reid et al,.2002; Callahan, 2002)*

Probe/primer sets	Sequences	Genome location
5' UTR real time RT PCR		
Forward Primer: (SA-IR-219-246f)	5' - CAC YTY AAG RTG ACA YTG RTA CTG GTA C – 3'	IRES
Reverse Primer: (SA-IR-315-293r)	5' - CAG ATY CCR AGT GWC ICI TGT TA – 3'	IRES
Probe: (FAM-TAMRA) (SA Multi2 – P – IR – 292 -269R)	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
Probe: (FAM-TAMRA)	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 using different one step RT-PCR protocols as follow:

3.1. TaqMan One step RT- PCR Master Mix reagents Kit (Applied biosystems)

The reaction mix contains forward primer SA-IR-219-246f (10 pmol), reverse primer SA-IR-315-293r (10 pmol), and probe SA multi2-P-IR-292-269r (6 pmol) in (2x) TaqMan Master Mix and (40x) RNase + Multiscribe in 25 µl volume. We use the follow temperature parameters: 48° C for 30 min. (RT step), 95° C for 10 min and 40° cycles at 95° C for 15 sec. and 60° C for 1 min.

3.2. QIAGEN OneStep RT-PCR Kit (QIAGEN)

Using the same probe and primers set in 25 µl reaction volume containing (5x) QIAGEN One Step RT-PCR buffer, Enzyme mix, d NTPs, MgCl2 (25 nM) and cycling parameters as described below: 50° C for 30 min. (RT step), 95° C for 15 min and 40° cycles at 95° C for 15 sec. and 60° C for 1 min.

3.3. VetAlert FMDV r RT PCR kit, Tetracore, USA

This is ready to use diagnostic kit, produced by Tetracore, USA and includes primers/probe set design to detect 3D region of FMDV genome. [1] PCR mix should be proceed after dehydration in follow temperature parameters: 60° C for 10 min (RT) and 45° cycles - 95° C – 2 sec., 60° C - 30 sec.

We tested a panel from test samples (FMDV type O1 Losana and Asia1 PAK) in 10 fold dilutions to asses the analytical sensitivity of different on One step r RT PCR assays. All samples were tested in duplicates to assess the intra-assay reproducibility.

1. Virus isolation test

Cell lines

IBRS-2 and ZZ-R 127 (foetal goat tongue, FLI, Riems, Germany)

Viruses

FMDV, type O1 Losana
FMDV, type A5 Alier

Results and Discussion

There was high correlation between SVANODIP FMDV – Ag devises and Indirect Sandwich ELISA result. The two tests are enough sensitive for detection of FMDV in test samples with total agreement of 77, 4 %. 1:125 dilutions of FMDV positive controls were still detectible (weak positive) on chromatographic devises. There was no false positive reaction with SVDV samples corresponding

with high specificity of SVANODIP FMDV – Ag devises. The results are shown in Table 2 and Figure 2.

Figure 2. SVANODIP FMDV – Ag devises – pos. results

Table 2. Ag ELISA and SVANODIP results - tests sensitivity

Sample (cell culture supernatant)	Dilution factor	Ag ELISA results	SVANODIP results
FMDV O1 Lousana	1:5	pos	pos
	1:25	pos	pos
	1:125	pos	wp
	1:625	wp	neg
FMDV A5 Alier	1:5	pos	pos
	1:25	pos	pos
	1:125	pos	neg
	1:625	neg	neg
FMDV Asia 1	1:5	pos	pos
	1:25	pos	pos
	1:125	pos	wp
	1:625	wp	neg
SVDV	undiluted	neg	neg

The definitive FMD diagnosis should be based on complex laboratory testing including indirect sandwich ELISA and RT-PCR methods combine with virus isolation on cell culture (IB-RS, BHK, pig or lamb kidney) as a "gold standard" method, recommended by OIE.

Indirect sandwich ELISA (IAH) has been used in our laboratory as a basic method for identification and serotyping of FMDV. In last several years antigen ELISA is supplemented by real time RT-PCR for conformation of positive result. It is more sensitive and can guarantee fast laboratory confirmation detecting FMD virus genome even when the field samples doesn't content live virus or virus amount below ag ELISA detection limit.

We tested a panel of samples (10-fold dilutions of 2 viral strains - FMDV, type O1 Lozano and Asia1 PAK), to asses the analytical sensitivity of different one step r RT PCR assay. As a positive were classified all samples with Ct value less than 38. The results are shown on table 3. All positive samples were detected in dilutions at 10^{-4}. One step protocols shows low sensitivity compare with classic two step protocol. Ct values on QIAGEN One-Step RT-PCR was vastly high than in TaqMan one step RT- PCR Kit, (AB). So we need further optimization of one step protocols for FMDV detection.

The results are described in the table below compared with Ct-values on two step 5' UTR TaqMan r RT-PCR. (Table 3, Figure 3).

Table 3. Comparative study of One step r RT-PCR protocols (Ct values)

Sample	Dilution factor	Ct values			
		Two step 5' UTR RT PCR (IAH)	QIAGEN One-Step RT-PCR Kit	TaqMan one step RT- PCR Kit, (AB)	Vet Alert One step PCR kit, Tetracore
O1 Losana cell culture	(PC)	16.60	19.69	28.54	14.98
O1 Losana cell culture	10^{-1}	19.07	25.02	31.15	15.08
O1 Losana cell culture	10^{-2}	22.03	29.04	34.96	17.14
O1 Losana cell culture	10^{-3}	25.35	32.17	39.13	21.83
O1 Losana cell culture	10^{-4}	29.02	35.38	≥ 40.0	24.78
O1 Losana cell culture	10^{-5}	31.49	≥ 40.0	≥ 40.0	28.24
O1 Losana cell culture	10^{-7}	34.02	≥ 40.0	≥ 40.0	31.79
Asia1/PAK/ cell culture	(PC)	18.40	19.99	27.13	15.78
Asia1/PAK/ cell culture	10^{-1}	21.98	25.40	28.54	17.34
Asia1/PAK/ cell culture	10^{-2}	25.16	30.38	32.93	22.56
Asia1/PAK/ cell culture	10^{-3}	29.34	31.98	36.93	24.25
Asia1/PAK/ cell culture	10^{-4}	31.35	≥ 40.0	≥ 40.0	17.02
Asia1/PAK/ cell culture	10^{-5}	33.73	≥ 40.0	≥ 40.0	31.51
Asia1/PAK/ cell culture	10^{-7}	37.35	≥ 40.0	≥ 40.0	34.32

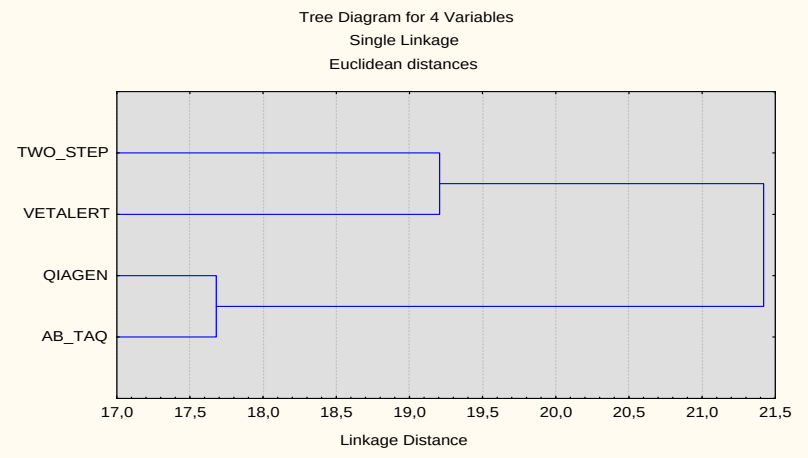


Figure 3. Comparative distance of one step r RT-PCR protocols

Virus isolation we perform inoculating IBRS-2 and ZZ-R 127 permanent cell lines in parallel with two different types of FMDV – O1 Losna and A5 Alier. Specific CPE on IBRS-2 cells we observed usually in 24h – 48h, in most cases after performing of 1 "blind" passage. In ZZ-R 127 cells the CPE appears between 18 – 24h, without need of strain adaptation. The CPE was confirmed on Ag ELISA. The results show the higher sensitivity of ZZ-R 127 than IBRS-2 cells for FMD virus isolation. (table 4., fig. 4,5).

Table 4. CPE of FMD virus on ZZ-R 127 and IBRS-2 cell lines (+ - 20% of monolayer, ++ - 40 – 50% of monolayer, +++ - 70% of monolayer и ++++ - 100% destruction of monolayer)

FMDV serotype, ELISA titre	Cell culture					
	ZZ-R			IB-RS 2		
	1-passage	2- passage		1- passage	2- passage	
	24	24	48	24	24	48
FMDV O1 Losana, 1:25	+++	++++	-	+	++++	-
FMDV A5 Alier, 1:25	+++	+++	++++	-	++	++++

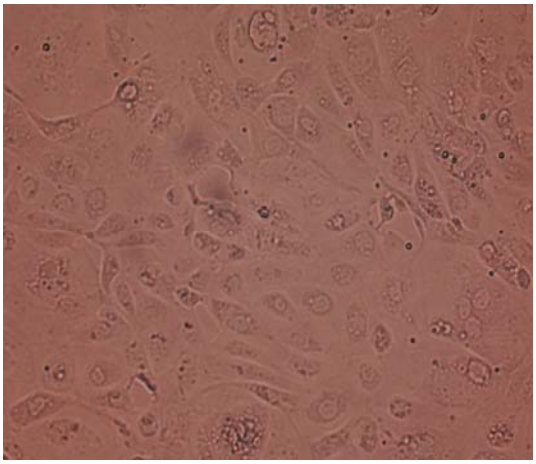


Figure 4. ZZ-R 127 cell line monolayer

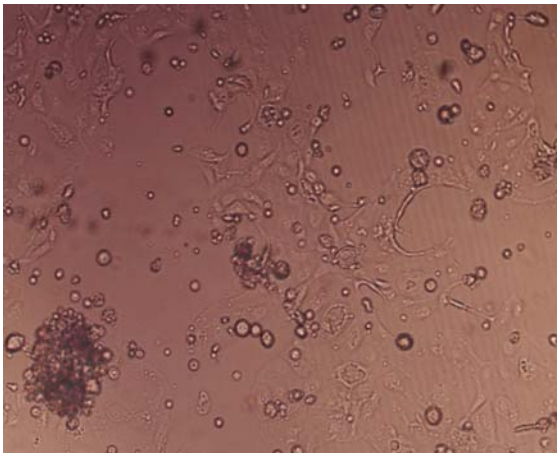


Figure 5. FMDV CPE on ZZ-R 127 cell line monolayer (type O1 Losana, 14h)

Laboratory diagnosis of FMD is based on detection of virus or viral antigen/genome in tissue or vesicular fluid or EDTA blood samples using Ag ELISA and r RT-PCR followed by isolation of FMD virus in tissue culture.

Every NRL define its own approach for FMD diagnosis in accordance with OIE Manual for Diagnostic tests and vaccines requirements. Laboratory confirmation of suspect FMD cases in our NRL involves routine methods as Ag

ELISA, real-time RT-PCR and Virus isolation tests, which are sensitive and specific for FMD virus detection.

Lately the meaning is given on decentralized diagnosis and faster identification of secondary FMD outbreaks. In support of this policy the SVANODIP FMDV – Ag test can be used as a key element of early detection and early warning system. It is rapid and easy to perform, enough specific and sensitive for direct detection of FMDV antigen on a field. It is appropriate for preliminary diagnosis in case of FMD suspicion and will support the final confirmation of the disease by choosing of appropriate samples for laboratory diagnosis.

Indirect sandwich ELISA was used routinely for detection and serotyping of FMD virus under laboratory conditions.

To improve laboratory diagnosis we adapted real time RT-PCR assay to supplement the conventional virological tests (ELISA and VNT, approved by OIE for FMD virus detection). Real time PCR still can not replace the conventional methods of virus isolation on cell culture "gold standard" but it provide faster diagnosis and has some practical advantages as high sensitivity and specificity 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 capture ELISA detection limit. Implementing one step real time RT PCR protocol combines the reverse transcription and amplification steps using single buffer system reduces the risk of cross contamination of test samples and allows getting the results in a short time period.

Optimizing the diagnostic scheme for food-and-mouth disease virus we should have also a reliable cell system for virus isolation in case of suspicion and laboratory confirmation of FMD case. The ZZ-R 127 cells, developed by *Brehm et al.* (2008) can be used as a sensitive cell system for FMD virus isolation, which allows fast isolation and confirmation of field isolate in case of disease suspicion. The diagnostic tests used routinely in our lab for detection of FMD virus can be classified as follow:

Virological methods	
indirect sandwich ELISA for detection of FMDV and SVDV	- work with inactivated virus - diferentiation from SVDV - results in 24h
real time RT-PCR assay	- high sensitivity and specificity - results in 6 -8 h
Virus isolation on permanent cell lines (BHK – 21, IB – RS or ZZ-R) or primary cell cultures (pig and lamb kidney)	- require strict bio security measures - time consuming (4 – 10 days)
LFD	- easy to perform - results in 20 min - field application - high Se

Poboljšana strategija dijagnostikovanja slinavke i šapa u Bugarskoj

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Rezime

Slinavka i šap je veoma ozbiljna, izrazito zarazna bolest kopitara koja pogađa veliki broj životinjskih vrsta, domaćih i divljih. Različite zemlje imaju različite statuse sa stanovišta slinavke i šapa koji zahteva određeni pristup u strategiji dijagnostikovanja i kontrole. Razvijeni su mnogobrojni dijagnostički testovi i procedure kako bi se unapredila laboratorijska dijagnoza ove bolesti. Cilj ovog istraživanja je bio ocena savremenih dijagnostičkih alata i kapacitet i sposobnost laboratorija da otkriju virus slinavke i šapa ili viralni genom u uzorcima sa terena i ćelijske kulture korišćenjem sledećih postupaka Ag ELISA, TaqMan real-time RT-PCR i izlocaija virusa u kombinaciji sa hromatografskim - LFD (lateral flow devises) testovima.

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