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DIFFERENTIAL DIAGNOSIS OF DISEASES, CAUSING ORAL LESIONS IN CATTLE WITH RELATION TO THE DIFFERENTIAL DIAGNOSIS OF FOOT AND MOUTH DISEASE

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Summary

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The differential diagnostics of diseases causing oral lesions in cattle can be a reason for problems in the clinical and the post-mortem pathological diagnostics. Many infectious and non-infectious diseases, different from foot-and-mouth disease (FMD), may be associated with the formation of crusts on the nasal mirror, erosions, ulcerations, necroses and sometimes formation of vesicles on the oral mucosa. There are very few signs or lesions in oral infections in cattle that may be pathognomonic for FMD. Sometimes the observed changes and clinical symptoms, based upon them, are not sufficient to be given a definitive and precise diagnosis. Such a diagnostics of FMD, a disease that provoke colossal losses to the national economy, ultimately requires clear identification and determination of the reason at the very early stage. High economic impact of FMD and the strategic geographical location of our country determine the strong need of research and permanent monitoring of the epizootic situation for FMD, both on regional and on global level to assess the risk of spread in Bulgaria. Intense international trade exchange and the integration of European countries in a single community for free trade and cross-border movement of people, animals and products, derived from them, determine the need of adequate preventive measures to protect the health of animals, humans and environment.

Key words: differential diagnostics, foot-and-mouth disease (FMD), oral lesions

INTRODUCTION

The causative agent of FMD is a virus that belongs to family *Picornaviridae*, genus *Aphthovirus*. There are seven serological types and over 65 subtypes of the virus. The FMD is a highly contagious, acute viral infection that affects cloven-hoofed animals. The disease in the various animal species is manifested by different clinical signs. In domestic animals, the most visible are the symptoms in cattle, while in sheep and goats FMD usually occurs with weak clinical symptoms and sometimes without obvious or hardly noticeable non-specific signs. The virus is characterized not only by its high stability and survival in the environment, but also with the ability to persist for a shorter or a longer period in the lymphoreticular tissue (nasal pharyngeal area and tonsils) of the reconvalescent animals. The differential diagnostics of those diseases that cause oral lesions in cattle can provoke problems in the clinical and the post-mortem pathological diagnostics (Holliman, 2005).

Many infectious and non-infectious diseases, different from foot-and-mouth disease (FMD), may be associated with the formation of crusts on the nasal mirror, erosions, ulcerations, necroses and sometimes with a formation of vesicles on the oral mucosa. There are very few signs or lesions in oral infections in cattle that may be pathognomonic for FMD. Sometimes these observed changes and clinical symptoms based upon them are not sufficient to be given a definitive and precise diagnosis. Therefore the diagnostics of FMD, ultimately requires its clear identification and determination at very early stage.

The prevention and the eradication of FMD depend on the applied system for monitoring and early detection of the infected animals or upon the detection of virus carriers. The early field recognition of that infection requires a good orientation for clinical symptoms of FMD and the sending of the most appropriate samples to the diagnostic laboratory.

According the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2011) the final diagnosis of FMD requires a demonstration of: a live virus, a viral antigen or genome in clinical specimens; for in unvaccinated animals – specific antibodies against the structural or nonstructural proteins of the virus.

This article describes three different cases from the practice of the National Reference Laboratory for FMD, including the last epizootic outbreak of foot-and-mouth disease in Bulgaria during the 2011 year. The cases were associated with the differential diagnostics of diseases causing oral lesions in cattle, which are related to the diagnostics of FMD in cattle.

MATERIAL AND METHODS

Virological tests

- Indirect (dual) sandwich ELISA for FMDV and SVDV antigen detection in field samples (mouth ulcers, vesicles, vesicular fluid, etc.).

This method allows direct serotype identification of the isolated field strain, and its differential diagnostics from swine vesicular disease virus (SVDV) (Hamblin *et al.*, 1984; Roeder *et al.*, 1987; Ferris & Dawson, 1988). The assay was performed using the standard operating procedure.

- Real time RT-PCR assay

Real time RT-PCR is a quick and reliable method for qualitative or quantitative determination in real time of a target sequence of the FMDV's genome based on the detection of the fluorescent signal. Reading of the signal is carried within the analysis, and allows tracking of the result in real time without the need of waiting for completion of the analysis (end-point analysis). One step TaqMan real time RT PCR protocol was performed using two different probe/primer sets as follow (Callahan *et al.,* 2002, Reid *et al.,* 2002) (Table 1). Total RNA was manually extracted with QIAamp Viral RNA Mini kit, (QIAGEN) and semi automated extraction with Maga-Bio 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 (2×) TaqMan Master Mix and (40× RNase + Multiscribe (TaqMan one step RT-PCR, Applied Biosystems) in 25 μL volume. We use the follow temperature parameters: 48 °C for 30 min (RT step), 95 °C for 10 min (enzyme activation) and 40° cycles at 95 °C for 15 s and 60 °C for 1 min (DNA amplification).

Table 1. Primers and probes, used for RT-PCR analysis

Probe/primer sets	Sequences	Genome location
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
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
Probe:	5'– TCC TTT GCA CGC CGT GGG AC – 3'	3D

* Reid *et al.,* 2002; ** Callahan *et al.,* 2002

Three reference FMDV strains (O1 Lousana, A5 Alier and Asia 1 PAK) in 10 times dilutions were used. 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.

- A commercial ELISA kit was used for detection of BVDV antigen (IDEXX HerdCheck ELISA BVDV Ag).

Serological tests

- ELISA for detection of antibodies against the non structural protein (NSP ELISA) of food and mouth disease virus (Georgiev *et al.,* 2001). The used kit was produced by Priocheck FMDVNS-Prionics (Lelystad B.V., Netherlands).
- Liquid-phase blocking ELISA (LPBE), produced by WRL/IAH Pirbright, UK (Hamblin *et al.,* 1986). The test is applied for detection of antibodies against different FMDV viral types strains. It was used as a confirmatory test for NSP positive samples according standard operating procedure using a cut off of 50% PI.
- Liquid phase blocking enzyme immunoassay for detection of antibodies of foot-and-mouth disease virus (Serotype O) -Mono-O Priochek.
- Commercial abELISA kit for detection of antibodies against non structural protein NS3 of bovine viral diarrhea virus (BVDV), produced by IDEXX. The optical density was reported with spectrophotometer (Dynatex) with a wavelength of 450 nm, according the recommendation of the producer and a detection of parameters for a validation of the test.

RESULTS

Clinical and epidemiological studies

Case 1. During the summer of 2010 a message for a sick cow and a calf in a farm with 50 dairy cows from Nov Pat district of Vidin city was received. Both, clinical examination and epidemiological investigation were performed. The cow was 3,5 years old. An intense salivation, erosions on the hard palate and the base of the tongue, gingivitis and reduced milk production were observed (Fig. 1). The body temperature was normal. The calf was only with a salivation, gingivitis and softened stools. The interdigital space, the

coronary edge, the udder and the teats had no changes during the clinical examination. No lameness was observed during walking. The appetite was maintained. The other cows in the farm did not show any changes in their physical condition and productivity.

Fig. 1. Erosive gingivitis and salivation in a cow, Nov Pat district, Vidin city.

Different samples for laboratory tests were taken as follows:

- Blood samples from the sick cow, the calf, other 10 cows from the same herd.
- Oesophageal-pharyngeal fluid (OP-sample) from the cow and the calf.
- A blood sample from a clinically healthy pig grown in a barrack within the farm, which was fed milk from the local dairy point.

Case 2. On 15.02.2011 the National Reference Laboratory received samples as follows: 20 blood sera and 6 samples for virological examination (lingual epithelium and oesophageal-pharyngeal secretion sample) from cows in a farm with 200 dairy cows from Stefan Karadjovo village, region Yambol. The detailed clinical examination in some animals showed intense salivation with a presence of strands of vitreous-transparent saliva (Fig. 2), erosions on the hard palate, the tongue and the gingiva (Fig. 3 & 4), but without a drop in milk yield. Body temperature was normal. There were no changes in the interdigital space, the coronary edge, the udder and the teats. No lameness was observed during the walking and the appetite was maintained. The other cows in the farm did not show any clinical signs and changes of their productivity. About a week the different wounds and lesions in the oral cavity of the cows had been irrigated with disinfectant solutions and treated with Granulin-a drug formulation for local use. Stefan Karadjovo village is well known from the history, because in 1991 there was a case of FMD there. Therefore the increasing epizootic process in the Burgas region gave a real reason that farm could be suspected for appearance of FMD.

Fig. 2. Intense salivation in a cow.

Fig. 3. Erosions on the hard palate and the tongue of a cow.

Fig. 4. Deep lesions on the tongue and gingivitis in a cow.

The later epidemiological investigation established the probable reason for erosions were traumatic injuries due to the consumption of crude and inappropriate feed.

Case 3. Twenty one buffaloes from Republic of Turkey, resided on the Rezovska municipality territory (Fig. 5) during the FMD epizootic case in the beginning of 2011, did not show any clinical signs of the disease. Two parcels with samples in the National Reference Laboratory for foot-and-mouth disease and swine vesicular diseases were received as follows:

- On 21.01.2011 – 6 blood sera for serological test from the buffaloes mentioned above.
- On 25.01.2011 – 8 blood sera from buffaloes for serological test, 8 samples for virological examination (lingual epithelium, retropharyngeal samples and epithelium from the hard palate), oesophageal-pharyngeal secretion (OP sample) from one buffalo with old lesions in the oral cavity (suspicious for passed recent FMD infection). The animals were in good clinical condition (Fig. 6). The herdsmen had used the better conditions for grazing on the Rezovo municipality's territory, because the average daytime temperatures at that time of the year (mid-January) were more than 12 ^{o}C.

Fig. 5. The pasture near Rezovo village (arrow).

Fig. 6. All buffaloes were in good clinical condition.

Laboratory tests

Case 1. The results from the laboratory tests are presented in Table 2. The virological and serological tests for FMDV were negative in all samples. The result for SVDV from the pig's sample was also negative. There were only one positive result from the serological study (Ab ELISA – BVDV) and two positive results from the virological test (Ag ELISA – BVDV), both, in the samples from the cow and from the calf, which determined the BVD diagnosis (in accordance with the observed clinical signs in the field).

Case 2. Negative results for the presence of antibodies against structural and non-structural proteins of the FMDV were received from the study of blood sera with NS ELISA and Mono – O - type ELISA Prionics. All OP samples tested by rRT-PCR were also negative (Fig. 7).

Case 3. The laboratory tests with LBPL ELISA of blood sera for the various serotypes of FMDV showed antibodies against O-, A-, Asia1-types. The most remarkable and the higher titers were against the O-type (Table 3). The testing with Mono – O - type ELISA confirmed the observed result (Table 4). Probably the buffaloes had been vaccinated in Re-

Table 2. Results from the laboratory tests

Animal species	Laboratory methods used and results obtained				
	Ag ELISA BVDV	Ab ELISA BVDV	Ag ELISA FMDV (SVDV)	LPBL ELISA FMDV	rRT-PCR FMDV
Cow	+	–	–	–	–
Calf	+	+	–	–	–
Pig	–	–	–	–	–

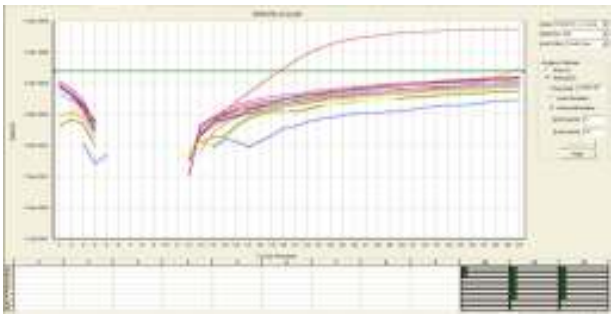

Fig. 7. RT-PCR results from suspicious samples obtained from villlage St. Karadzhovo on 16 February 2011.

Table 3. Serological results from WRL IAH Pirbright test of buffaloes, imported from Turkey

Buffalo No	Type-O/VNT	Type-A/VNT	Type-Asia1/ VNT	Final results
1	POS/512	POS/32	POS/128	POS
2	POS/128	POS/40	POS/16	POS
3	POS/512	NEG/<8	NEG/8	POS
4	POS/512	POS/64	POS/64	POS
5	POS/512	POS/178	POS/45	POS
6	POS/512	POS/45	POS/45	POS
7	POS/512	POS/355	POS/64	POS

public of Turkey (the end of October and the beginning of November 2010). These vaccines (O, A, Asia1 types) are administered from years in this country.

Table 4. Serological results for FMD from Mono-O-type prionics linear titration ELISA test of buffaloes, imported from Turkey

Buffalo No	Mono-O-type prionics linear titration
1	1:1024
2	1:256
3	1:1024/1:2048
4	1:1024
5	1:1024
6	1:256

Table 5. FMDV Mono-O-type prionics linear titration ELISA and RT-PCR results

Buffalo No	Mono-O-type prionics linear titration	One step rRT-PCR
1	1:1024	Negative
2	1:128	Negative
3	1:1024	Negative
4	1:64	Negative
5	1:256	Positive (Ct=31.58)
6	1:256	Negative
7	1:128	Negative
8	1:128	Negative

Most of the animals had antibodies to non-structural proteins of FMDV, but due to the lack of data for the age of the animals and the number of the vaccinations, these data could not be discussed, although in virgin (non-vaccinated) animals they are considered to be a result from a passed circulation of "wild" virus of any of the types FMDV or an evidence of infection with it, because they are not type-specific.

The results from the molecular-biological investigations of the samples from the epithelium, OP secretion and hard palate showed only one positive result in one sample (buffalo No 5), corresponding to a serum sample with Mono – O - type ELISA titer (Prionics linear titration) of 1:256 (Table 5).

DISCUSSION

Clinical signs in cattle with oral lesions can be a result of many diseases or infectious agents different from FMD in cloven-hoofed animals and may lead to disputable diagnostic results. These are a number of viral diseases such as viral vesicular stomatitis (VVS), papular stomatitis in cattle (Orf), mucosal disease-bovine viral diarrhea virus (BVDV), malignant catarrhal fever in cattle (MCF), rinderpest, bluetongue, bovine rhinotracheitis (IBR), enzootic haemorrhagic disease in deer (EHD), and a number of diseases or con-

ditions with non-contagious nature. In all cases, especially when it concerns for an individual animal, the laboratory testing for confirmation or exclusion of the diagnosis FMD (including demonstration of viral antigen/nucleic acid or serologically) is obligatory.

In the case with the cow and the calf from the farm in Nov Pat district, Vidin city, the lesions were localized only in the mouth of the cow and specific antibodies against BVDV were found both in the cow and the calf. The basic method for diagnostics of FMD used in most of the European laboratories is indirect "sandwich" ELISA (agELISA). Antigen ELISA technique prove 70–80% from the positive samples. At the same time of the testing the type of viral strain (O, A, Asia-1, SAT-1, SAT-2, SAT-3) could be determined. This method also detects the antigen of swine vesicular disease (SVDV) and has an important differential diagnostic significance in the practice for testing of field samples, because of the similar clinical signs in both diseases.

The definitive negative results from the molecular test and the serological test of suspected animals for FMD were crucial for the rejection of this diagnosis. The positive result with ELISA tests for antibodies and antigen of BVDV gave rise for definitively confirmation of this diagnosis.

The main argument related to the cows from the Stefan Karadjovo village for quick rejection of FMD diagnosis was the strong negative result from the rRT-PCR test. Negative result for the presence of antibodies against structural or nonstructural proteins of the FMDV in the early stages of infection could mislead the investigator. The combined use of Ag ELISA and rRT-PCR allows establishing positive samples, which contain insufficient FMDV under the diagnostic threshold of Ag ELISA method. Additional information in relation to the problem dated 10 days before treatment of the oral lesions required another test in order to confirm the reliability of serologically negative results with NS ELISA and O-ELISA Prionics tests. Here, in contrast to the cases, when there are a few cases of affected animals, the capacity for collection of samples, suitable for laboratory test, is larger.

Another advantage gives the ability for obtaining indicative data from the clinical examination of a larger number of animals. The absence of lesions on the legs and the udder and particularly the absence of a fever and reduced productivity did not support the diagnosis of FMD. Nevertheless the laboratory confirmation/rejection of the diagnosis is obligatory and crucial (Polihronova *et al.,* 2011).

The rRT-PCR method can be used both as a confirmation and mass screening for identification of hidden virus infection (carrier status). According the choice of primers for serotyping of FMDV it can be used for determination of the virus type.

Probably the buffaloes from Rezovo had had some levels of protective immunity against FMD, which was due to regular scheduled vaccination in Turkey with trivalent vaccines during the last three months of 2010 year. This immunity had different strengths of antibodies to the various components of the vaccine type (O, A, Asia-1) with values ranging from zero, unsatisfactory, non-protective to satisfactory and protective. Distinctly the higher titers against type O of the FMDV should be considered as a result of the entrance of the animals in an infected environment of active infection with circulating "wild" O-type FMDV followed by further upgrading of the immunity. Such high titers (some animals had titers of

1:1024 and 1:2048 binary logarithm upon a base 8, detected by monospecific O-type FMDV ELIS of Prionics) could not be reached even at a revaccination. Only one of the buffalo calf which showed low titers of the protective O-type antibodies had old lesions on the tongue, probably resulting after hidden FMD infection.

CONCLUSIONS

Mucosal disease-bovine viral diarrhoea (BVD) and other diseases or conditions from the non contagious nature causing stomatitis and oral lesions in cattle may lead to controversial clinical diagnostic results and the most important contagious disease for our country – FMD can be omitted.

The clinical signs in cattle with oral lesions could result from different diseases or infectious agents different from FMD. It is necessary to examine the largest number of available animals and additional data have to be colected, which could direct the epizootiological investigation and the virological laboratory tests. In all cases, especially when it concerns individual animals, it is obligatory to run laboratory tests for confirmation/rejection of the FMD diagnosis, including demonstration of viral antigen/nucleic acid or serological test.

Probably the Turkish buffaloes in the region of Rezovo had had some level of immunity as a result of the recent vaccination in November 2010 in Turkey. This immunity in serotypes A and Asia-1 with different protective levels varied in individual animals from unsatisfactory and non-protective to satisfactory and protective which have been recorded by ELISA.

The distinctly higher titres against serotype O of FMDV, established by ELISA, were probably a result from the entrance of the animals in an environment of active infection with circulating "wild" O-type virus and followed by further upgrading of the immunity (booster effect) of the buffaloes with high and protective titers (1:1024 or 1:2048 binary logarithm upon a base 8), recorded with mono-O-type FMDV ELISA of Prionics.

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