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MORPHOCLINICAL ASPECTS AND DIAGNOSIS REGARDING BLUETONGUE OUTBREAKS IN ROMANIA

ASPECTE ANATOMOCLINICE ŞI DIAGNOSTICUL DE LABORATOR ÎN FOCARELE DE BLUETONGUE DIN ROMÂNIA

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ABSTRACT | REZUMAT

Bluetongue is a non-contagious, insect-borne disease, caused by the virus of the genus *Orbivirus, Reoviridae family*, and affects domestic and wild ruminant and camelid as well, with a variety of clinical symptoms, starting from an unapparent evolution to serious and deadly cases, this polymorphism of anatomo-clinical expressions being dependent by a series of factors linked to the viral strain and host (species, breed, age). Clinical signs and lesions of the disease are produced by the vascular lesions consecutive viral replication in vascular endothelium of small blood vessels, like infarcted tissue, hemorrhages, edema and disseminated intravascular clotting.

From the appearance of the cases in august 2014 to last cases in autumn of 2015, the laboratory investigations performed by the National Reference Laboratory for Arbovirosis of the Institute for Diagnosis and Animal Health and the national network of County sanitary veterinary and for food safety laboratories have identified cases of bluetongue, serotype 4, in 401 cattle, 334 sheep, 7 goat, 7 bisons and 1 muflon out of 1006 cases tested during 2014 and in 34 cattle and 3 sheep out of 54104 cases tested in 2015.

Key words: Bluetongue, serotype 4, Romania

Bluetongue este o boală infecțioasă necontagioasă, produsă de un virus încadrat în genul *Orbivirus*, familia *Reoviridae*, ce afectează rumegătoarele domestice și sălbatice, precum și camelidele, cu o varietate de simptome clinice, pornind de la o evoluție inaparentă și până la cazuri grave și mortale. Acest polimorfism al aspectelor anatomo-clinice este dependent de o serie de factori legați de tulpina virală și de gazdă (specie, rasă, vârstă). Semnele clinice și leziunile sunt determinate de leziunile vasculare consecutive replicării virale în endoteliul vascular al vaselor mici de sânge, respectiv infarcte tisulare, hemoragii, edeme și coagulare intravasculară diseminată.

De la apariția primului caz de boală din august 2014 și până la ultimele cazuri din toamna anului 2015, investigațiile de laborator realizate în cadrul Laboratorului Național de Referință pentru Arboviroze din cadrul Institutului de Diagnostic și Sănătate Animală și în rețeaua laboratoarelor sanitar veterinare și pentru siguranța alimentelor județene au identificat infecția cu serotipul 4 de virus bluetongue la 401 bovine, 334 ovine, 7 caprine, 7 zimbri și 1 muflon din 1006 cazuri testate în 2014 și la 34 de bovine și 3 ovine din 54104 cazuri testate în anul 2015.

Cuvinte cheie: bluetongue, serotip 4, România

The onset of bluetongue needs an epidemiological chain consisting of 3 simultaneous links: the animals of a susceptible species, bluetongue viruses (BTV) and the competent vectors. As a result, the global distribution of bluetongue viruses follows the distribution of the competent vector species (2). These vectors are represented by the insects from *Culicoides* genus, *Diptera* order, *Ceratopogonidae* family. The favorite area of the culicoids midges are the warm and wet places, with standing water and swamps, with high content of organic matter. Due to the influence of the distribution and of the biological cycle of vectors on the epidemiology of bluetongue, in temperate regions, the disease has a seasonal evolution - at the end of summer and autumn (5), whereas in the subtropical regions, the diseases occurs during the whole year (the rainy season).

Unlike other arboviruses, bluetongue viruses are not vertically transmitted among generations of vectors, i.e. there is no trans-ovarian transmission with the egg, but it is described the phenomenon of the persistence in the ecosystem of the bluetongue viruses (*overwintering*) during the cold season, when, despite the interruption of the contact between the vector in-

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sects and the susceptible population of ruminants, the virus survives over the winter. (6)

After an infecting bite of the culicoid vectors, BTV are transported through the dendritic cells to the regional lymph node, where the primary replication occurs. From this level, BTV associated to blood cells reach the target organs of the secondary viral replication, especially lung and spleen. The viral replication sites are represented by the vascular endothelial cells as well as mononuclear phagocytes (monocyte, macrophage). Unlike the primary viremia, in the secondary viremia the BTV can be found only associated to trombocytes and red blood cells, and are located in the invaginations and folds of the membrane. Due to this phenomenon, BTV is protected from the influence of the humoral immune factors, so the viremia in ruminants coexists with the presence of high titers of specific antibodies protecting the host against reinfection with an homologue serotype. (4)

In the viremic blood of ruminant species, the infectious virus can be identified for a period between 14 and 90 days, depending on the species. The viral RNA persists up to 145 days. So, the lifespan of the red blood cells will determine the duration of the viremia. Thus, the sheep and goats are viremic up to 54 days after the infection, and in cattle even 90–100 days. (4)

The disease is subclinical in cattle and in wild ruminants and obvious clinical manifestations were recorded in sheep, especially in fine wool breeds. The goats become infected less frequently with BTV and rarely express clinical illness, also. There is even less signs of disease cases in bison and mouflon.

The lesional picture of the disease are due to the increased vascular permeability and inflammatory reactions produced by the virus mediated excess production of some mediators. Thus, the infection of endothelial cells by BTV induces the synthesis of interleukins (IL-1, IL-6, IL-8) and cyclooxygenase 2 and interferon-1, who are the mediators involved in the pathogenesis of viral hemorrhagic fever in general. (1)

As a result, the main lesions of the BTV infection are the damages of vascular endothelia of the small blood vessels of tissues – arterioles and venules, the occurrence of vascular thrombosis causing the infarction of irrigated tissues, and the increased vascular permeability that will produce edema in tissues. In the pathogenesis of the disease there is also a coagulopathy of consumption, expressed as a disseminated intravascular coagulation, characteristic for the serious forms of the diseases,with abundant hemorrhages, described mainly in sheep. Moreover, the phenomena of activation lung endothelial cells, which imply an increased synthesis of vasoactive and inflammatory mediators, are said to be much more accentuated in ovine than in cattle. (7)

MATERIALS AND METHODS

During the two years considered in this paper, were performed 54054 serological tests from whole blood samples, 3033 RT-PCR tests from EDTA blood and 2244 RT-PCR tests from organs of cattle, sheep, goats, bisons, 1 lama, 1 deer and 1 mouflon.

A range of clinical data of from the sick animals from whom the samples were taken are available, as well as several details of the morphopathological picture of the animals sampled for organ samples. The diagnostic tests were performed in the County sanitary veterinary and food safety laboratory, as well as in the National Reference Laboratory (NRL) for Arbovirosis, active within the Institute for Diagnosis and Animal Health. The samples from the very first outbreaks were sent to the European reference laboratory in Pirbright, UK, for the confirmation of the disease and for additional investigations.

The methods used in laboratory (County laboratory and NRL Arbovirosis) were: competitive / blocking ELISA (Ingezim BTV Compac 2.0 – Ingenaza, Bluetongue Virus Antibody Test Kit – VMRD Inc, Pourquier Bluetongue Competition – Pourquier Institute, IDEXX Laboratories), Real time RT-PCR - SuperScript. III One-Step RT-PCR System with Platinum Taq DNA Polymerase" (Invitrogen), virus isolation on cell cultures – VERO (ATCC CCL–81, kidney cell line from *Cercopithecus aethiops*),staining method HE and HEA. SR EN ISO 17025/2005 has been implemented in all laboratories as a referential for the quality management system. Methods used at the EuRL Pirbright: BTV group – specific qRT-PCR (targeting Seg-10), BTV 4 real-time RT-PCR assay (targeting Seg-2) and virus isolation on cell cultures.

RESULTS AND DISCUSSION

The onset of the disease, in Romania, was unexpected, far from the border with Bulgaria that had experienced outbreaks with BTV 4. The first suspicion of disease was in the county of Buzău, in a bovine. The case was subsequently confirmed by laboratory tests.

The clinical signs most frequently recorded in the outbreaks were: conjunctivitis with periorbital edema, congestion of muzzle and lips, erosions and crusts at the cutaneous-mucosal junction, petechiae on the

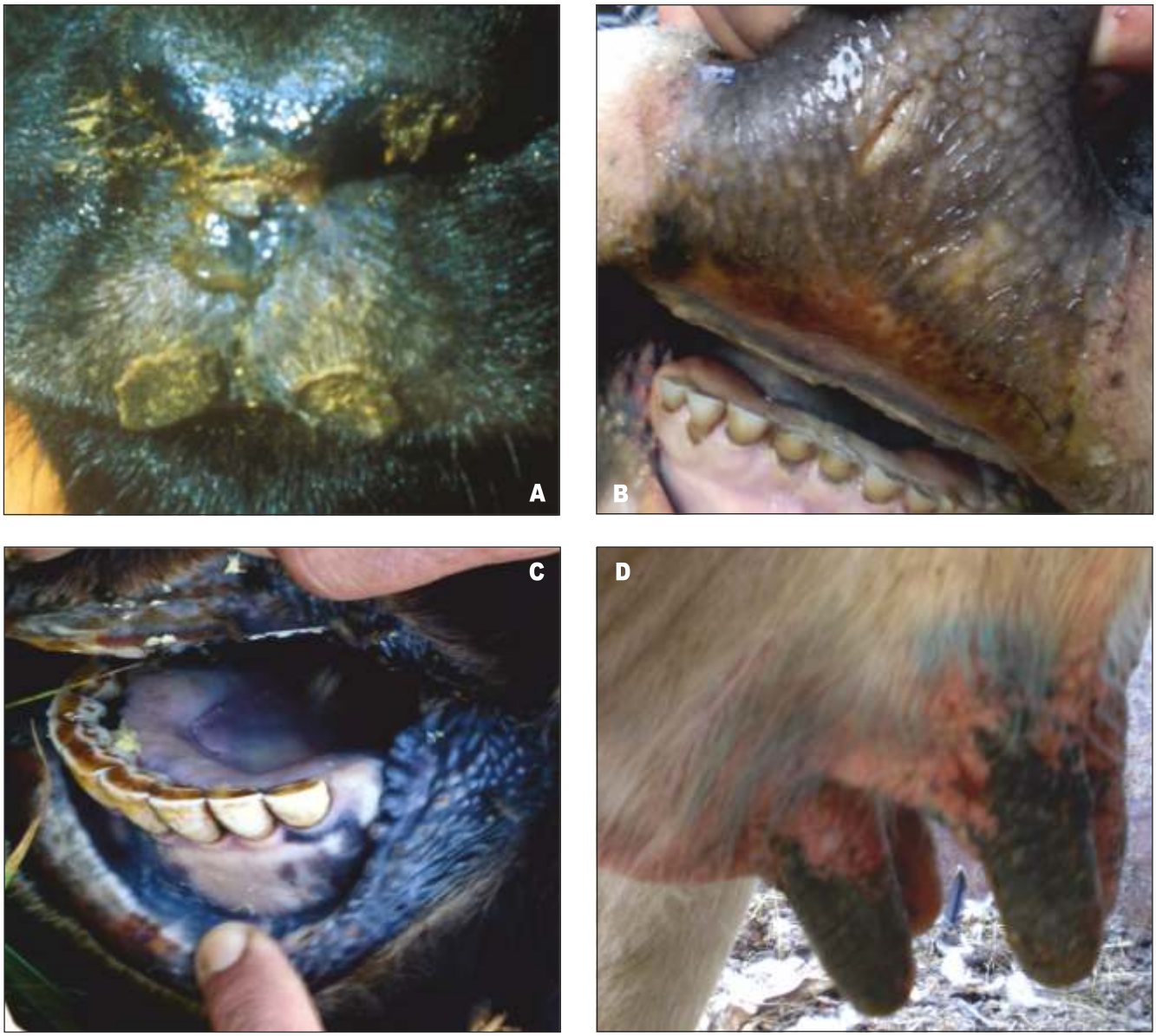


Fig. 1. Frequently observed clinical signs in Bluetongue: (**A**): Ovine from the County of Buzău: Skin erosions covered by crusts on muzzle and nose, mucous nasal discharge; (**B**): Cattle in Buzău County: Multifocal hemorrhages and superficial erosions on muzzle and nostrils, erosions of gums, swollen tongue, cyanosis, nasal discharge; (**C**): Bison, Hunedoara County, multifocal hemorrhages and superficial erosions on muzzle, hemorrhages and edema on lips and gums; (**D**): Cattle, Vrancea county. Skin hemorrhages on ventral abdomen and udder accompanied by skin necrosis and erosions.

nostrils, serous and sero-mucous nasal discharge,cyanosis and edema of tongue, petechiae and ecchymoses on teats with ulcers and necrotic lesions, interdigital petechiae and ecchymoses. Less frequent clinical signs reported are coronitis, generalized edema, ocular discharges, hemorrhages and ulcers on tongue, petechiae and ecchymoses on the scrotum, perineal and inguinal region, and a lower milk production.

The clinical expression of the disease is extremely variable, and is primarily dependent on the host species. In the published literature the clinical signs are more frequently described in sheep, while in cattle, goats and most of wild ruminants, the evolution of the disease is predominant asymptomatic or subclinical.

Despite this, the serotype 4 present in Romania caused obvious clinical symptoms in cattle and bison in most of the cases.

Hemorrhagic diathesis of the mucosa and organs is a characteristic of the morpho-pathological aspects of the disease, and is the result of virus-mediated lesions on small blood vessels, with subsequent vascular thrombosis, infarcted tissues and hemorrhages.

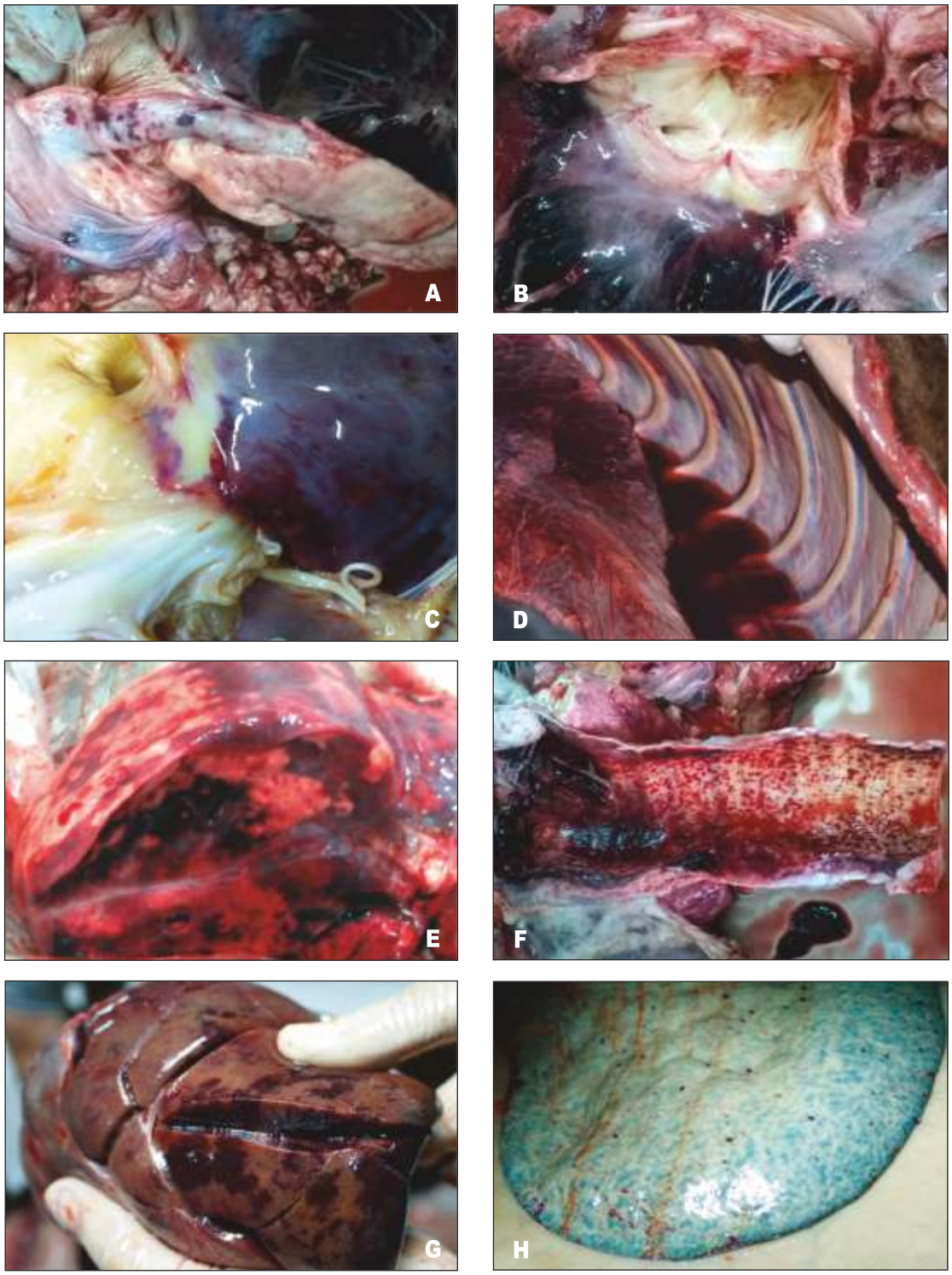


Fig. 2. Frequently observed lesions in Bluetongue: (**A**): Sheep, (**B**), (**C**), (**E**), (**F**): Cattle; (**D**), (**G**), (**H**): Bison.
(**A**): heart and aorta artery, hemorrhages in the form of petechiae and suffusions in the endocardium and aorta artery; (**B**): heart and aorta artery, subendocardial hemorrhages in the left ventricle; diffuse hemorrhage at the level of the tricuspid valves of the aorta artery; multiple hemorrhages at the level of papillary muscles; (**C**): Heart, hemorrhages at the base of pulmonary artery, hemorrhages in the form of petechiae and suffusions with trend of confluence in the endocardium of right ventricle; (**D**): thoracic cavity, hemorrhages in the lung, at the level of the ribs pleura, massive blood accumulation; (**E**): section through the lung, hemorrhage; (**F**): Trachea. Petechiae and suffusions with trend of confluence at the level of tracheal mucosa, blood clots at tracheal bifurcation; (**G**): Section through the kidney, Haemorrhages to the surface and in the depth of the kidney cortex; (**H**): Spleen with the disseminated petechiae across the surface.

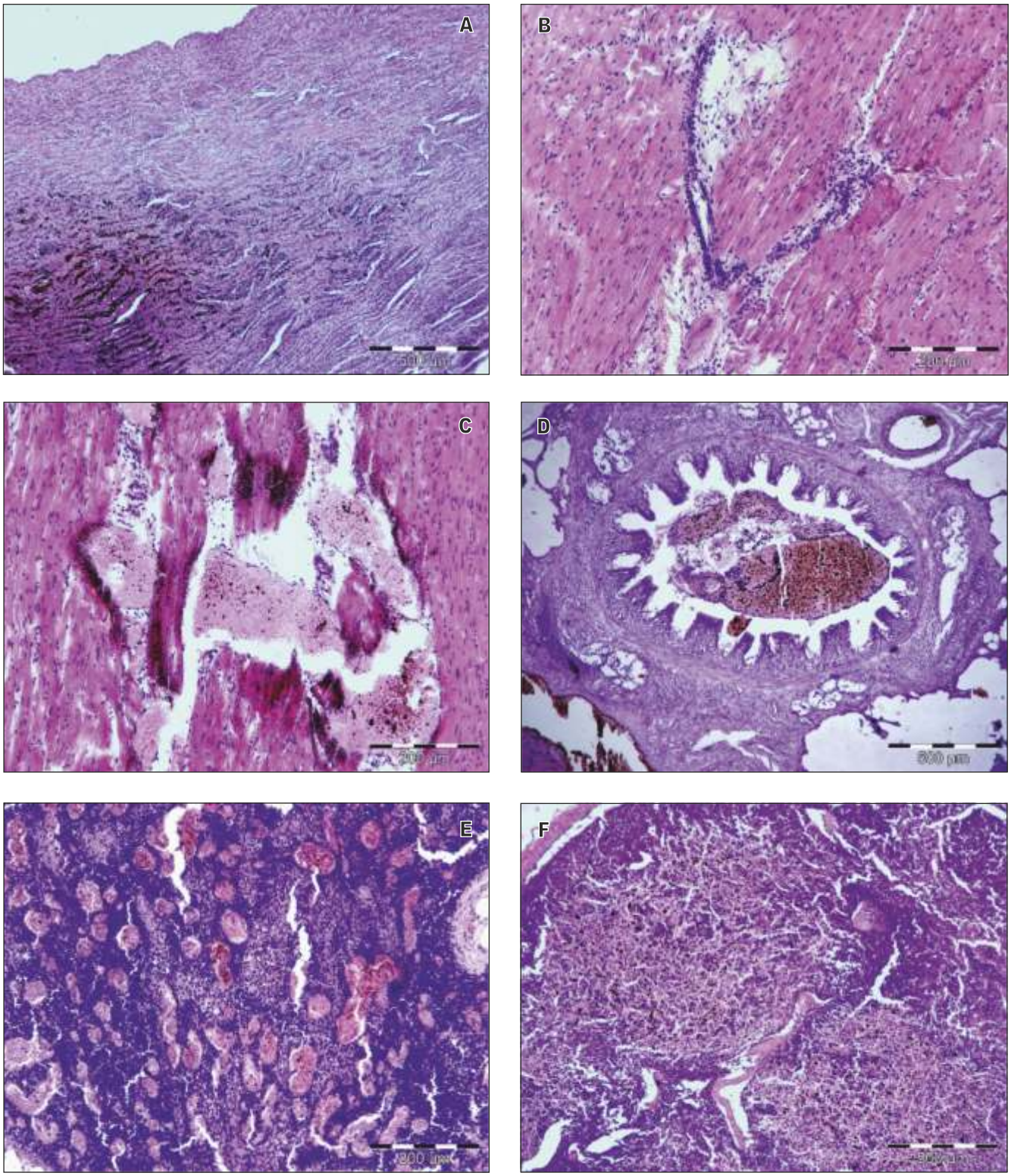


Fig. 3. Section stained with HE and HEA showing frequently observed histological lezions in Bluetongue: (**A**): Fragment of pulmonary aorta. Haemorrhagic foci in the middle tunic, HEAx40; (**B**): Heart. Perivasculitis accompanied by the small haemorrhages, oedema and local hialinizations of the cardiomyocytes; (**C**): Heart. Parietal endocardum, haemorhagic - necrotic foci with lysis of cardiomyocytes and a beginning of local infarction, HEx100; (**D**): Lung, bronchia with festooned and hiperplasic epithelium; haemorrhagic – necrotic detritus in bronchial lumen, HEx40; (**E**): Lymph nod. Lymphocitosis in cortical area, proliferation of dendritic cells and macrophages in yhe medullary area, acute hyperaemia, HEx100; (**F**): Spleen, haemorrhagic – necrotic foci in the cortical spleen; HEx40.

Table 1

Active and passive surveillance for Bluetongue, in Romania, in 2014-2015

No.	Species	2014				2015			
		No. serological tests	No. RT-PCR tests		No. positive cases	No. serological tests	No. RT-PCR tests		No. positive cases
			EDTA blood	Set organs			EDTA blood	Set organs	
1.	Cattle	663	1321	151	364	50156	939	10	34
2.	Sheep	331	692	1895	292	2057	36	43	3
3.	Goats	10	25	31	7	837	16	8	0
4.	Llamas	0	0	0	0	0	1	0	0
5.	Bisons	2	1	6	7	0	0	0	0
6.	Mouflons	0	1	0	1	0	0	0	0
7.	Stags	0	0	0	0	0	1	0	0
Total:		1006	2040	2083	671	53050	993	61	37

Characteristic aspects of the disease are the hemorrhages located at the base of pulmonary artery, as well as in the papillary muscles.

The histopathological investigations highlighted vascular lesions expressed by arteritis, with bleeding infiltrations in the middle tunic in most cases. A constant lesion was the bleeding infiltrations at the base of pulmonary artery and the heart valves. Perivasculitis, microhemorrhages, necrosis and infiltrates with lymphocytes and histiocytes of different intensities in heart, lung, lymph nod and spleen were found inconstantly.

At the National Reference Laboratory for Arbovirosis were carried out virus isolation on cell cultures and genome detection by RT – PCR on the samples (EDTA blood and organs) from the very first outbreaks and the EuRL has confirmed lately the results of these laboratory investigations. The situation of serological investigations and of the viral genome testing during the evolution of the disease in Romania is presented in Table 1.

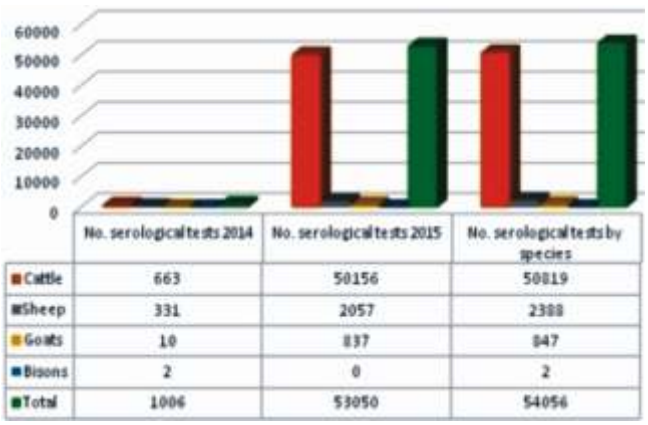


Fig. 4. Serological investigations for BT in Romania, in 2014–2015

In 2014, at the onset of the disease, the testing was carried out mostly with tests of viral genome detection on blood sample and organs. Afterwards, in 2015, the serological surveillance, including that of sentinel animals, was performed on a much larger number of samples. A graphic representation of the serological surveillance and BTV detection by PCR are presented (Fig. 4, Fig. 5). The surveillance for BT in 2014 was performed with serological tests on a low number of samples before the onset of the first outbreaks in August, as this type of surveillance is specific for a disease free territory. In 2015, the serological surveillance included also the sentinel animals for detection of possible BTV outbreaks.

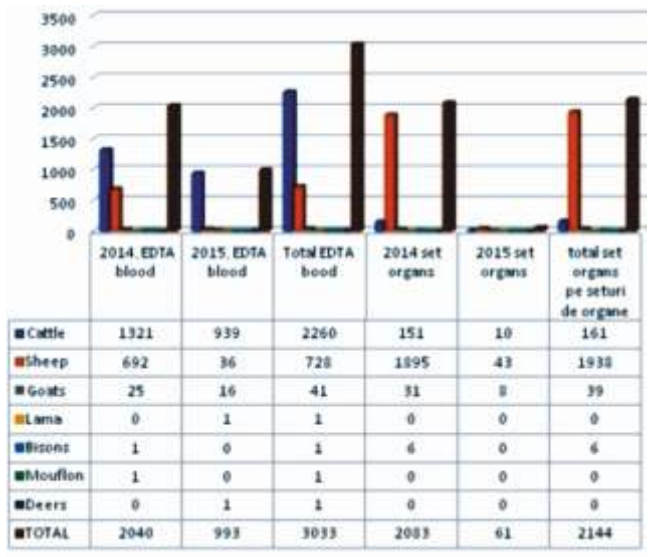
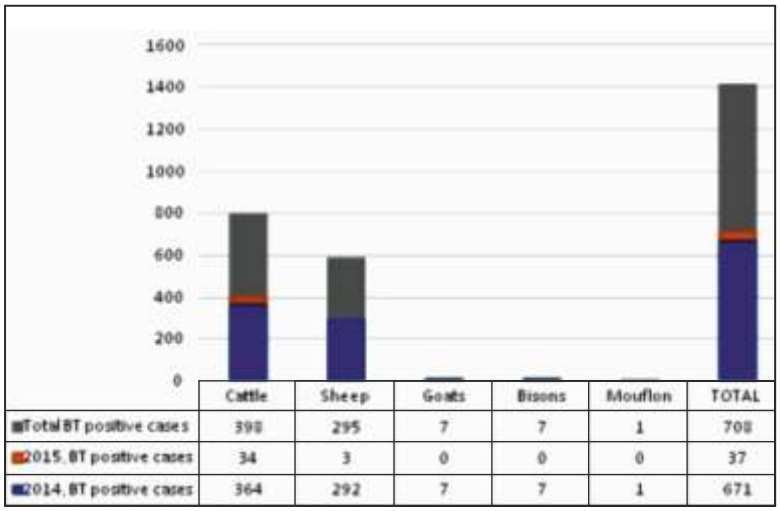


Fig. 5. Detection of BTV genome by RT-PCR in Romania, in 2014-2015

In Romania, the diagnosis of bluetongue was performed with molecular biology tests for the detection of the viral genome.

In 2015, the detection of the viral genome was used for the confirmation of cases with positive serology.

The infection with BTV was found in 708 animals, of which 398 cattle, 295 sheep, 7 goats, 7 bisons and 1 mouflon (Fig. 6). Samples from the very first outbreaks were sent to the **EuRL – The Pirbright Institute** (Ash Road, Pirbright, Surrey, GU24 0NF, UK) for confirmatory and additional tests confirming the evolution of BTV serotype 4 in Romania.



	Cattle	Sheep	Goats	Bisons	Mouflon	TOTAL
Total BT positive cases	398	295	7	7	1	708
2015, BT positive cases	34	3	0	0	0	37
2014, BT positive cases	364	292	7	7	1	671

Fig. 6. Total of BT cases in Romania, in 2014-2015

EuRL Pirbright sent the following comments: "**Sequencing and phylogenetic analyses of Seg-2 from Romanian isolates confirmed identification of BTV-4. These analyses showed that Seg-2 of Romanian groups with other strains of BTV type 4 from Sudan, Portugal, Spain, Corsica, Sardinia and Italy (with 95.6–94.1 nt sequence identities), thereby demonstrating that it belongs to the major Western topotype of BTV-4**."

Fig. 7. The map of the regional distribution of bluetongue outbreaks in the period 2014–2015. Scale: approx. 1/18.000.000 (OIE report)

Epidemiological data indicate the evolution of BTV serotype 4 in about 200 outbreaks in Greece during May–July, 2014. Bulgaria has recorded its first outbreak on July 4th 2014, in the southern area. Until the beginning of September, the disease was recorded in 21 out of 28 territorial administrative units.(Fig. 7) (3)

CONCLUSIONS

The fighting of infections produced by the bluetongue viruses is difficult, as it is difficult to fight the vectors and/or to prevent their access in the populations of susceptible animals.

The eradication of the disease is also a long-term process, depending on the animal density, the weather conditions and the geographical characteristics of a given the territory.

Irrespective of the mortality, the losses produced by the disease are due to the long convalescence, with low milk and wool production, as well as low reproductive performance and also abortion cases.

The trade restrictions linked with the occurrence of the disease in an administrative territory are another cause of losses. When the disease eradication decision includes also the specific prophylaxis, it is necessary to add the costs for the vaccination.

For the serotype 4, in bovine, the lesions are similar with those from sheep, contrary to what is described in the published literature.

Unlike other bluetongue serotypes, the evolution of the serotype 4 in Romania had the effect of affecting the bovine in an unexpectedly high percentage, with obvious clinical manifestations and high mortality.

The diagnostic methodology applied,in accordance with the flowcharts stipulated in the bluetongue diagnostic manual used in the NRL of IDAH, allowed the isolation of the causal agent of the bluetongue cases produced by the serotype 4 virus.

"**Romanian isolates together with isolates from neighbouring countries collectively represent an on-going BTV outbreak, caused by intro-**

duction of a novel western-topotype strain of BTV-4 into the eastern Mediterranean and Balkan region / Europe during 2014. Although the high nt identity observed indicates a very recent ancestry for Seg-2 of these viruses, it does not preclude the possibility of larger sequence variations in other genome segments, that could be caused by genome segment exchange/reassortments between more distantly related parental strains" – EuRL Pirbright.

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