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# INVESTIGATION ON MALIGNANT CATARRHAL FEVER VIRUS IN WILD RUMINANTS

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**ABSTRACT**

Malignant catarrhal fever (MCF) is a fatal disease responsible for the mortality of domestic, wild ruminant species and those inhabiting zoological garden.

The aim of this study is to investigate pathohystological and molecular biological peculiarities of MCF viruses isolated in large and small wild ruminants from the zoos of Sofia and Varna.

Different samples originated from 14 dead and alive wild animals (4 bisons, 3 gaurs, 2 yaks, camel, 2 cameroon goats, 1 hippopotamus, and 1 capricorn) were used for investigations.

Typical pathological changes have observed as mild circumferential limbal keratitis, disseminated foci of consolidation of pulmonary lobes, hemorrhagic cystitis, superficial necroses in small intestine and petehiae and haemorrhages in fore stomach.

Bbronchointerstitial pneumonia with hyaline membrane formation, lymphocytic-histiocytic endarteritis and phlebitis, hemorrhages in urinary bladder, hypoplasia of spleen, necrosis of the superficial epithelium of intestine and ulceration, in lymph node subcapsular lymphoid and histiocytic trafficking, neutrophils and mononuclear cells and carbonate uroliths in kidneys, subendocardial and subepicardial haemorrhages and huge necrosis in hard musculature were observed.

By conventional and nested polymerase chain reaction (PCR) for MCF virus of organ samples and cell cultural MCF virus isolates bands with molecular weight of 422 bp and 238 bp respectively were observed.

**Key words:** malignant catarrhal fever, pathohistological signs, PCR and wild ruminants.

## Introduction:

Malignant catarrhal fever (MCF) is sporadic fatal viral disease in cattle, buffaloes and wild ruminants characterized with high mortality (over 90 %). The disease is characterized with profuse nasal and ocular discharge, inflammation, exudation and ulceration in the mucous membranes of the respiratory tract and the mouth, keratoconjunctivitis with graying of the cornea and peripheral lymph nodes enlargement (Plowright, 1990). There are two epidemiological forms - African and European. The causative agent of African form of MCF (WD-MCF, AHV-1) is alcelaphine herpes virus 1 (AHV 1) belonging to the family Alcelaphinae (Plowright et al, 1960; Roizman et al, 1992). The European form of virus (SA-MCF, OHV-2 MCF) is ovine herpes virus 2 (OHV-2) (Goetze et Liess, 1929; Goetze, 1930; Li et al, 1995; 1996, Collins et al, 2000) and reservoir and source of the infections are the sheep.

Wide specter of sensitive animals to OHV-2 MCF has been determined – American bisons (Ruth et all., 1977, Reid et al., 1984, Schultheiss et al., 2000, O'Toole et al., 2002, Beresowski et al., 2005, Li et al., 2006, Cunha et al., 2012, Nelson at all., 2013), deers (Crawford et al., 2002, Klieforth et al., 2002, Keel et al., 2003, Palmer et al., 2013), American elk, buffalos (Tham 1997, Costa et al. 2009), different breeds of antelopes (Benetka et al., 2009, Taus et al., 2014), elephant seals (Goldstein et al., 2006), goats (Jacobsen et al., 2007) and sows (Loken et al., 1998) and different types of

exotic animals in zoo parks including giraffes (Hänichen et al., 1998, Li et al., 1999, 2013 Okeson et al., 2007, Campolo et al., 2008, Cooley et al., 2008, Bratanich et al., 2012).

In the fall of 2014 year in the zoos of Sofia and Varna were observed wild animals - gaurs, bisons, camel, antelopes, yaks, capricorn and hippopotamus with the MCF symptoms. By epizootological, clinical and pathoanatomical investigations MCF viral infection has confirmed (Hristov and Peshev, 2014). The purpose of this research is pathohystological and molecular biological investigations of MCF viruses isolated from those wild ruminants

**Materials and Methods**

Clinical-pathohistological studies and viral laboratory examinations of 14 wild ruminants (4 bisons, 3 gaurs, 2 yaks, camel, 2 cameroon goats, 1 hippopotamus, and 1 capricorn) from the Sofia and Varna zoos have performed. Tissue samples from liver, lungs, kidneys, heart, spleen and small intestines were collected for histological examinations. The materials were fixed in 10% neutral formalin, embedded in paraffin and processed routinely for histological examination (Lillie, 1965). The samples were sliced with thickness 5–6 μm and colored with hematoxylin and eosin.

Peripheral blood leucocytes from buffy coat, 10 % suspension of internal organs - lungs, speen, liver, brain, kidneys in phosphate buffered saline (PBS) with pH 7.2, or saline were used for MCF viral isolation. Different primary and permanent cell cultures – primary rabbit kidney (RK), embrional bovine trachea (EBTR), sheep fetal timus (SFT), Madin Darbi bovine kidney (MDBK) have infected with 0.2 mL of 10 % virus suspension and roller cultivation has been used. Several passages of viruses in cell culture were performed after observation of visible cytopathic effect (CPE). Titration of the virus isolates were accomplished by the method of Reed and Muench (1938). The type of DNA was proved by treatment with iod desoxiuridine (IDUR) and ether for existence of lypoprotein membranes.

To extract DNA pieces of internal organs and cell cultures, previously infected with 10% organ suspension from spleen, lymph nodes, liver, lungs and kidneys) buffy coat and brain samples were used. DNA was obtained by the described methods of Maniatis et al, (1982) with phenol, chloroform, isoamyl alcohol (25:24:1) and by kits GiAmp, Giagen (Pvt Ltd), Bioline and Robosceen, (Germany) for DNA extraction, according to manuals of the manufacturers. Classical and nested PCR for proving of the MCF viruses was applied (Li et al. 1995). Primers, described by Baxter et al. (1993), which were homologous to a portion of the DNA of sheep herpesvirus 2 (OHV 2) were used for confirmation of the MCF isolates. In both, first and second round of the reaction, the DNA was in quantity 150–200ng/μL (tabl. 1).

**Table 1: Nucleotide sequence of used primers, positions in the MCF genome and size of the products obtained after PCR.**

| Primer | Nucleotide sequences | Position 5'–3' | Product [bp] |
|---|---|---|---|
| 556 | 5'- AGTCTGGGTATATGAATCCAGATGGCTCTC-3' | 38–68 | 422 |
| 755 | 5'- AAGATAAGCACCAGTTATGCATCTGATAAA-3' | 460–431 | 422 |
| 556 | 5'- AGTCTGGGTATATGAATCCAGATGGCTCTC-3' | 38–68 | 238 |
| 555 | 5'- TTCTGGGGTAGTGGCGAGCGAAGGCTTC-3' | 275–247 | 238 |

Hot start Fideli Tag PCR master mix (2X) was applied for performing of PCR with the equipment for PCR QB. In the first round of PCR 12.5 μL Hot start Fideli Tag PCR master mix (2X),

primers 556 и 755 (10 pM) in quantity 0,5 μL, DNA matrix 150 ng/μL in quantity 1 μL, and distilled water up 25 μL were used. For the second step of PCR were used the parameters of the first round of PCR. As a target for a second round of PCR were used the obtained DNA in the first round of PCR in quantity between 150–200 ng/μL. The condition for both steps of PCR were: pre-denaturation to 95 ºC for 5 min, followed by 34 cycles, consisted of denaturation to 94 ºC for 1 min, attachment of the primers to 60 ºC for 1 min and elongation to 72 ºC for 2 min, final extension at 72 ºC for 7 min. For visualization of the reactions were used 5 μL from amplified DNA product and 4 μL of gel loading buffer. The electrophoresis was performed for 30–60 min at 90 V, 155 mA in a 2 % agarose gel and staining with ethidium bromide (10 mg / mL). DNA molecular weight marker 100 bp (Boehringer Manheim) was used for determination the size of the amplicons. The positive reaction was conirmed when the products of the first round of PCR were with size 422 bp, and after nested PCR were with the size 238 bp.

As controls for properly passing of the reaction with the selected primer pairs were used positive for bovine MCF DNA probes from lymph nodes, spleen and buffy coats, kindly donated by prof. Groshup, Germany. DNA obtained from buffy coats and brain samples from cattle without clinical signs of MCF and distilled water were used as the negative controls.

**Results**

In the clinical studies were found fever, lack of appetite, spiking of hair, redness of the conjunctiva, serous-purulent discharge from the nose, teeth grinding, ataxia, in some animal's bloody diarrhea, depression, inability to straighten, bed-sores and death at the 24–48 h.

Corneal opacity in both eyes and mucous purulent exudate from the nasal cavities, petechiae and ecchymosis in the tracheal lumen was observed in the pathological examinations of three bison (Bison bison). Mediastinal lymph nodes were enlarged and juicy. After section of abdominal cavity, the surface of the omentum was with numerous hemorrhages. Liver was pale, swollen with rounded edges and with yellowish and yellow-brown zones. The gallbladder was extremely enlarged and filled with around 600–700 ml fluid. Externaly, the forestomach and abomasum were without lesions and the hyperemic areas were observed on the mucous surface after opening. Numerous petechiae and ecchymoses were found under the serous surface of the small intestines and on the mucosa, more extensive longitudinal hemorrhages were visible. The most severe hemorrhages in the jejunum and ileum were observed. The content in the colons was dark brown in color, but there were not hemorrhages on the mucosa. The spleen was flat with atrophy and the white pulp was not visible after the cutting. Mesenteric lymph nodes were small. The urinary bladder was filled with urine and the blood vessels were dilated. Kidneys were with more light and pale areas. Consolidation and interstitial emphysema in the lungs were observed. Numerous petechiae and ecchymoses and extensive hemorrhages around adipose tissue of pericardium were found. Coronary and interventricular vessels of epicardium were with extensive hemorrhages and numerous pale zones in cardiac muscle were observed. Histopathological examination revealed total necrosis of the intestinal villi in the small intestines, hemorrhages in intestinal lumen, dilated blood vessels in lamina propria (Fig. 1 A), subserosal hemorrhages and hemorrhages in muscular layers of small intestines (Fig. 1 B). The spleen was with severe hypoplasia of the white pulp and a lot of siderocytes in the red pulp (hemosiderosis) (Fig. 1 C). In portal tract and midzone of the liver swollen and numerous multivacuolated hepatocytes were observed (Fig. 1 D). In the heart were observed subendocardiac and subepicardiac haemorrhages and large necroses with calcification in the cardiomyocytes (Fig. 1 E). Neutrophils

and mononuclear accumulations of cells were found in the glomerulus and renal tubules and carbonate uroliths (Fig. 1 F, G) were observed in the lumen of renal tubules. In the lungs was observed extensive purulent pneumonia.

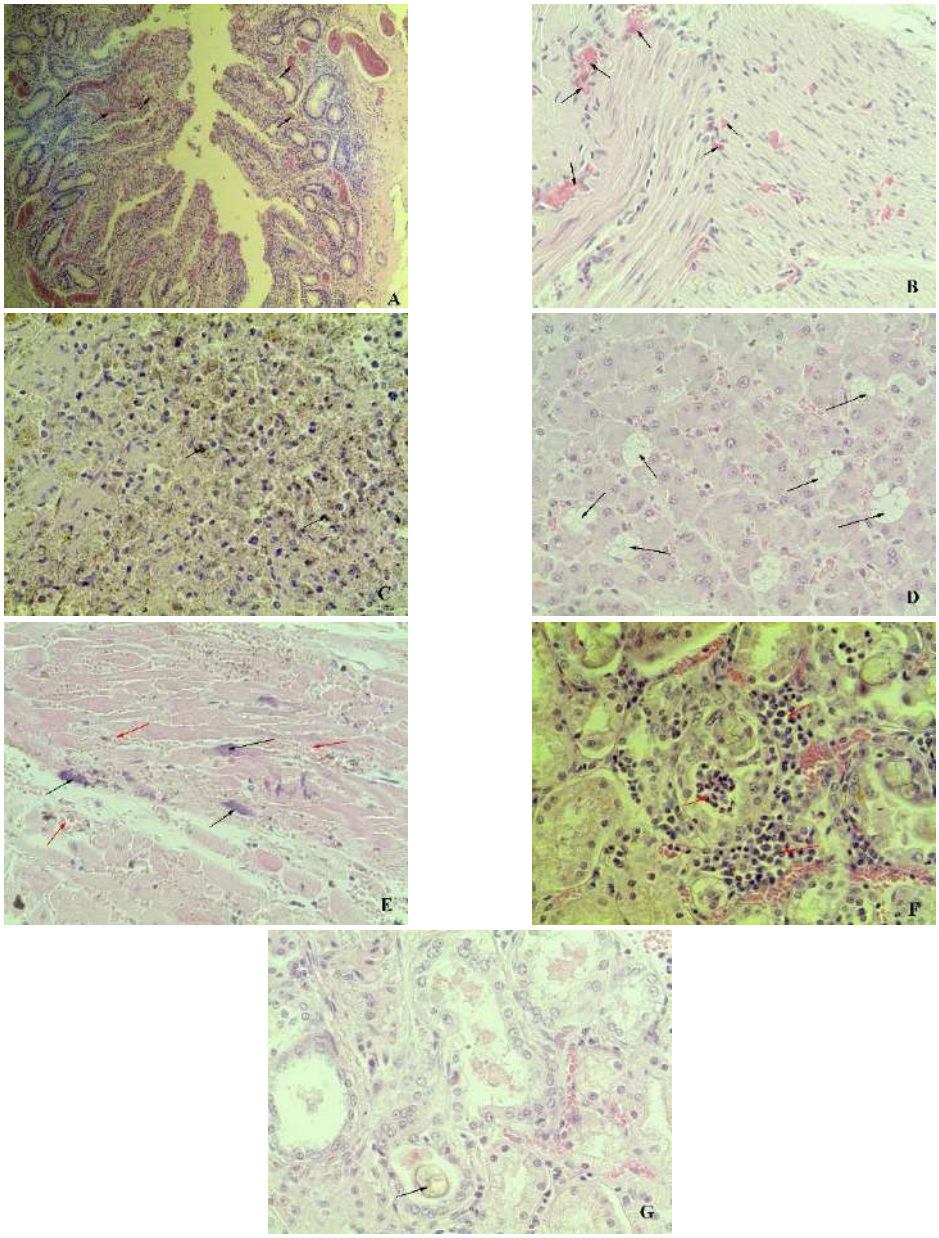


**Figure 1: Hystopathological changes in investigated organs of bison with clinical symptoms of MCF**
**A: total necrosis of intestinal villi and hemorrhages; B: hemorrhages in the inner and outer muscle layers of small intestine; C: severe hypoplasia of the white pulp of spleen and numerous siderocytes in the red pulp (hemosiderosis); D: swollen hepatocytes and numerous multivacuolated hepatocytes in the midzone; E: numerous massive necroses (red arrows) in the myocardium and areas with calcification (black arrows) of the cardiomyocytes; F: neutrophils and mononuclear cells accumulations in kidneys in the glomerulus; G: carbonate uroliths in the lumen of renal tubules of kidneys. Magnification: 400 x, HE.**

With organ suspensions of spleen and lymph nodes from gaur, bison, yak, camel, hippopotamus with clinical signs of MCF and from buffy coat of bison were infected various cell cultures. Viruses of MCF were successfuly cultivated on the cell cultures MDBK. After titration of viruses the titer from gaur isolate was log 106,6 TCID50/mL, for bison and yak isolates – log 107.3, for camel isolate – log 107.5 and for hippotamus isolate – log 107.0. After treatment of virus isolates with IDUR a reduction of titers with 2 to 4 log10 was found. In heterologous strain "Svetovrachene" no reduction in viral titers after treatment with IDUR. Lipoprotein virus envelope after using 20 % ether was not found.

DNA from infected MDBK cell cultures with manifested CPE was proved by classical and nested PCR. The amplification product from the first PCR was with size of 422 bp, and 238 bp after nested PCR (Fig. 2).

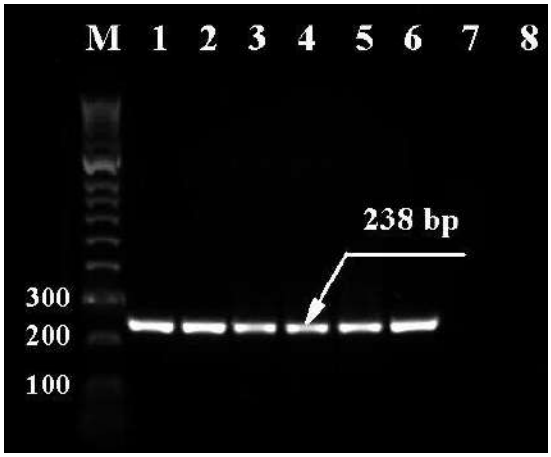


**Figure 2: Nested PCR for MCF virus cell culture isolates. M molecular weight marker 100 bp line 1: spleen, yak Varna; line 2: lymph nodes, bison; line 3: lungs, camel; line 4: spleen, hippopotamus; line 5: positive control Germany; line 6: spleen, capricorn; line 7: negative control, brain, hippopotamus; line 8: negative control distilled water.**

## Discussion

The pathogenesis of MCF in bison, as in other species, remains unclear. It is generally assumed that the small fraction of infected with OvHV-2 lymphocytes induces proliferation and deregulation of uninfected cells (Bridgen et al., 1992; Buxton et al. 1984).

Reports of epidemics from MCF in zoo gardens are rare (Zimmer et al., 1981, Heuschele et al., 1982, Castro et al., 1984, Cooley et al., 2008, Li et al., 2013), but wild ruminants are considered reservoir and source of MCF virus, because of excretion of infectious virus by wild newborn calves up to 3 months (Castro et al., 1981). In wild ruminants, predominant form of MCF is acute and chronic while subclinical forms are rare.

In the present study some of the characteristic gross lesions as keratitis, nasal and eye discharge and rhinitis were observed in all investigated wild animals (Heuschele 1982, Plowright, 1986). Intestinal tract was affected from duodenum to the rectum. The most severe hemorrhages were observed in the jejunum and ileum (Hristov and Peshev, 2014). Similar lesions (stomatitis, pharyngitis, esophagitis and necrohemorrhagic typhlocolitis) are observed by O'Toole and Li (2014) in terminally affected bison. Arteritis and phlebitis were not observed histologically although they are more

typical in cattle with fibrinoid necrosis in tunica media (Schultheiss et al., 2000). The moderate histological lesions of MCF may be masked by rapid autolysis due to high fever in the time of death. Most probably the observed hystopathological changes in investigated tissues from the organs of wild animals with MCF clinical symptoms are connected to the viral MCF replication.

After appearance of clinical symptoms of MCF OvHV 2 is detectable in multiple tissues (Hänichen et al., 1998, Berezowski et al., 2005, Li et al., 2006, Campolo et al., 2008, Cooley et al., 2008, Cunha et al., 2012). But in Indian gaur (Bos gaurus gaurus) and Javan banteng (Bos javanicus javanicus) was found AHV 1 as a cause for MCF (Hänichen et al., 1998). In northern elephant seals with clinical MCF symptoms after DNA sequencing is identified a new Gammaherpesvirus similar to Porcine lymphotropic virus 2, Alcephalineherpesvirus 1 (malignant catarrhal fever virus from wildebeest), and Chlorocebus rhadinovirus 1 from African green monkeys (Goldstein et al., 2006).

After infection of cell cultures from bovine fetal kidney with herpesvirus with size 114 nm isolated from two species of wild ruminants is observed formation of syncytia and intracellular inclusions of CC (Plowright, 1968, Castro et al., 1984). The observed CPE by us from different isolates depends on the type of wild animals (Hristov and Peshev, 2016). In gaur and bison different in size syncytium were observed. Most probably this difference is a result from different type of viruses. To prove this hypothesis in the present study we performed biochemical and molecular biological confirmation of viral isolates.

CPE was inhibited by 5-iodo-2-deoxyuridine (IUdR), and the infectivity of the virus is inhibited after treatment with ether or chloroform. (Taus et al., 2014). The evidence that the isolated viruses are herpesviruses is presence of the proved viral DNA and lipoprotein envelope after processing with IDUR and ether.

After the molecular biological studies of the isolated pathogens by classical and nested PCR, after multiplication with the specific primers the positions of bands after first and second rounds of reaction were the same as that of primary samples from dead animals and the positive control from Germany. That is also evidence that the cause of disease of investigated animals is MCF virus.

In Europe MCF is induced mainly of OvHV 2 and CaHV 2. Currently, viruses causing OvHV 2 MCF are not isolated on cell cultures, despite numerous attempts. AlHV-2 is isolated from the topi (Damaliscus lunatus jimela). From the blood of wildebeest "in vitro" has been cultivated virus strain of MCF a stage where it can be propagated serially in primary cell cultures of thyroid gland. Main effects of the viral DNA multiplication are intracellular inclusion bodies and syncytia. Through this study is confirmed the successful isolation and adaptation of MCF viruses from wild large and small ruminants from the zoos in Sofia and Varna city.

## Conclusions

1. The observed hystopathological change are typical for MCF in wild ruminants.
2. Molecular biological methods applied for the study of the isolated viruses confirmed that they are herpesvirus, causing MCF.

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