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Studies on Malignant Catarrhal Fever in Wild Ruminants

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Abstract

Wild and domestic animals inhabiting Sofia and Varna zoos showing clinical symptoms of malignant catarrhal fever (MCF) were investigated. Clinical and pathological studies were carried out with 16 wild and 8 domestic ruminants. Typical clinical symptoms for MCF were confirmed. Samples for virus isolation and molecular biological investigations were obtained from dead animals. Ten percent suspensions from the lymph nodes, spleens, lungs, brain samples, and buffy coats in phosphate buffered saline (PBS) were used for viral isolation. Primary and permanent cell cultures: rabbit kidney, Madin Darby bovine kidney (MDBK), embryonic bovine trachea (EBTR). Bovine trachea (TTr) and VERO cells were used. Viruses with peculiarities of herpesviruses were isolated from bison , yak, camel, Cameroon goats and hippopotamus on MDBK cell culture. The isolated strains were confirmed as MCF viruses by classical and nested polymerase chain reaction (PCR). The size of the amplicons after classical PCR was 422 bp, and 238 bp after nested PCR.

Thirty seven serum samples originated from 17 sheep, 13 Cameroon goats, 5 mouflons and two bison were investigated serologically by a competitive-inhibition ELISA for spread of MCF. In 21.4% of sheep sera, 30.7% of Cameroon goat sera, and 50% of bison sera antibodies against MCF were found. The total distribution of MCF antibodies in wild and domestic ruminants was 27%.

Key words: Malignant catarrhal fever, wild ruminants, PCR, serological investigation, competitive inhibition ELISA

Резюме

Изследвани са диви и домашни животни обитаващи зоопарковете в София и Варна и показали клинични признаци на злокачествена катарална треска (ЗКТ). Клинични и патологоанатомични изследвания са извършени на 16 диви и 8 домашни преживни животни. Потвърдени са типичните за ЗКТ клинични симптоми. От умрелите животни са получени проби за вирусна изолация и молекулярно биологични изследвания. За вирусна изолация са използвани 10% суспензии от лимфни възли, слезки, бели дробове, мозъчни проби и съсирена кръв във фосфатно буферен разтвор (PBS). За вирусна изолация са използвани първични и постоянни клетъчни култури (КК) - заешки бъбрек (ЗБ), Мадин Дарби говежди бъбрек (МДБК), ембрионална бовинна трахея (ЕБТР) и бъбреци от зелена африканска маймуна (VERO) клетки. Вируси с характеристики на херпесвируси са изолирани от бизон, гаур, як, камила, камерунска коза, хипопотам върху КК МДБК. Изолираните щамове са потвърдени като вируси на ЗКТ чрез класическа и нестед полимеразно верижна реакция (PCR). Ампликоните след класическата PCR бяха с големина 422 bp, а след нестед PCR с големина 238 bp. За установяване на разпространението на ЗКТ чрез компетитивна инхибираща ELISA са изследвани серологично 37 серумни проби от 17 овце, 13 камерунски кози, 5 муфлона и два бизона. В 21,4% от овчите серуми, 30,7% от камерунските кози и 50% от бизоните бяха установени антитела срещу вируса на ЗКТ. Общото разпространение на антителата срещу вируса на ЗКТ при изследваните животни беше 27%.

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Introduction

Malignant catarrhal fever (MCF) is a fatal virus disease in wild and domestic animals. The disease has been observed in different captive ruminants in zoological collections. In some species, such as bison and some deer, MCF is acute and highly lethal, capable of affecting large numbers of animals. In farm-reared white tiled deer and American bison, the disease is a major threat and causes high economical losses. The etiological agent of MCF belongs to subfamily *Gammaherpesvirinae*, genus *Macavirus* from *Herpesviridae* family. Five gamma herpesviruses associated with MCF are ascribed to the genus *Rhadinovirus* - *Alcelaphine herpesvirus*-l (AlHV-1) (Plowright *et al.,* 1960; Roizman, 1992), Alcelaphine herpesvirus-2 (AlHV-2) (Klieforth *et al.,* 2002), Ovine herpesvirus-2 (OvHV-2) (Li *et al.,* 1995; 1996; 1999; Muller Doblies *et al.,* 1998; Collins *et al.,* 2000), Caprine herpesvirus-2 (CpHV-2) (Crawford *et al.,* 2002; Keel *et al.,* 2003; Li *et al.,* 2001*,* 2003) and one virus, yet unnamed, causing MCF in white-tailed deer (MCFV-WTD) (Li *et al.,* 2000).

Two forms of MCF are known: African or wildebeest associated MCF (WD-MCF), and European or sheep associated MCF (SA-MCF). The carriers of MCF viruses in Africa are wildebeest (AlHV-1), in Europe are sheep (OvHV-2), and goats (CpHV-2). With accumulation of data on genomic peculiarities of different MCF agents, it has been determined that the viruses causing bothforms of MCF are closely related, however, they are different viruses (Bridgen *et al.,* 1989; Reid, 1991; Ensser *et al.,* 1997; Coulter *et al.,* 2001). In ruminants inhabiting zoos, the MCF virus spreads from the wildebeest or sheep to susceptible animals. Wildebeest calves are infected by intrauterine transmission from the female or shortly after birth by horizontal transmission of the virus and spread the MCF infection for a period of 3-4 months. The way of infection of new born lambs is not fully clarified. Lambs are infected within 3-6 months of birth by aerosol transmission from older sheep and shed the virus until 9 months of age. The epizootology of caprine herpesvirus 2 is similar to OvHV-2. In European breeds of cattle (*Bos taurus*), the MCF disease is seen sporadically and is highly fatal. In other species, bison, Bali cattle, and cervid species (eg, white-tailed and Pere David‘s deer), the disease is highly contagious. Bison and deer are more susceptible to the disease than cattle, and the virus can remain in a latent form. Therefore, recrudescence of the infection is possible. The MCF infection is lifelong in surviving animals.

In the autumn of 2014, clinical signs typical of MCF in wild big and small ruminants with high mortality rate were observed in Sofia and Varna zoos. The aim of the study is to identify the cause of the high mortality in wild animals and to characterize the agents responsible for the mortality.

Material and methods

An epidemiological investigation was performed in two zoos - Sofia and Varna. The time of outbreaks of disease, the number and type of affected wild animals, the duration of observed clinical symptoms and the time of deaths after onset of disease, the import of new animals in the zoo, contacts between wild and domestic animals, the type of rearing, type of foods and water supply and performed veterinary measures as treatment against bacterial and parasitic disease, disinfection, etc. were investigated.

A clinical investigation was performed with 15 wild and 8 domestic animals - 2 yaks, 4 bison, one camel, 3 gaurs, 2 capricorns, 2 Cameroon goats and one hypopotamus. During the anamnestic studies, changes in the animals' general state, clinical symptoms typical of the disease, temperature, changes in the eyes, central nervous system, respiratory tract, superficial and mesenteric lymph nodes, skin and mucous membrane were examind. The animals with clinical symptoms typical of MCF were pathologically studied after death - one yak, four bison, one gaur, two Cameroon goats and one hypopotamus. The materials for histological investigation were conserved in 10% buffered formalin and afterwards processed according to the methods of Lillie (1965).

Virological samples from the spleen, lymph nodes, lungs, liver, kidney, brain, and buffy coats from diseased wild and domestic ruminants were investigated. The samples were kept at 4^{o}C till delivery in the laboratory and were preserved with glycerin. The samples were squeezed and a 10% organ suspension was prepared in phosphate buffered saline (PBS) pH 7,2. Penicillin 200 UI, Streptomycin 200 µg/mL or Gentamycin 20µg/mL antibiotics were added to the samples Afterwards the samples were centrifuged at 4000 rpm/15 min. Clear supernatant was used for inoculation of primary and permanent cell cultures. Primary rabbit kidney, primary lamb testicle and permanent cell culture Madin Darby bovine kidney (MDBK), embryonic bovine trachea (EBTR), bovine trachea (BTr) and green monkey kidney (VERO) were used for iso-

lation and identification of MCF viruses. Minimal Essential Medium (MEM), with Hank's, or Earle salts, Penicillin 100 IU/mL, Streptomycin 100γ/mL antibiotics, L glutamine, 0.2 M/L, sodium bicarbonate 7.5% for pH 7.4 of media and fetal calf serum (FCS) 10% were added as growth mediums. The maintenance medium was the same as the growth medium, but with a lower amount of FCS – 2%. The tubes with cell cultures were inoculated in three ways: on cell suspension, on a 24-hour-monolayer, or onto a fresh 3-4-hour-monolayer with dimethyl sulfoxide at a concentration from 0.5 to 1%. The tubes were placed for roller cultivation.

Titrations of isolated viruses were performed by the methods of Reed and Muench (1938). The biochemical investigation of isolates - type of nucleic acids, presence of lipid envelope and sensitivity to low and high pH was carried out using the methods of Payment and Trudel, (1993). *Paramyxovirus parainfluenzae* 3, strain *Svetovrachene*, was used as a heterologous strain, which was treated in the same manner as the virus isolates from wild ruminants.

The isolated viruses were confirmed as MCF viruses after classical and nested polymerase chain reaction (PCR) by the methods described by Li *et al.* (1995). For determination of the MCF virus type (AlHV 1 or OHV 2), PCR with primers multiplying AlHV 1 was also used (Hsu *et al*., 1990).

Thirty seven serum samples from wild and domestic zoo-inhabiting animals were investigated serologically. The investigation was performed by a microvirus neutralization test as described by Dilovski *et al.* (1982) against malignant catarrhal fever virus isolated from gaur with virus titer $10^{6,7}$ $TCID_{50}$/mL. The same samples were investigated by a competitive-inhibition ELISA by the methods described by Li *et al.* (1996).

Results

The epizootological investigation determined that 12 big and small ruminants were affected. In the same year, antelopes had been imported from a Romanian zoo, and a camel from a private owner. After six months one bison was found dead, and as the disease continued another 11 animals died within a period of two months. The bison and other big ruminants inhabited a place which was in close proximity with the premises inhabited by the antelopes and domestic sheep. The big and small ruminants were looked after by the same staff fed with the same food. Special immune-preventive actions, apart from deworming, were not carried out.

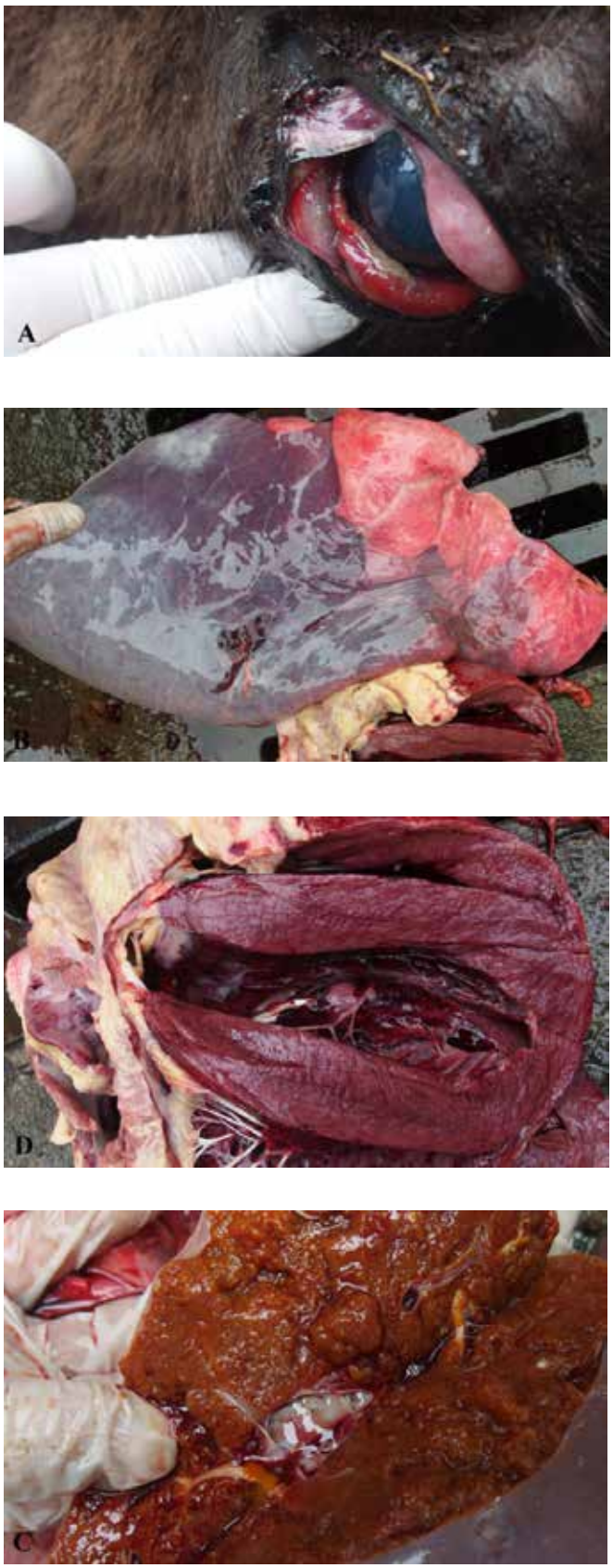

Fig. 1. Changes in cornea (A), lungs (B), liver (C) and hard muscle (D)

Fever, lack of appetite, spiking of hair, redness of the conjunctiva, serous and later purulent discharge from the nose, teeth grinding, ataxia, bloody diarrhea, depressed condition, inability to stand up, bedsores and subsequent death within 24-48 h were found in bison and big ruminants (gaurs and yak).

The pathology examination found mucopurulent exudates in the nasal cavities and bilateral graying of the cornea (Fig. 1 A). No damage to the mucosa of the buccal papillae and palate was observed in the oral cavity. Upon opening the trachea, petechial and ecchymosis hemorrhages, enlarged mediastinal lymph nodes were found. The lungs were stained blue and were sealed (Fig. 1 B). The liver was enlarged; the edges were rounded with a yellowish to orange color (Fig. 1 C). The gall bladder in some cases was the size of a small child's head. Mesenteric lymph nodes were not enlarged. The spleen was atrophic with necrosis, and upon resection there was no white pulp. On the epicardium and at the base of the heart multiple petechial and wider bleeding was visible, and the blood vessels were strongly injected with blood (Fig. 1 D).

Multiple petechial and more extensive longitudinal bleeding (Zebra stripes) was found in the mesentery of the small intestine (Fig. 2 A), the most severe in the first and middle sections of the small intestine (Fig. 2 B). There was no bleeding in the colon, and the intestinal content was of dark brown color. The kidneys were of dark red color (Fig 2 C), the urinary bladder was with urine and injected blood vessels. The rumen and abomasum did not appear to have any visible changes, but resection revealed reddened mucous membrane with a lot of petechial hemorrhages.

The samples obtained from diseased and dead animals were used to infect primary and permanent cell cultures. The best condition for the MCF virus growth was observed on the MDBK cell line after more than 21 passages. The visible cytopathic effect initially was formed 6-7 days after inoculation. The cytopathic effect was visible earlier with the increase in the number of passages. At 24th h after infection with the camel isolate, cell rounding and grapelike cluster formations were visible, the changes in the cell monolayer were augmented at 48th h and a large part of cells were detached in the maintenance medium at 72nd h (Fig 3. A, B and C). Large and small syncytia were observed from the bison and gaur isolates after 24th h. With the progression of sub-cultivation, the viral growth increased, CPE accelerated and after 48-60th h the number of

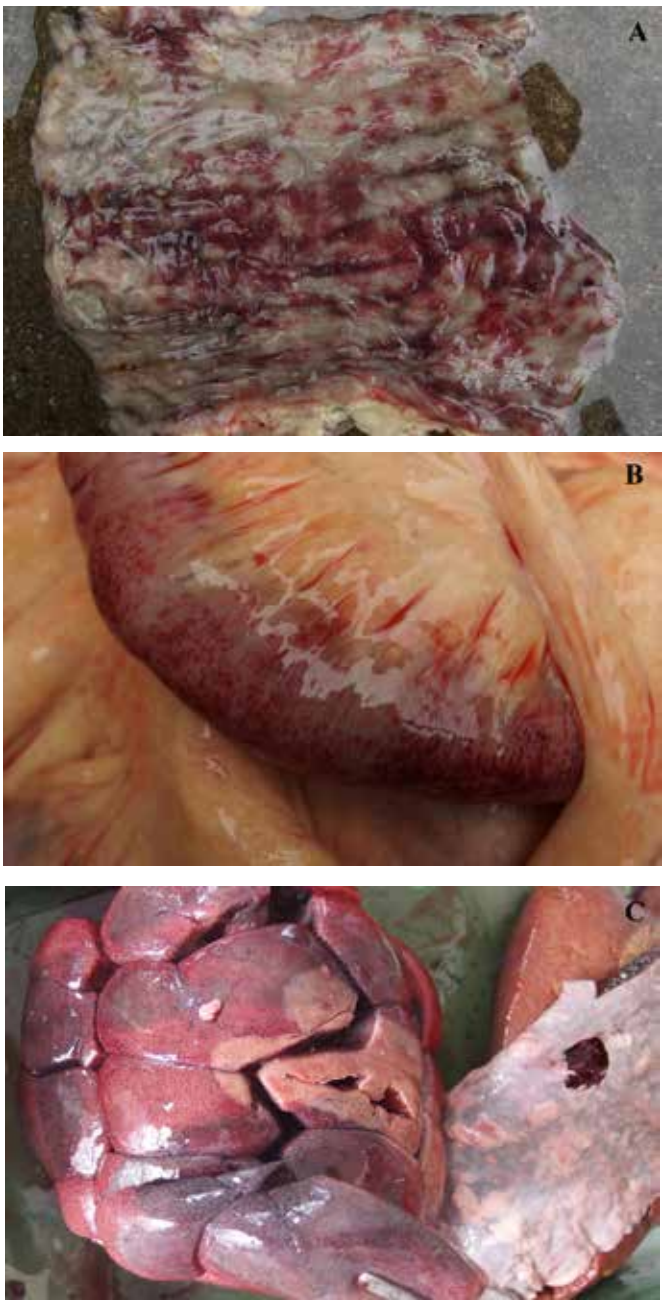

Fig. 2. Changes in small intestines (A and B) and kidney (C)

syncytia also increased and approximately 50% of the monolayer was affected. Full destruction of the cell monolayer was observed at 72nd h.

Viral titers of the isolated viruses on cell culture from gaur, camel, yak and two bison varied between log10^{6,6} TCID50/mL and log10^{7,3} TCID50/mL. The viral titer of the heterologous RNA paramyxovirus parainfluenza 3 (Pi-3) strain *Svetovrachene* was log10^{5,33} TCID50/mL. Viral titers decreased by 2 - 3 log10 after treatment of the viruses with 5-jod 2-desoxiuridine 5-iodo-2-deoxyuridine, while the titer of heterologous Pi-3 *Svetovrachene* strain did not change. CPE was not observed after treatment of all isolated viruses with

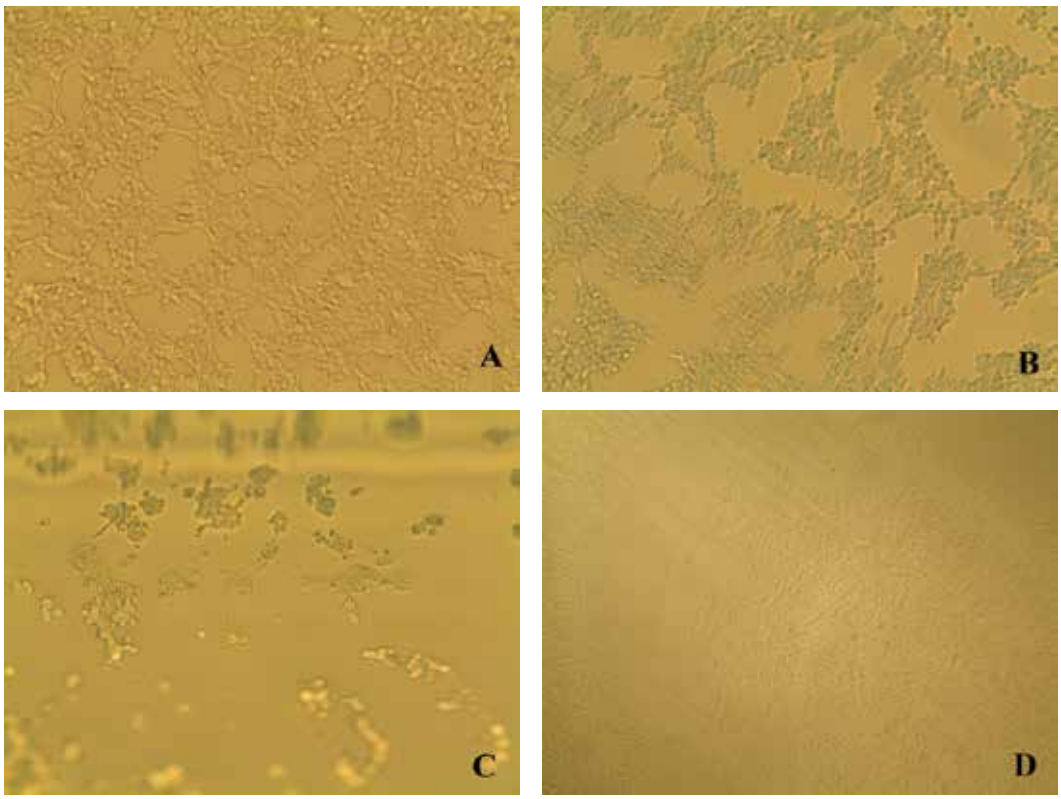

Fig. 3. Cytopathic effect of 21 passage after infection of MDBK cell culture with 10% lung suspension from camel with MCF clinical symptoms: A – 24th, B - 48 th, C - 72nd hours after cell culture inoculation and D – MDBK negative control. Magnification 200X

20% ether and following inoculation of MDBK cell culture.

DNA was received from all native samples obtained from the investigated wild ruminants and cell cultures with visible CPE. The amplification products obtained by nested PCR with primers and procedures for OHV 2 after the first round of reaction were obtained amplicons with the size of 422 bp, and after the second round the size of 238 bp (Fig. 4). Isolated DNAs were not amplified by PCR with primers multiplying AlHV 1 for all isolates.

Thirty seven serum samples originated from 17 sheep, 13 Cameroon goats, 5 mouflons and two bison were investigated serologically by a competitive-inhibition ELISA for spread of MCF. In 21.4% of sheep sera, 30.7% of Cameroon goats sera, and 50% of bison sera antibodies against MCF were found. The total distribution of MCF antibodies in wild and domestic ruminants was 27%.

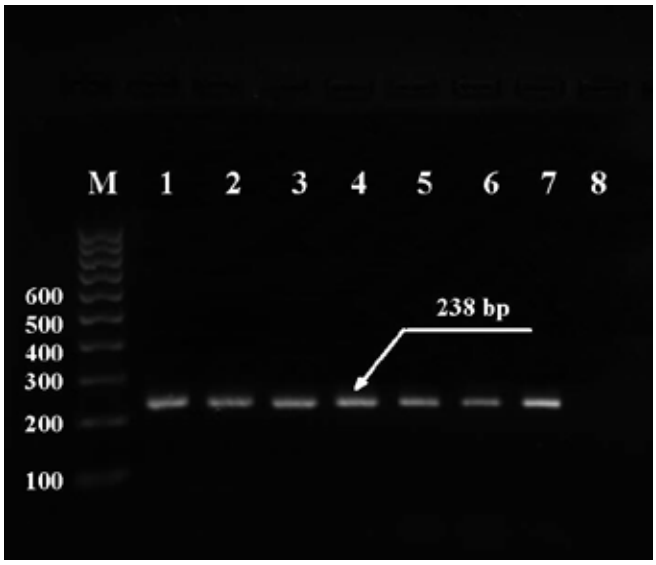

Fig. 4. PCR proving the MCF virus in native and MDBK cell culture samples. M molecular size marker 100 bp. MCF DNA originated from cell culture: Line 1- buffy coat bison, Line 2 – spleen gaur Line 3 – lungs camel, Line 4 – spleen hippopotamus, and native samples: Line 5 – lungs camel, Line 6 – spleen bison. Line 7 – positive control Germany, Line 8 – negative control none infected cell culture MDBK

Discussion

Wild animals are a reservoir and source of MCF virus (Castro *et al.,* 1981). It has been proved that the infectious virus is spread by young newborn calves up to 3 months old (Plowright, 1968; Castro *et al.,* 1984). In our investigation the ani-

mals were at different age. All animals investigated by us with clinical symptoms of MCF had been in direct contact with sheep and in indirect contact with wild life blackbuck antelope (*Antelope cervicapra*), antelope kana and capricorn (Hristov and Peshev, 2014). Papers on the epizooties of MCF in zoo parks are rare (Castro *et al.,* 1981; Campolo *et al.,* 2008). Our study proved that in Sofia zoo there is MCF epizoothy. This assumption was confirmed by an ELISA study, in which different types of animals with antibodies against MCF were found.

Plowright *et al.* (1963), Plowright (1968), Castro *et al.* (1982) have found large-sized syncytium and inclusion bodies in bovine embryonic testis cells and bovine fetal kidney of isolates originating from an Indian gaur and Greater kudu with typical clinical signs of MCF. Likewise, we have observed growth of infected MDBK cell culture with samples from organ suspensions from gaur. The observed CPE in MDBK cell culture inoculated with organ suspension from bison with typical signs of MCF (Hristov and Peshev, 2015) is different from camel isolates. The different type of CPE cannot be explained with different titers of virus isolates, which varied between log $10^{6,6}$ for gaur to log $10^{7,3}$ for bison and log $10^{7,0}$ $TCID_{50}$/mL for camel. Most probably, the viral isolates from bison and gaur are genetically different than camel isolates.

Reduction of the viral titers with 2 and 3 log10 after application of biochemical methods – iodine-deoxyuridine treatment for proving viral DNA on the cell culture isolates is the evidence that the investigated isolates are DNA viruses. It is proved by the results obtained by classical and nested PCR. Till now data on the isolation of OHV 2 virus was missing. The negative results from PCR specially designed for AlHV used in this study confirm that the cell culture MCF isolates in our investigation are OHV 2 viruses. Additional genetic investigations are needed to confirm the type of these isolates.

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