ISOLATION AND IDENTIFICATION OF MALIGNANT CATARRHAL FEVER VIRUS IN CELL CULTURES

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Summary

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Malignant catarrhal fever (MCF) is a fatal disease syndrome responsible for mortality in domestic and wild ruminant species. MCF is caused by gammaherpesviruses – the ovine herpesvirus type 2 causing sheep-associated malignant catarrhal fever (SA-MCF) and alcelaphine herpesvirus type 1 known as wildebeest-associated MCF (WA-MCF). The present study described the cultural peculiarities of MCF virus isolated from different samples originated from dead and alive wild animals from the Sofia Zoo. MDBK, EBTR, RK, MA-104 and VERO cell cultures were used for the isolation of MCF virus. The best growth of viruses was observed on MDBK cell cultures. The CPE was characterised with forming of the syncytium and destruction of the monolayers 2–3 days after the virus adaptation. The CPE was different for obtained isolates. The isolates from gaur formed a bigger cell syncytium than that in the cell cultures infected with buffy coat from bisons where a cell syncytium of smaller size and shape was observed. The virus identification was performed by biochemical methods and PCR.

Key words: cell cultures, cytopathic effect, malignant catarrhal fever (MCF), PCR, wild ruminants

INTRODUCTION

Malignant catarrhal fever (MCF) is a sporadic fatal viral disease in cattle, buffaloes and other wild ruminants. The disease is characterised with heavy nasal and eye discharges, inflammation, exudation, appearance of ulcers of the respiratory tract and mouth mucosa, keratoconjunctivitis with graying of the cornea and enlargement of peripheral lymph nodes (Plowright, 1990). Two different epizootiological forms of disease are known – African and European. The African form of MCF is observed after contact of the cattle with wild ruminants and is named as wildebeest-derived MCF (WD-MCF) (Daubney & Hudson, 1936). Sheep are source of the infection with the European form of MCF which is hence named sheep associated MCF (SA-MCF).

The etiologic agents of both MCF forms are members of the genus *Macavirus*, subfamily Gammaherpesvirinae, fa-

mily Herpesviridae. Etiological agents of MCF are classified as a separate subgroup of *Macavirus* genus or MCF-subgroup due to similarity of the genomic structure and the high homology in basal DNA sequences of the different agents of genus *Radinovirus* (Coulter *et al.,* 2001; Li *et al.*, 2001a).

The causative agents of WD-MCF are different viruses – Alcelaphinae herpes virus 1 (AHV-1) isolated from wild animals belonging to family Alcelaphinae (Plowright *et al.,* 1960; Roizman *et al.*, 1992) and Alcelaphinae herpes virus 2 (AHV-2) isolated from topi (*Damaliscus lunatus jimela*) (Taus *et al.,* 2014). The etiological agent of SA-MCF is the ovine herpes virus 2 (OHV-2) (Li *et al.,* 1995; 1996; Muller-Doblies *et al.,* 1998; Collins *et al.,* 2000).

MCF is broadly spread in domestic and wild ruminants; the clinical and pathological signs are not significantly different. Transmission of the OHV-2 from sheep to cattle or bison or transmission between cattle and bison was found (Plowright *et al.,* 1960; Schultheiss *et al.,* 1998; 2000). American bison are very sensitive to SA-MCF (Ruth *et al.,* 1977; Nelson *et al.,* 2013). Clinically depression, nasal and eye discharges, conjunctivitis, keratitis and diarrhoea are observed. The morbidity in flocks vary from 3 to 53.8% and the mortality is 100%. After autopsy ulcerative lesions are observed in all gastrointestinal tract compartments, the trachea, bronchi. Microscopically necrotising vasculitis without thrombosis in almost all investigated organs is determined. This fatal disease is characterised with lympho-proloferation, vasculitis and ulcers of bison's mucosa. SA-MCF was proved by PCR in American bison and red deer without clinical symptoms (Powers *et al.,* 2005).

Widespread infection among different animals is observed by diagnostic tests for antibody detection – agar gel immunoprecipitation and complement fixation but these tests were with low sensitivity (Rossiter & Jesset, 1980; Rossiter *et al.,* 1980; Rossiter, 1985). Furthermore after using indirect immunofluorescence test, a cross reaction with other bovine herpesviruses is observed (Plowright, 1986). Serum neutralisation test was also used for detection of MCF in wild animals (Hamblin & Hedger, 1984; Heuschele *et al.,* 1984). Recently used serological tests are more sensitive – indirect competitive enzyme-linked immunosorbent assay (c-ELISA) (Li *et al.,* 1994; 1995), competitive inhibition ELISA in which MAb 15-A against a specific conserved viral epitope against OHV-2, AHV 1 and new MCF isolated strains from white-tailed deer (Li *et al.,* 2000) is used; direct c-ELISA (Li *et al.,* 2001b).

Polymerase chain reaction (PCR) is a sensitive test for molecular diagnostics of MCF. PCR for proving AHV 1 DNA was developed by Hsu *et al.* (1990). Baxter *et al.* (1993) have developed specific nested PCR based on targeting a DNA fragment in the ORF 75 of OHV 2 and have confirmed the mortality in 21 bison (*Bison bison*) infected with SA-MCF (Berezowski *et al.,* 2005).

In zoos, among American bison (*Bison bison*) reared in close contact with sheep (*Ovis aries aries*) Campolo *et al.* (2008) have determined clinical symptoms similar to those in acute form of MCF. By real time PCR the authors have proved OHV 2 DNA in blood, nasal and eye secretions and *post mortem* tissue samples. In an Argentinian zoo, Bratanich *et al.* (2012) have determined OHV 2 DNA in sample from lymph nodes and spleen of five bison with clinical symptoms of MCF using

PCR. Taus *et al.* (2014) observed typical clinical and pathological changes for disease, different from those for SA-MCF, in 2 of 6 bison inoculated with AHV 2.

For *in vitro* cultivation of MCF virus primary bovine thyroid (BTh) cell culture, bovine kidney (BK), bovine embryonic kidney (BEK) and calf testicle (CT) cells are used (Plowright *et al.*, 1960; Plowright & Ferris, 1961; Castro *et al.*, 1982). Castro *et al.* (1982) have isolated a herpesvirus on primary BTh cell culture from buffy coat cells obtained from Indian gaur (*Bos gaurus*) and greater kudu (*Tragelaphus strepsiceros*) with MCF symptoms and cultured the virus on BEK. Five isolates obtained from wild ruminants with MCF signs in San Diego wild animal park have been cultured on foetal aoudad (*Ammotragus lervia*) kidney (FAK) cell culture and after electron microscopy they were identified as herpes viruses with size of 118 to 220 nm (Castro *et al.*, 1984).

In the Sofia zoological garden, MCF signs with high mortality were observed among the wild ruminants – gaurs (*Bos gaurus*, Indian bison), bison (*Bison bison*), camels (*Camelus dromedarius*), antelopes (Blue wildebeest, *Conochaetes taurinus*), yak (*Bos grunniens*), capricorn (*Capra ibe*x), and hippopotamus (*Hippopotamus amphibius*). MCF infection was proved by the epizootological, clinical, pathoanatomical and laboratory investigations. The purpose of the current study was to investigate the isolation, cultural peculiarities and identification of MCF virus in cell cultures.

MATERIALS AND METHODS

Samples

Samples obtained from 14 dead small and big wild ruminants at different ages (3 gaurs, 4 bison, 2 yaks, 1 camel and 2 ca-pricorns, 1 hippopotamus from the Sofia zoo and one yak from the Varna zoo) with clinical symptoms of MCF (Hristov & Peshev, 2014) were used for virus isolation. Investigated samples were peripheral blood leukocytes from buffy coat, 10% suspension from internal organs – lungs, spleen, liver, brain, kidneys in phosphate buffered saline (PBS, pH 7.2) or saline. Native samples were identified for MCF virus DNA by PCR.

Cell cultures

For isolation and identification of cultural peculiarities of MCF viruses primary and permanent cell cultures: primary rabbit kidney cells (RK), permanent cell culture Madin Darby bovine kidney (MDBK), embrional bovine trachea (EBTR), monkey kidney (VERO), and monkey kidney (MA-104) were used.

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

All organ samples obtained from above described animals with clinically confirmed MCF infection were used for inoculation of cell cultures. At least three blind passages on non-infected cell culture were performed with infected cell cultures and after the cytopathic changes of the monolayers, the isolated agents were identified for MCF virus DNA by PCR. Sam-

ples without cytopathic changes on monolayers after three passages were determined as MCF-negative. The virus titres were calculated by Reed & Muench (1938) methods. The cytopathic effect (CPE) was evaluated as one plus when 25% of the monolayer was affected.

The type of nucleic acid was determined by the methods of Payment & Trudel (1993). As heterologous strain, Svetovrachene paramyxovirus parainfluenzae 3 strain was used. It was treated in the same manner as virus isolates from wild ruminants.

Polymerase chain reaction (PCR) for virus detection

For preparation of DNA cell cultures infected with 10% organ suspension (spleen, lymph nodes, liver, lung, and kidney), buffy coats and brain samples showing CPE were used. DNA was isolated by the method of Maniatis *et al.,* (1982) with phenol, chloroform, isoamyl alcohol (25:24:1) and via the diagnostic kits for DNA isolation GiAmp, Giagen (Pvt Ltd), Bioline and Robosceen (Germany) according to the description of the manufacturer.

The nested PCR were performed according to the method of Li *et al.* (1995), modified and adapted to conditions of our laboratory. The specific primers for DNA region of OHV 2 (Table 1) were used at 200 ng/µL for the first and the second round of the reaction (Baxter *et al.,* 1993).

Li *et al.* (1995) have performed nested PCR using desoxynucleotide phosphates (DNTPs) and polymerase enzyme. In our modification the Hot start Fideli Tag PCR master mix (2×) was used as source for DNTPs and polymerase enzyme. PCR were performed in PCR termocycler QB in two steps and the following reagents were used: 12.5 µL Hot start Fideli Tag PCR master mix (2×), 0.5 µL of primers 556 and 755 (10 pM), 1 µL DNA template 150 ng/µL, distilled water to 25 µL. After application of Tag DNA polymerase and Hot start Fideli Tag PCR master mix the following parameters for amplification were used: pre denaturation at 95° C for 5 min, 34 cycles each consisting of denaturation at 94° C for 1 min, annealing at 60° C for 1 min and elongation at 72° C for 2 min. Final elongation were performed for 7 min at 72° C. The DNA from the first step of reaction at quantity between 100–150 ng/µL were used as template for the second round of PCR as well as the same parameters from the first round of reaction. For visualisation of reaction, 5 µL of amplified products and 4 µL of gel loading buffer were used. The amplicons were analysed by gel electrophoresis for 30–60 min at 90 V, 155 mA in 2% agarose gel stained with ethidium bromide (10 mg/ mL) and their size were determined with size marker DNA 100 bp (Boehringer Manheim). Positive reaction was admitted when the products from the

Table 1. Nucleotide sequencing of used primers, their position in genome and the size of obtained products in bp after nested PCR

Primers	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

second round were with size of 238 bp. Positive DNA samples originating from lymph nodes, spleen and buffy coats from MCF positive bovine, kindly donated from Prof. Groshup from Germany were used as controls for the correct performance of the reaction. Distilled water and DNA samples obtained from buffy coats spleen and brain from cattle without MCF clinical symptoms were used as negative DNA controls.

RESULTS

The viral agent was well developed in primary as well as in permanent cell cultures. On the rabbit kidney primary cell culture, CPE was observed by the fourth-fifth day and the total destruction of monolayers was observed after 6–7 days. CPE was not observed after 7 passages.

On the permanent cell culture VERO and MA 104 the virus growth was successful between three and five subcultivations. CPE was observed after 5 days and the total destruction of the monolayer was visible by the 7th–8th day. On the permanent cell culture EBTR, the CPE was visible earlier – after 3–4 days and the total destruction of monolayer was established by the 5th–6th day. CPE was not observed after 9 passages.

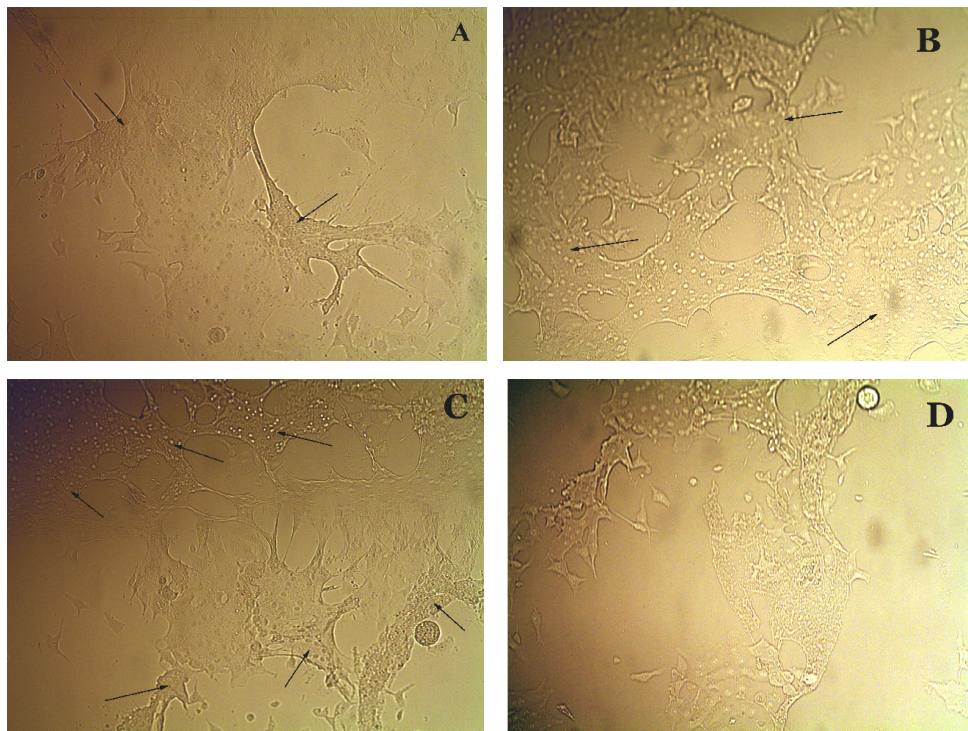The best condition for the MCF virus growth was observed on the MDBK cell

Fig. 1. Cytopathic effect of 19 passages after infection of MDBK cell culture with 10% spleen suspension from a gaur with clinical symptoms of MCF on the 24th (A), 48th (B), 60th (C) and 72nd hour (D) after cell culture inoculation. Arrows indicate large syncytia. Magnification 200×.
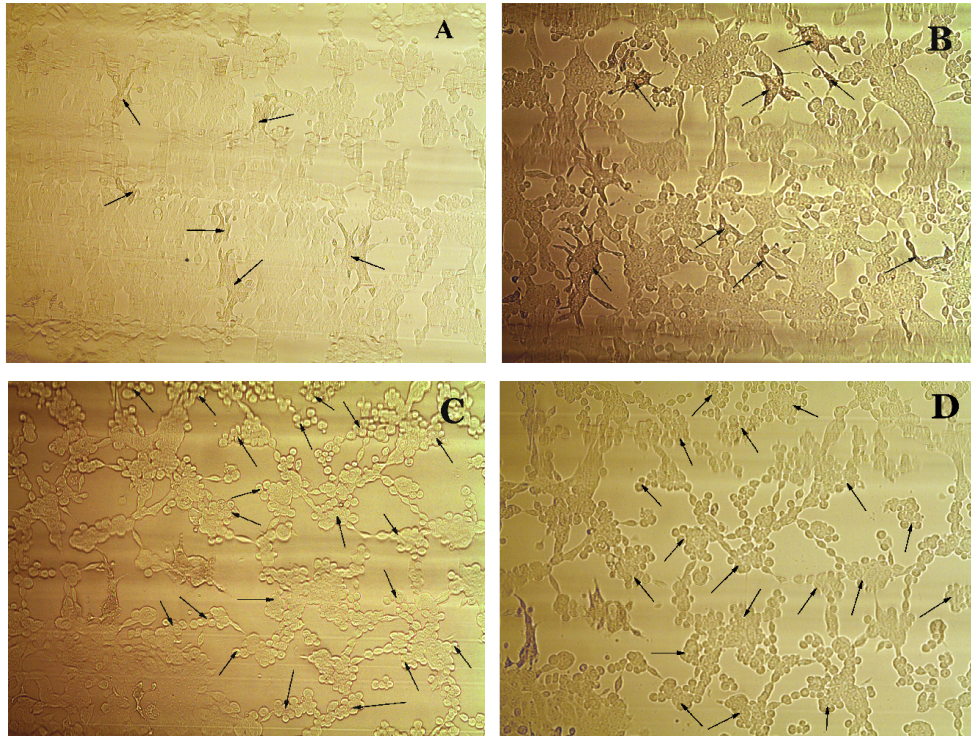
Fig. 2. Cytopathic effect of 19 passages after infection of MDBK cell culture with buffy coat from a bison with clinical symptoms of MCF on the 24th (A), 48th (B), 60th (C) and 72nd hour (D) after cell culture inoculation. Arrows mark small syncytia and rounding of cells. Magnification 200×.

line and more than 19 passages were carried out. CPE was observed up to 10 passages and total destruction of monolayers was found 6–7 days after inoculation. Observed CPE of investigated isolates was characterised with three types of changes on the monolayers. In the camel isolate rounding of cell and grapes formations were visible 24 h after infection and by 48th h, a large part of cell monolayers was detached in the maintenance medium. Large syncytia were observed from gaur isolates by the 24th h. As subcultivation progressed, the viral growth was increased, CPE was accelerated and after 48–60 hours, the number of syncytia has also increased and approximately 50% of the monolayers were affected (Fig 1B and

1C). Full destruction of cell monolayers was observed by the 72nd h. (Fig 1D).

On bison buffy coat isolates, the CPE were characterised with syncytia of small size (Fig. 2A). The number of the syncytia was increased and beginning of the cells rounding was observed on the 48th h (Fig. 2B). By the 60th–72nd hour, the number of syncytia has decreased (Fig. 2C), the cell monolayers were tearing and plenty of cells were detached in the maintenance medium (Fig. 2D).

Viral titres of the isolated viruses from gaur, camel, yak and two bison on cell culture varied between $\log10^{6.6}$ TCID$_{50}$/mL and $\log10^{7.3}$ TCID$_{50}$/mL. The virus titre of the heterologous RNA Svetovrachene paramyxovirus parainfluenza 3 (Pi-3)
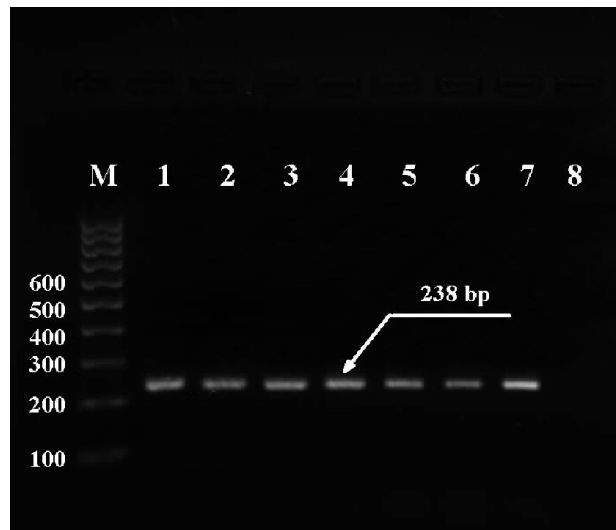
Fig. 3. PCR for detection of the MCF virus in native and MDBK cell culture samples. M: molecular size marker 100 bp. MCF DNA originating from cell cultures: Lane 1 – buffy coat, bison; lane 2 – spleen, gaur; lane 3 – lungs, camel; lane 4 – spleen, yak, and native samples: lane 5 –capricorn spleen, lane 6 – hippopotamus spleen. Lane 7 – positive control; lane 8 – negative control (non-infected MDBK cell culture).

strain was $\log 10^{5.33}$ $TCID_{50}$/mL. After treatment of the viruses with 5-iodine-2-deoxyuridine, reduction of virus titres by 2 $\log_{10}$ and 3 $\log_{10}$ was observed for gaur and bison isolates respectively, while the titre of the heterologous Svetovrachene Pi-3 strain was not changed. CPE was not observed after treatment of all isolated viruses with 20% ether and following inoculation of cell culture MDBK.

DNA was obtained from all native samples from investigated wild ruminants and cell cultures with visible CPE. The amplification products obtained by nested PCR after the first round of reaction were 422 bp of size and after the second - 238 bp of size (Fig. 3).

DISCUSSION

Papers for MCF epizooties in zoo parks are few (Castro *et al.,* 1981; Campolo *et*

al., 2008), but evidence that the wild animals are reservoir and source of MCF virus (Castro *et al.,* 1981). It was proved that infectious virus has been spread from young newborn calf up to 3 months of age (Plowright, 1968; Castro *et al.,* 1984). In our investigation the animals were at a different age. The youngest studied bison was 7-months old while the oldest animal was a 20-year-old female bison. All diseased animals with the clinical symptoms of MCF have not been with direct contact with sheep, but with indirect contact with wild blackbuck (*Antelope cervicapra*), eland and capricorn (Hristov & Peshev, 2014).

Only one from observed three different cytopathic effects of isolates from camel, gaur and bison with typical clinical symptoms of MCF is described. Plowright *et al.* (1963), Plowright (1968), Castro *et al.* (1982) have found big syncytia and

inclusion bodies in bovine embryonic testis cells and bovine foetal kidney of isolates originating from an Indian gaur and Greater kudu with typical clinical signs of MCF. We have observed growing of infected MDBK cell culture with samples from organ suspensions from gaur (Fig. 1).

The observed CPE in MDBK cell culture inoculated with organ suspension from bison with typical signs of MCF is characterised with small syncytia and intranuclear inclusion bodies (Fig. 2). CPE was totally different in camel isolates. It was characterised only with rounding of cells and grapes formation of cells. The different type of CPE cannot be explained with different titres 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 there is another reason for the different CPE. Till now data for isolation of OHV 2 virus are missing and the used PCR proved AHV 1 as well as OHV 2 virus. Most probably the camel viral isolate is different than gaur and bison viral isolates. Additional investigations are needed for confirmation of the type of viral isolates as AHV 1 or OHV 2.

Pierson *et al.* (1973) have isolated the MCF virus from gaur after the 12th passage with preserved virulence for cattle. According to the authors isolation of the viruses is successful only in whole blood obtained during the febrile phase of the disease, subsequent treatment and inoculation in cell cultures and receptive animals. In this study the MCF virus was successfully isolated from whole bison blood in the febrile phase of the illness, from both buffy coats and internal organic suspensions from dead large and small wild ruminants as Castro *et al.* (1982) did.

During the initial passages of viruses, the CPE appeared later, most probably due to the high quantity cell-associated viruses, while after the 19th passage the quantity of cell-free virus in the media was enough for early appearance of CPE – at the 24th h. The monolayer was fully dropped after 2–3 days. That is due to the adaptation of viral agents as a result of the interaction of viral and cell receptors with passage progression in this type of cell cultures.

Cultivation of MCF viruses is very difficult by using cell-free virus in BK cells, but the bovine testis cells are highly sensitive to viral reproduction (Mushi & Plowright, 1979; Plowright, 1984). The BT cells produced up to $10^{5.0}$ TCID$_{50}$/mL cells-free infectious viruses (Patel & Edington 1980; 1981). We used different types of cell cultures and found that Madin Darby bovine kidney cells are the most sensitive for development of MCF viruses. Because of the long refractive growths of syncytia the cytopathic changes in this cell culture are easily detected.

After a determined number of subcultivations, the viruses from different wild ruminants were adapted and grew with high titres. The isolation from buffy coat from bison was easier than the isolation from internal organs, probably because of the higher quantity and less cell-associated MCF virus, than in the internal organs. Nevertheless, we isolated MCF viruses from internal organs of dead zoo wild large and small ruminants – gaur, yak (*Bos grunniens*), capricorn (*Capra ibex*), camel and hippopotamus. In the available literature, there are no data for MCF infection in the last two wild animal species.

Reduction of the viral titres by 2 and 3 log10 after application of biochemical methods – iodine-deoxyuridine treatment for proving viral DNA on the cell culture isolates evidenced that the investigated isolates were DNA viruses. In the treat-

ment of viruses with ether and their subsequent inoculation in cell culture CPE has not developed, indicating the existence of a lipoprotein envelope, which is a further evidence that the isolates are herpesviruses. This statement was confirmed also by the results obtained by nested PCR. Additional investigation is necessary for confirmation of the MCF type despite that isolates were from wild ruminants because the animals in the zoo were in an indirect contact with sheep and wild ruminants.

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