

Immunofluorescence and cytopathological features induced by Caprine herpes virus 1 in MDBK cells

I.N. SIRAKOV^{1*}, R. PESHEV¹, M. ALEXANDROV², E. GARDEVA²

¹National Diagnostic & Research Veterinary Institute, 15 "Pencho Slaveykov" Blvd., 1606 Sofia, BULGARIA.

²Bulgarian Academy of Scientist, Institute of Experimental Pathology and Parasitology, "Akad. Georgi Bonchev" str., bl.25, 1113 Sofia, BULGARIA.

*Corresponding author: insirakov@gmail.com

SUMMARY

Polyclonal antibodies against Caprine Herpes virus 1 (CpHV-1) were prepared and used for development of a direct immunofluorescence test for detection of CpHV-1 infection. The test was evaluated using infected and non infected MDBK cells. Specific asymmetric perinuclear immunofluorescence was observed since 2 hours post infection and lasted up to the end point investigated in monolayers obtained at the 36th hour post infection. Infected and uninfected MDBK cells were analyzed in parallel following May-Grünwald Giemsa and double fluorochrome staining with acridine orange (AO) and propidium iodide (PI). The dynamics of the cytopathological changes showed cell shrinkage, cell loss and marked increase in the apoptotic index after the 12th hour post infection. Infected cells were rearranged into many syncytia in which live and apoptotic cells could be present at the 24th up to the 36th hour post infection. The present investigation clearly indicated that immunofluorescence test is an appropriate method for the detection of CpHV-1 infection within infected MDBK cells, while the double fluorochrome staining with AO and PI showed a number of advantages for morphological evaluation of the cytopathological changes induced by CpHV-1.

Keywords: Caprine herpes virus 1, MDBK cells, immunofluorescence, fluorochrome, apoptotic cells, syncytium, viral replication.

RÉSUMÉ

Immunofluorescence et caractéristiques cytopathologiques induites par l'herpès virus Caprin 1 dans les cellules MDBK

Des anticorps polyclonaux contre l'herpès virus caprin 1 (CpHV1) ont été préparés et utilisés pour mettre au point un test d'immunofluorescence directe de l'infection virale. Ce test a été évalué sur des cellules MDBK en culture infectées ou non. Une fluorescence périmoléculaire asymétrique spécifique a été observée dès la deuxième heure après l'infection et elle a duré jusqu'à la dernière monocouche analysée obtenue à la trente-sixième heure de l'infection. Des cellules MDBK infectées ou non ont été parallèlement analysées par coloration au May-Grünwald-Giemsa ou par une double coloration fluorescente par l'orange d'acridine (OA) et l'iodure de propidium (IP). Les modifications cytopathologiques, condensation cellulaire, perte cellulaire et forte augmentation de l'index apoptotique, ont été maximales 12 heures après infection. Les cellules infectées se sont regroupées en de nombreux syncytia qui pouvaient comporter des cellules vivantes et apoptotiques entre la 24^{ème} et la 36^{ème} heure. Cette étude montre clairement que ce test d'immunofluorescence directe permet la détection du CpHV1 dans les cellules MDBK infectées, tandis que la double coloration en fluorescence par OA et IP permet d'évaluer les modifications cytopathologiques induites.

Mots clés : Herpes virus caprin 1, cellules MDBK, immunofluorescence, fluorochrome, cellules apoptotiques, syncytium, réplication virale.

Introduction

Caprine herpes virus-1 (CpHV-1) is distributed worldwide and causes serious economic losses [3, 11, 29, 31]. CpHV-1 infection is characterized by different clinical symptoms depending on the age of the animal. Young, 1- or 2-week-old kids show symptoms of enteritis and severe generalized disease, and most of them die [12, 23]. In adult male and female goats the infection is most commonly subclinical, or is displayed with mild clinical symptoms [2]. The virus typically affects the respiratory and genital tracts. Pneumonia, vulvovaginitis, and balanoposthitis may develop, causing infertility and abortion [3, 7, 10, 12, 25, 26, 31]. New data indicate that CpHV-1 may induce apoptosis in caprine peripheral blood mononuclear cells and Madin-Darby bovine kidney (MDBK) cells [14, 18]. This, however, has not been investigated cytologically.

The disease is diagnosed by virus isolation, electron microscopy, PCR, RT-PCR, or serologically, with the serum neutralization test, immunofluorescence methods (IF) and ELISA

[3, 5, 6, 15, 21, 27-30, 32]. Among these, immunofluorescence methods have been reported as most suitable for detection of CpHV-1 in cell cultures and foetuses [13, 32, 33]. However they still find limited application. Hence, the aim of this study was to develop a direct IF method for quick diagnostics of CpHV-1 infection in cell cultures and to assess the cytopathological changes induced by CpHV-1 in MDBK cells based on double staining with two vital fluorochromes.

Material and Methods

VIRUSES AND CELL CULTURES (CC)

The CpHV-1 strain E/CH, kindly provided by Pr. M. Engels, the Bovine herpes virus 1 (BHV-1) strain K 22, the Bovine herpes virus 4 (BHV-4) strain Movar and the Aujeszky's disease virus were used at titres of 10^{5.5}, 10^{7.3}, 10^{6.5} and 10^{5.3} cell culture infective dose 50% (CCID₅₀), respectively. The virus titre was determined by the method of Reed and Muench [20].

The CpHV-1 reference strain McK was used as a positive control in PCR.

The continuous cell line MDBK (CCLV 1992) was cultured in Eurobio Dulbecco's modified Eagle's medium + Sigma Minimum Essential Medium Eagle with Hank's salt and L-glutamine in a 1:1 ratio, 5% foetal bovine serum, Penicillin 100 UI/ml, and Streptomycin 100 µg/mL. Cells were maintained in the same medium, but with 3% foetal bovine serum, following virus adsorption for 2 hours at 37°C and monolayer washing.

ANTI-CPHV-1 AND FITC-CONJUGATED ANTI-CPHV-1 ANTIBODIES

Serum from a latently CpHV-1 infected goat in which the virus neutralization titre was 9 log₂ was used. The serum was negative for antibodies to *Brucella melitensis*, *Chlamydia abortus*, *Coxiella burnetii* and *Leptospira pomona*. It was obtained 29 days following immunosuppression with Dexamethasone 0.4% (Caliercortin – Laboratories Calier S.A.) as described by BOUNAVOGLIA *et al.* [2]. The conjugation was done at the National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria.

IMMUNOFLUORESCENCE ASSAY

MDBK cells were grown to confluence on specially prepared glass lamellas (10×20 mm). They were inoculated with the CpHV-1 strain E/CH (10^{5.5} CCID₅₀) and after 1, 2, 4, 5, 6, 12, 24, 36, and 48 hours, they were fixed for 20 minutes in cold acetone (-20°C). Then cells were equilibrated in PBS pH 7.6, for 10 minutes and air-dried at room temperature. They were stored at -70°C for later use. Lamellas were washed twice in PBS and incubated for 1 hour in optimally diluted fluorescein isothiocyanate (FITC)-conjugated anti-CpHV-1 antibodies then washed three times in PBS. Evans blue (0.1%) was used for contrast staining. Lamellas were washed three times in PBS and mounted in buffered glycerol (pH 8). Fluorescence microscope Zeiss (AXIO, Imager.A1, Germany) was used with light source and FITC excitation/emission filter combination.

As additional controls for the specificity of the observed staining, non-inoculated cell cultures and cell cultures inoculated with BHV-1 strain K 22, BHV-4 strain Movar, Aujeszky's disease virus, and heterologous immunofluorescence conjugates (parainfluenza 3 virus and classical swine fever virus) were used. Blocking test was carried out with serum which was positive for anti-CpHV-1 antibodies at a titre of 8 log₂ and negative for antibodies to *Brucella melitensis*, *Chlamydia abortus*, *Coxiella burnetii* and *Leptospira pomona*. Additionally, lamellas inoculated with the CpHV-1 strain E/CH were incubated with FITC-conjugated anti-BHV-1 monoclonal antibodies (Bio-X Diagnostics, Belgium, EU).

CYTOLOGICAL ASSAY

Duplicate cytological samples from non-inoculated and inoculated MDBK cell culture were washed in PBS. Then samples

were double-stained with two vital fluorochromes or were air-dried at room temperature and stained with May-Grünwald-Giemsa. The double vital staining solution was prepared by adding 100 µL of 1 mg/mL propidium iodide (PI, Sigma) and 100 µL of 1 mg/mL acridine orange (AO, Sigma) to 10 mL of PBS. Lamellas (in triplicate) with infected monolayer were removed, washed in PBS and a drop of staining solution was pipetted onto them. To classify cells by colour and chromatin morphology the following criteria were used:

- Viable non apoptotic cells (green non-fragmented nucleus);
- Viable apoptotic cells (green fragmented nucleus or green condensed chromatin);
- Non viable apoptotic cells (red pyknotic nucleus or red condensed chromatin);
- Non viable non apoptotic cells (red non-fragmented nucleus);
- Necrotic cells (red intact nucleus).

The total cell count in 5 random photographed fields per slide was scored. The investigations were performed on fluorescent microscope Leika DM 500B (Wetzlar, Germany) equipped with the FITC combination of filters. Apoptotic index and percentage of necrotic and dead cells were determined by the following criteria and formulas [4]:

$$\text{Apoptotic index (\% of apoptotic cells)} = 100 \times (\text{LA} + \text{DA}) / (\text{LN} + \text{LA} + \text{DN} + \text{DA});$$

$$\text{\% of necrotic cells} = 100 \times \text{DN} / (\text{LN} + \text{LA} + \text{DN} + \text{DA});$$

$$\text{\% of dead cells} = 100 \times (\text{DN} + \text{DA}) / (\text{LN} + \text{LA} + \text{DN} + \text{DA}),$$

where LN were live target cells with normal nuclei, LA live cells with apoptotic nuclei, DN dead cells with normal nuclei and DA dead cells with apoptotic nuclei.

ELECTRON MICROSCOPY

Cells were harvested by low-speed centrifugation of cell culture supernatants (1 500 g for 20 minutes at 4°C) from virus isolates. The low-speed supernatant was pelleted at 109 000 g for 1 hour at 4°C. The obtained high-speed pellet was suspended in a minimal volume (0.5 mL) of sterile distilled water and centrifuged at 1 500 g for 20 minutes at 4°C. Formvar and carbon coated (400 mesh) copper grids were floated on 50 µL drops of concentrated virus suspension and negatively stained with 2% sodium phosphotungstate (NaPT), pH 6.8. Samples were imaged using a JEM 1200 EX electron microscope at an accelerating voltage of 80 kV and 6000-75000X instrument magnification.

DNA ISOLATION AND PCR

To detect the presence of intracellular CpHV-1, MDBK cells were analyzed 15, 30, 45, 60, 90, 105, 120 and 180 minutes following inoculation with CpHV-1 strain E/CH. Maintaining medium was removed and cells were washed 5 times with physiological solution. Cell monolayers were then mechanically collected using a cell scraper (Greiner bio-one, Germany); cells were centrifuged at 735g for 3 minutes at 4°C and suspended in phosphate buffer, pH 7.2. DNA was extracted with the QIAGEN kit (DNeasy[®] Blood & Tissue kit) according to the manufacturer's instructions.

PCR amplification was performed using HotStart-ITFideliTaq MasterMix (MM) (USB Corporation, Ohio, USA) and the following primer pair for the gC gene [9]: Primer 1 (nucleotides 759-779) 5'-AGGGCGCCGGTGGATGCTCTG-3' and Primer 2 (nucleotides 1172-1154) 5'-GGCGGGCGGTGCGTCGTGA-3'. The reaction mixture (25 μ L) contained 2 μ L DNA, 1 μ L of each primer (at a concentration of 10 pmol/ μ L), 8.5 μ L distilled H₂O, and 12.5 μ L MM. PCR was performed under the following conditions [28]: 1 cycle of denaturation at 96°C for 10 minutes, annealing at 65°C for 1 minute, elongation at 72°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 65°C for 1.30 minute, elongation for 72°C for 1 minute and a final elongation step at 72°C for 5 minutes. The amount of amplified DNA generated was determined by gel electrophoresis and Gene Quant II spectrophotometer (Pharmacia LKB Biochrom, UK).

Results

IMMUNOFLUORESCENCE ASSAY

Two hours after the inoculation of cell cultures with CpHV-1 strain E/CH ($10^{5.5}$ CCID₅₀), asymmetric perinuclear staining was observed (figure 1a). In the following hours the number of immunofluorescence-positive cells considerably increased. They formed cell syncytia 12 hours post infection which were more marked 24 hours after. Then from 36 to 48 hours after infection, there was a noticeable loss of cells from the monolayer. Isolated fluorescent syncytia with well defined fluorescent cytoplasmic bridges were observed (figure 1b). These

cytological modifications were specific of cell infection by the CpHV-1 strain E/CH as demonstrating by specificity tests and controls described above.

In the next step, lamellas infected with BHV-1, Aujeszky's disease virus and BHV-4 were incubated with conjugated anti-CpHV-1 antibodies. Cytoplasmic and perinuclear fluorescence of syncytia was observed 21 to 42 hours post infection. The strongest staining was seen in BHV-1 infected CC, and the poorest staining, in BHV-4 infected ones. When conjugated anti-BHV-1 antibodies were applied on CpHV-1 infected lamellae, the fluorescence was weaker than that observed for the reaction between conjugated anti-CpHV-1 antibodies and BHV-1.

CYTOLOGICAL ASSAY

Native and May-Grünwald-Giemsa-stained samples of infected MDBK cell cultures were observed microscopically. In both cases visible cytopathic effect was found 6 hours post infection: few cells exhibited fragmented or condensed chromatin (figures 2b and 2c). In addition to these changes, cell syncytia (figure 2d) of various sizes and with different numbers of nuclei in which apoptotic nuclear morphology (fragmented and/or hyper-condensed chromatin) was considerably more apparent were formed 24 hours post infection. In control cells, these nuclear changes were not present (figure 2a).

The results from the quantitative cytological analysis based on double staining with acridine orange and propidium iodide are shown in Table I. Whereas in non infected cells the proportions

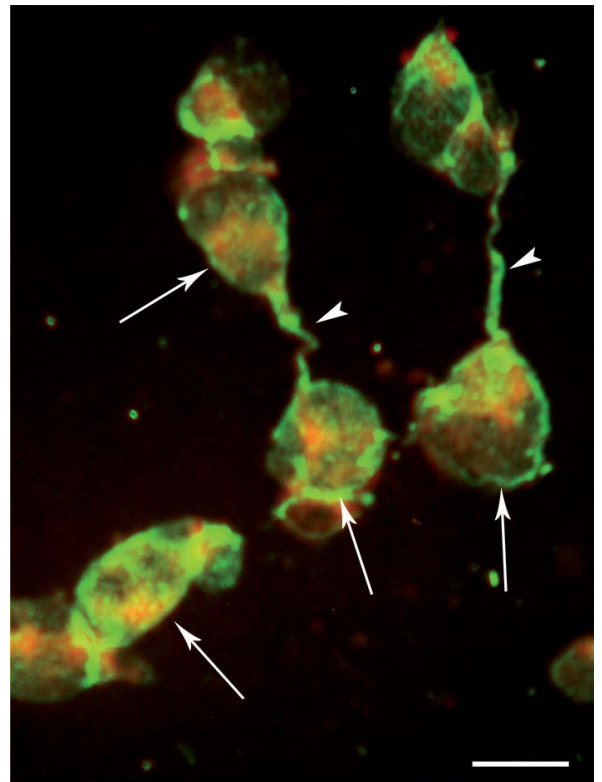
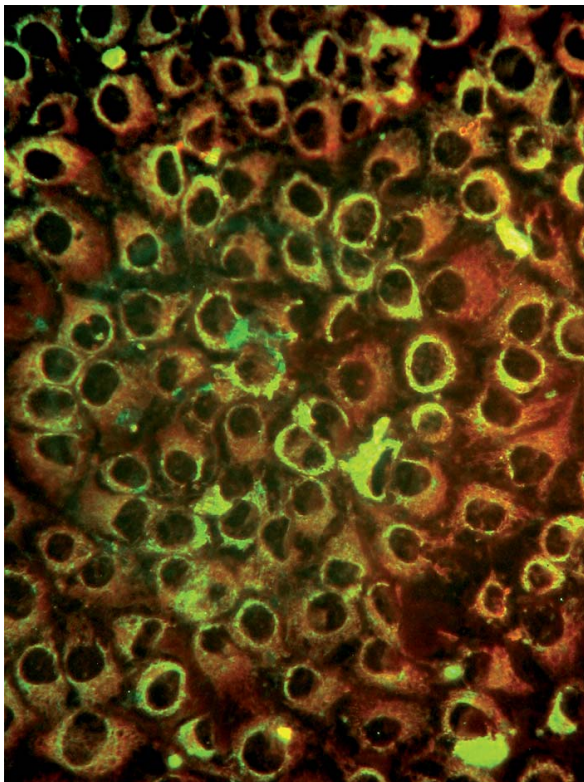


FIGURE 1: MDBK cells infected with CpHV-1 strain E/CH ($10^{5.5}$ CCID₅₀), direct immunofluorescence staining, bar length: 30 μ m. **a.** Perinuclear and cytoplasmic immunofluorescence 2 hours post infection; **b.** Cytoplasmic and nuclear fluorescence of syncytia (arrows) and cytoplasmic bridges (arrowheads) between them, 48 hours post infection.

of dead cells and apoptotic cells have remained below to 0.56% during 36 hours, some apoptotic viable cells appeared in cultures infected with the CpHV-1 reference strain E/CH since the 2nd hour. On 12 hour post infection, the counts of apoptotic cells exceeded 20% and dramatically increased on 36 hours. From 12 to 36 hours post infection, the proportions of dead cells became important (> 12.00%) and necrotic cells were detected at the 12th hour. In parallel, the total cell count in the five random fields decreased from 3345 to 1329 in a time-dependent manner, resulting from loss of cells from the monolayer as a consequence of virus replication.

In addition, electron microscopy has confirmed the presence of CpHV-1 in the inoculum used for infection of all fluorescent and cytologically assayed cell cultures (figure 3).

DNA ISOLATION AND PCR

The PCR amplification yielded a specific intracellular 414 bp product of CpHV-1 gC gene at all the studied time intervals, except 15 minutes after cell infection (figure 4).

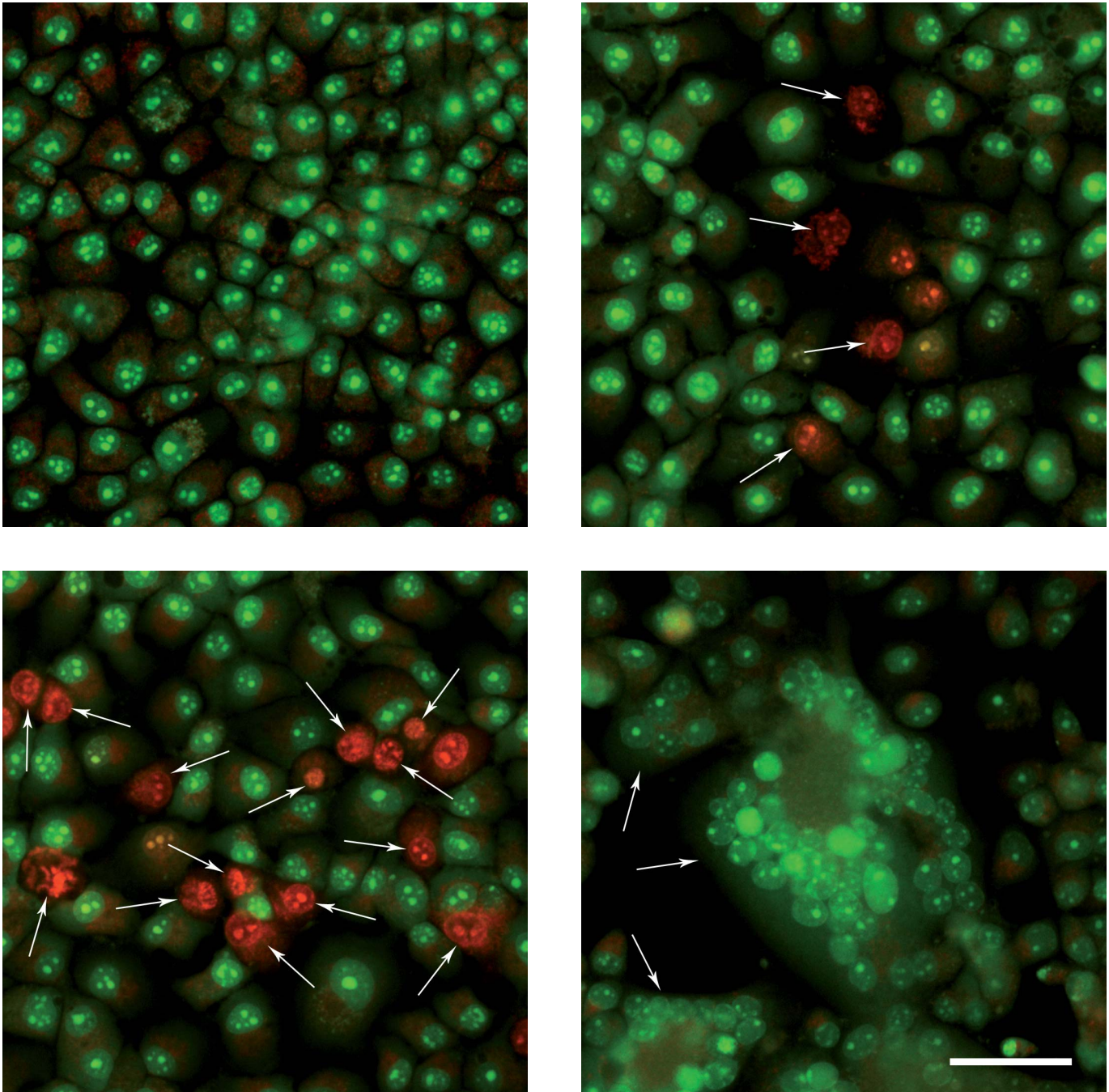


FIGURE 2: Double vital staining of MDBK cell culture with propidium iodide and acridine orange. **a.** Non inoculated cell culture after 60 hours of culture; **b.** MDBK cell culture 6 hours after infection with CpHV-1 strain E/CH ($10^{5.5}$ CCID₅₀). Note the presence of apoptotic cells (arrows); **c.** MDBK cell culture 12 hours after infection with CpHV-1 strain E/CH ($10^{5.5}$ CCID₅₀). Note the increased number of dead apoptotic cells (arrows) and decreased number of cells in the monolayer; **d.** syncytium of live nuclei (arrows) 36 hours after infection with CpHV-1 strain E/CH ($10^{5.5}$ CCID₅₀). Bar length is 50 μ m.

	Time (in hours) after infection						Cell control
	2	4	6	12	24	36	
Apoptotic cells (%)	2.43	5.76	6.41	26.28	22.12	53.05	0.56
Necrotic cells (%)	0.00	0.00	0.00	0.12	0.79	1.65	0.00
Dead cells (%)	0.05	0.09	0.69	12.69	12.19	24.3	0.00

The percentages of apoptotic cells (AP), necrotic cells (NC) and dead cells (DC) were determined following the respective formulas: $AP = 100 \times (LA + DA)/(LN + LA + DN + DA)$, $NC = 100 \times DN/(LN + LA + DN + DA)$ and $DC = 100 \times (DN + DA)/(LN + LA + DN + DA)$ where LN were live target cells with normal nuclei, LA live cells with apoptotic nuclei, DN dead cells with normal nuclei and DA dead cells with apoptotic nuclei.

*Note: Because, over 95% cells from monolayer have been fell into medium at 48 hours, the apoptotic index and percentage of necrotic and dead cells were not determined.

TABLE I: Proportions of apoptotic, necrotic and dead cells 2 to 36 hours following infection of MDBK cells with CpHV-1 reference strain E/CH (10^{5.5} CCID₅₀). Results are expressed as mean determined from 5 random photographed fields per slide.

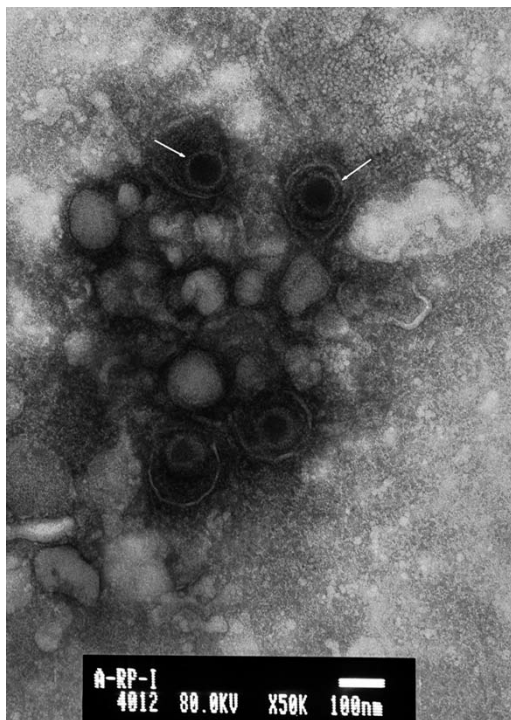


FIGURE 3: CpHV-1 strain E/CH concentrated by differential centrifugation. Negative contrast with sodium phosphotungstate, pH 6.8. Viral particles are noted with arrows.

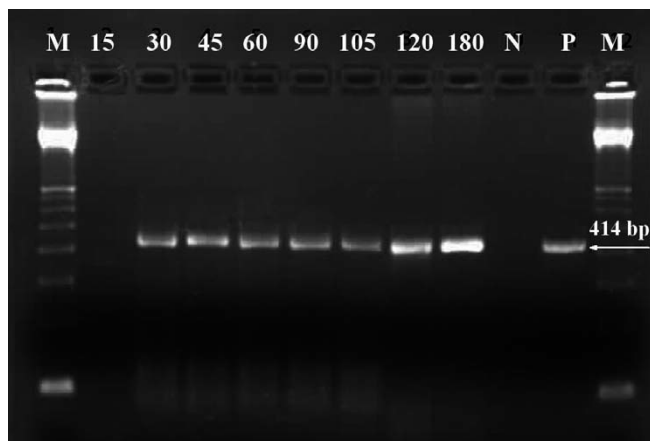


FIGURE 4: PCR products according to time (in minutes) after infection of MDBK cell monolayer with CpHV-1 strain E/CH. M: molecular weight marker; N: negative control from non inoculated cell culture; P: positive control CpHV-1 strain McK.

Discussion

The results from this study clearly showed that the IF method developed here could be considered as highly specific and suitable for quick diagnostics of CpHV-1 in primary isolates from cell cultures.

In studies on Herpes simplex virus and Herpes virus saimiri the absence of cytoplasmic fluorescence has been explained with the presence of immature viral particles in the cytoplasm and mature ones in the nucleus [16, 19]. Contrary to their observations, perinuclear staining was detected in the present study as early as 2 hours post infection. This could most probably be due to intensive synthesis of structural viral proteins in cytoplasm during the latent phase of virus replication, when the synthesis of structural proteins takes place immediately after DNA replication and late mRNA translation [17]. This suggestion was further supported by the detection of a specific PCR product 30 minutes after infection, which is indicative of the presence of intracellular viral DNA. These results were in agreement with the 2-4 hours duration of the latent phase reported by Berrios and Mc kercher [1].

The increased immunofluorescence staining observed 6 hours after infection in the present study was most likely due to the progeny virions after a long eclipse period of 5-6 hours [1, 6]. This was also demonstrated by the increased number of apoptotic cells as a result of activation of caspases-8 and -9, which are involved in the two distinct apoptosis pathways – death-receptor mediated pathway and mitochondrial pathway [14]. The diffuse staining of the monolayer 12 hours after infection represented the exponential phase of viral replication. Exponential growth has been observed between 6 and 12 hours post infection by some authors [6], or between 10 and 12 hours post infection by others [1]. In agreement with these findings, the results obtained by IF assay and AO staining confirmed that the exponential phase occurred about 12 hours post infection. As additional supporting evidence, a sharp increase in the apoptotic index was noted. Similar data have been obtained by other authors for BHV-1 [24], except that in our experiments the changes occurred earlier because of the shorter CpHV-1 replication cycle. The observed staining of cytoplasmic bridges from syncytia indicated that there was passage of virus particles through them.

The degree of fluorescent staining of the heterogeneous herpes viruses incubated with conjugated anti-CpHV-1 antibodies

was in good correspondence with the reported antigenic relationships between them [22]. These cross-reactions most probably resulted from the presence of anti-gB antibodies, as the gB gene is conservative and with high sequence homology among the members of the herpes virus group [8]. Since ENGELS *et al.* [6] have reported a greater degree of neutralization of CpHV-1 by BHV-specific antiserum than of BHV-1 by anti-CpHV-1 serum, a stronger fluorescence for the reaction between conjugated anti-BHV-1 antibodies and CpHV-1 than between conjugated anti-CpHV-1 antibodies and BHV-1 was expected. Interestingly, the opposite was observed most probably because the conjugated anti-BHV-1 antibodies used in the present experiments were monoclonal, while the conjugated anti-CpHV-1 antibodies were polyclonal. Further experiments are underway to test whether the developed method could be successfully used for diagnostics of CpHV-1 in pathological material.

Additionally, electron microscopy was performed to confirm the presence of virus particles in the inoculum in order to cytologically analyze the dynamics of CpHV-1 infection. The results showed similar changes to those described by other authors [6, 31], except for the typical herpes virus Cawdry type A inclusion bodies, which were not observed in the present experiments. To make a quantitative cytological analysis of cell death dynamics in the cytological samples, double staining assay with acridine orange / propidium iodide was used. It was obtained that the morphological criteria were considerably better defined as compared to May-Grünwald-Giemsa staining. Similar dynamics of the apoptotic index in CpHV-1 infected cell cultures have been described by PAGNINI *et al.* [18].

As a conclusion, the IF method developed in the present work could be considered as highly specific and suitable for quick diagnostics of CpHV-1 in primary isolates from cell cultures. Based on the results from the cytological assay, it could be concluded that CpHV-1 induces apoptosis in infected cells without forming Cawdry type A bodies. This, together with the formation of syncytia, could be considered as specific cytopathic changes typical of cell cultures.

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