

EFFECTIVE PRIMARY ISOLATION AND *IN VITRO* CYTOPATHIC EFFECT EVALUATION OF CANINE DISTEMPER VIRUS WILD-TYPE STRAINS

PROPAGAREA *IN VITRO* ȘI EVALUAREA EFECTULUI CITOPATIC PRODUS DE TULPINI SĂLBATICE ALE VIRUSULUI DISTEMPER CANIN

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ABSTRACT | REZUMAT

Canine distemper virus (CDV), currently named Canine morbillivirus, is a highly contagious pathogen that affects canids. It is well determined that CDV is a pathogen with multiple cell tropism, its host range including a great variety of species. Improving the understanding of the CDV wild-strains pathogeny, Vero cells derived from African green monkey kidney were used to isolate and phenotypically characterize the cytopathic effect. In this study were used eight CDV wild-strains collected during 2019-2020 from unvaccinated dogs with different clinical forms of distemper virus infection. The infected Vero cell monolayers were examined at 6h, 28h, and 48h post-infection for CPE and evaluation of the cell destruction. Differences in CPE on Vero cells inoculated with CDV wild-strains have been detected. Virus growth was detected first in Vero cells at 6h post-infection, whereas fusion of infected cells was most obvious at 24h post-infection. Our results indicate that Vero cells are sensitive to propagate CDV from clinical specimens and that the *in vitro* pathogenicity of the virus can be correlated with the clinical form.

Keywords: Canine distemper virus, Vero cell, cytopathic effect

Virusul distemper canin (VDC), denumit în prezent Canine morbillivirus, este un agent patogen extrem de contagios care afectează canidele. Acest agent viral cu tropism pentru diverse tipuri de celule, produce îmbolnăviri cu simptomatologie diversă la un număr mare de specii de animale. Pentru a îmbunătăți înțelegerea patogenității tulpinilor sălbatice de VDC, linia celulară Vero derivată din celule renale de maimuță a fost utilizată pentru izolarea și caracterizarea fenotipică a efectului citopatic. În acest studiu au fost utilizate opt tulpini sălbatice de VDC, colectate în perioada 2019-2020 de la câini nevaccinați cu diferite forme clinice. Culturile de celule Vero infectate au fost examinate la 6h, 28h și 48h post-infecție (p.i.) pentru evaluarea efectului citopatic. În urma studiului propagării *in vitro* a tulpinilor de VDC au fost detectate diferențe considerabile în ceea ce privește efectul citopatic produs de acestea pe linia celulară Vero. Replicarea virală a fost detectată de la 6 ore p.i., în timp ce fuziunea celulelor infectate a fost evidentă la 24 de ore după infecție. Rezultatele obținute în urma prezentului studiu indică faptul că linia celulară Vero poate fi utilizată pentru propagarea VDC din speciile clinice și că patogenitatea virusului *in vitro* poate fi corelată cu forma clinică evolutivă.

Cuvinte cheie: virusul distemper canin, linia celulară Vero, efect citopatic

Canine distemper virus (CDV) is included in the *Paramyxoviridae* family, the *Morbillivirus* genus, which comprises some of the most important pathogens in human history. Morbilliviruses have the propensity for infecting multiple host species, the host range of CDV being the widest of all morbilliviruses. Canine distemper virus is the only member of the genus where natural infections of both terrestrial and marine species have been recorded (2). CDV is a unique morbillivirus, being able to persist in target hosts living in low-density populations. Moreover, the virus is well-known for

its neurovirulence. Both properties of the virus may be correlated with the severity of the immune suppression and extended virus shedding (7).

Morbilliviruses are single-stranded, non-segmented, negative-sense, RNA viruses encoded by genomes of between 15,690 to 16,050 nucleotides in length (9). The CDV genome consists of 6 genes, which encode the large protein (L), nucleocapsid protein (N), matrix membrane protein (M), phosphoprotein (P), fusion protein (F), and hemagglutinin protein (H) (6). The H and F glycoproteins are responsible for viral attachment and entry process by binding to the host cells receptors (11). It has been confirmed that H protein displays antigenic variation and that the antibodies against H glycoprotein have a major role in the

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development of protective immunity against CDV (1). The F protein mediates the membrane fusion event, which allows the entry of the viral genome into the cytoplasm. The CDV cytolytic strains were demonstrated to spread through cell cultures both by producing infectious extracellular particles and by lateral cell-to-cell fusion. Accumulated evidence suggests that N-glycosylation of viral glycoproteins has a major impact on virus replication and virulence, such as attachment to cell membrane receptors and protecting against neutralisation by specific antibodies (5).

The aim of this study was virus isolation and *in vitro* evaluation of CDV cytopathic effect and cell fusion. The usage of cell lines represents a good alternative for the biological characterization of CDV field isolates, since there is no other effective method. Our results indicate that Vero cells are sensitive to propagate CDV from clinical specimens.

MATERIALS AND METHODS

Sampling

CDV wild-strains were collected during 2019-2020 from unvaccinated dogs with different clinical forms of distemper virus infection (Table 1). In all patients, the infection was confirmed using an in-clinical immunoenzymatic assay for CDV antigen detection (Vetexpert®One step test, Poland).

Cerebrospinal fluid was collected in sterile cryotubes (Nalgene) via cerebello-medullary cisternal puncture after the animal death and stored at -80°C . Ocular and nasal swabs were collected in sterile PBS from three dogs. The swabs were suspended in PBS, vortexed, and then centrifuged at 5000 rpm for 5 minutes and the supernatant was collected into a new sterile tube. Consequently, the antibiotics were added (100 unit/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin, Calbiochem, Germany) and after that, the flasks

were incubated at 37°C for one hour. The sample was then centrifuged at 8000 rpm for 10 minutes and the supernatant was collected into a new sterile cryotube which was stored at -80°C until further use.

Reagents

Considering the susceptibility of CDV, the Vero cell line was used as a candidate for the present investigation. Vero cells were cultivated in Dulbecco's modified Eagle medium (DMEM-Sigma, UK) supplemented with 10% foetal bovine serum (FBS-Gibco, Life Technologies Scotland), 100 unit/ml of penicillin, and 100 $\mu\text{g}/\text{ml}$ of streptomycin (Calbiochem, Germany), and grown in a 5% CO_2 atmosphere incubator at 37°C . The cells were regularly subcultured in a Nunc filter cap flask (Nuncclon, Denmark) by employing standard techniques. Trypsin-EDTA solution (10X) was purchased from Sigma, UK.

Virus isolation. Vero cells were seeded in Nunc filter cap flask of 25cm^2 . When confluency was 100% (Fig. 1) the culture media was removed and the cultures were overlaid with 0.5 ml of each virus suspension and incubated for one hour with shaking at 15 min intervals.

After CDV adsorption, the suspensions were removed and the cultures were washed once with DMEM media. The cultures were then overlaid with DMEM medium 10% FBS and incubated at 37°C in a 5% CO_2 atmosphere. An uninoculated flask of Vero cells was used as a negative control. The infected Vero cell monolayers were examined at 6h, 28h, 48h post-infection for cytopathic effect (CPE).

RESULTS AND DISCUSSION

Canine distemper is a worldwide distributed viral disease that is highly contagious and has high morbidity and mortality. Viruses with a broad host range

Table 1
Identification data regarding the dogs from which were collected the samples

Case no.	Year of sampling	Date of sampling	Dog Age	Dog Sex*	Dog Rase
1	2019	12.04.2019	1 year	F	Mixed breed dog
2		22.06.2019	4 months	M	German Shepherd
3		6.07.2019	1 year	M	Pekingese dog
4		8.08.2019	6 months	M	Bichon
5		8.12.2020	3 months	F	Mixed breed dog
6	2020	26.02.2020	3 months	F	Bichon
7		27.02.2020	4 months	F	Mixed breed dog
8		1.03.2020	3 months	M	Mixed breed dog

* male - M; female - F

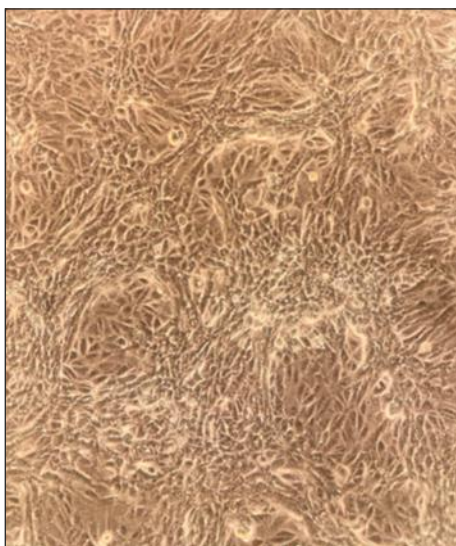


Fig. 1. Vero cells before inoculation, approximately 99% monolayer confluency, magnification x100

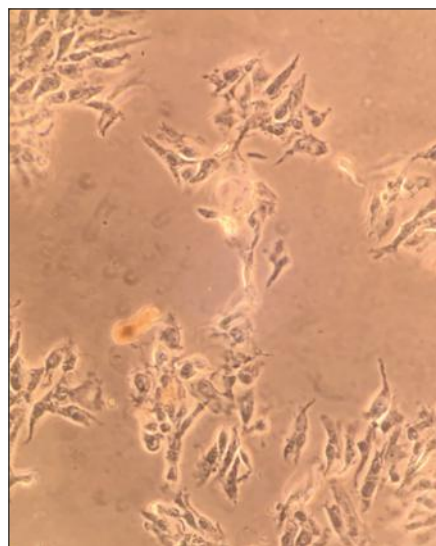


Fig. 2. Vero cells at 24h after inoculation with CDV (case no. 8): the destruction of 85% of the monolayer, magnification x 200

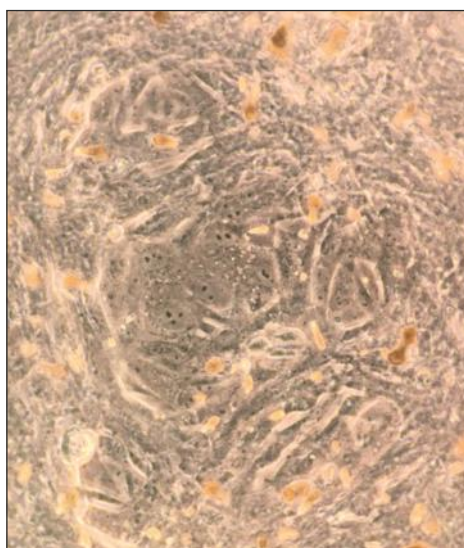


Fig. 3. Vero cells at 24h after inoculation with CVD (case no. 3): fusion of the infected cells, magnification x 200

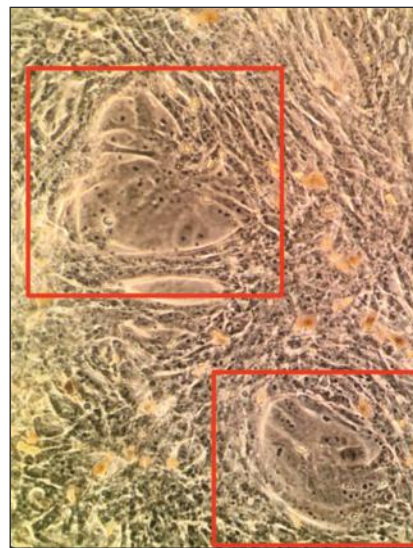


Fig. 4. Vero cells at 24h after inoculation with CVD (case no. 2): fusion of the infected cells, magnification x 200

such as CDV could persist in populations of great sizes and may potentially cause epidemics in endangered species. For canine distemper virus, the domestic dog population may represent a favourable homogeneous environment that permits optimization of CDV adaptation in this particular host (8). Wild-type CDV isolation and propagation have been often succeeded only by co-cultivation of infected tissues samples with peripheral blood mononuclear cells derived from SPF (specific pathogen-free) dogs. In this study was analysed the utility and consequences of an easier and less expensive alternative: CDV wild-type strains propagation on Vero cells. There are no reports on CDV isolation from the clinical samples of Romanian dogs

and it is a well-established fact that isolation of wild-type CDV is tedious and difficult. In our study the CDV - infected Vero cell monolayers were examined at 6h, 28h, 48h post-infection for CPE and evaluation of the cell death percentage (Table 2).

After 24h pi, samples from three dogs (case no. 6, 7, 8) caused CPE characterized by leading to the destruction of more than 80% of the cell monolayer (Fig. 2). Within 24 h after inoculation, one sample (case no. 3) caused CPE in Vero cells which was characterized by syncytium formation (Fig. 3). After 48h pi, samples from two dogs (case no. 2, 4) caused CPE characterized by the fusion of infected cells (Fig. 4).

A particularly noticeable CPE of CDV infection on

Vero cells is the syncytia or polykaryocytes forming. Syncytia represents multi-nucleate enlarged cells produced by fusion of infected cells, displayed in large cytoplasmic masses that have many nuclei (poly, many; karyon, nucleus). The H and F proteins of CDV may produce cell fusion when are expressed simultaneously in susceptible cell lines (10). The mechanism of fusion of infected cells results from the interaction between the host cell membranes and viral glycoproteins. Cell fusion represents a process through which a virus disseminates from an infected cell to an uninfected one. It was shown that the co-expression of the F and H glycoproteins was necessary and sufficient to induce cell fusion, and that the CDV H protein was the major factor determining cell tropism. In addition, reverse genetics technology suggested that the CDV tropism and the cytopathogenicity, also termed fusogenicity, in Vero cells were mainly determined by the H protein (5).

Table 2

Evaluation of cytotoxic infection of wild-type CDV on Vero cells

Case no.	Type of sample	Cell death (%)		
		6h pi	24h pi	48h pi
1	nasal discharge	1%	5%	10%
2	CSF	10%	20%	40%
3	eye discharge	15%	20%	40%
4	nasal discharge	10%	30%	50%
5	CSF	15%	30%	50%
6	CSF	70%	99%	100%
7	CSF	50%	80%	100%
8	CSF	50%	85%	100%

The eight Romanian isolates of CDV wild-strain presented quite distinct phenotypes behaviours on Vero cells from each other. The CDV strains were classified taking into account the growth ability and development of CPE in Vero cells. Virulent wild-type CDV replicated readily in Vero cells causing a high percentage of cell destruction at 6h post-infection. Virus growth was detected first in Vero cells at 6h post-infection, whereas fusion of infected cells was most obvious at 24h post-infection. A similar result was obtained recently by Cheng et al. (1), which propagated and evaluated the CPE produced on Vero cells by a wild-type strain of CDV recovered from a racoon dog. Moreover, effective primary isolation in Vero cells of wild-type canine distemper virus isolate from naturally infected free-ranging raccoons was also accomplished (4). Efficient replication on Vero cells was obtained also in the case of canine distemper virus isolated from lung tissue of a rhesus monkey (3). These facts may indicate that Vero cells are very useful for the classification of various CDV strains after propagation.

CONCLUSIONS

Based on these findings, there were highlighted significant differences in distemper virus CPE phenotypical characteristics and appearance. The first sign of viral infection was the destruction of the Vero cell monolayer. In the case

of virulent strains, cells in the monolayer shrieked rapidly, become dense, and detached within 24h post-infection. In the case of three CDV wild strains was identified characteristically CPE through the formation of syncytia. In our opinion virus isolation is important to confirm the diagnosis and investigate pathogeny.

CONFLICT OF INTEREST STATEMENT

The authors declared that there is no conflict of interest.

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