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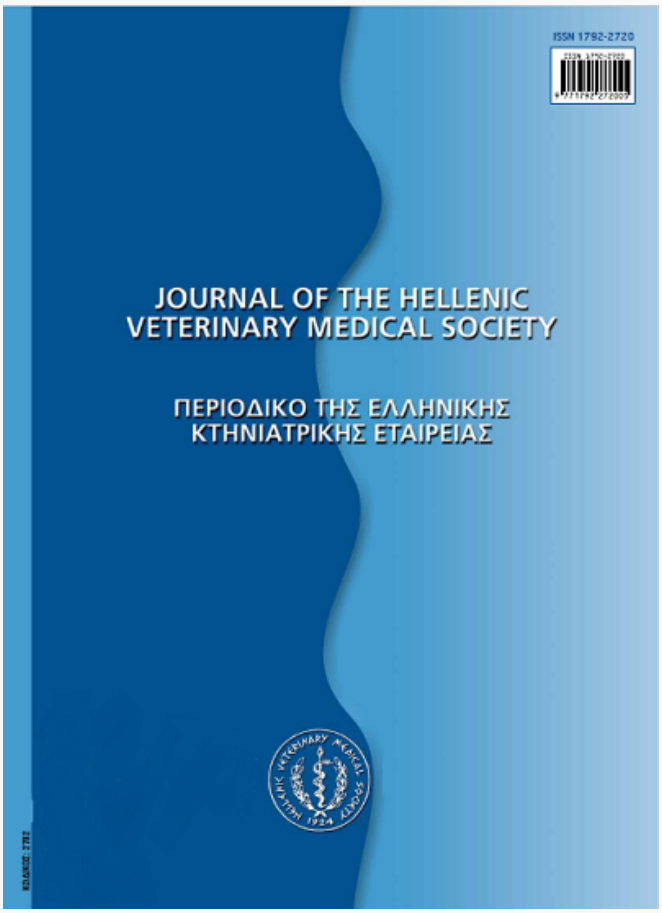

Adaptation of velogenic Newcastle disease virus in Vero cells: velogenicity of virus unaltered after adaptation

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Ερευνητική εργασί

Adaptation of velogenic Newcastle disease virus in Vero cells: velogenicity of virus unaltered after adaptation

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Προσαρμογή και διατήρηση της ιδιότητας ταχυγενούς στελέχους ιού της ψευδοπανώλους σε κύτταρα Vero

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ABSTRACT. In the present experiment, velogenic Newcastle disease virus (NDV) was adapted to Vero cells without the addition of exogenous trypsin. The cytopathic effect (CPE) in NDV-infected Vero cells was observed from passage 3 (P 3) onwards with concomitant increase in the haemagglutination (HA) and virus titres. The CPE was characterized by rounding of cells, their detachment from the surface and syncytia formation. The Vero-adapted NDV was confirmed by reverse transcription-polymerase chain reaction (RT-PCR), which detected the amplification of haemagglutinin neuraminidase (HN) gene from virus-infected cells. The mean death time (MDT) of 58 hours upon inoculation to embryonated chicken eggs and the intravenous pathogenicity index (IVPI) of 2.12 following inoculation to SPF chicken suggested that Vero adapted virus retained the velogenicity even after ten passages. Furthermore, the nervous signs and haemorrhages in the pro-ventriculus of birds that died at day 5 post inoculation with P10 virus confirmed that the Vero-adapted NDV was a neurotropic viscerotropic velogenic strain.

Keywords: Newcastle disease virus, adaptation, Vero cells

ΠΕΡΙΛΗΨΗ. Στην παρούσα εργασία, ταχυγενές στέλεχος του ιού της Ψευδοπανώλους (Newcastle disease virus, NDV) προσαρμόστηκε σε κύτταρα της συνεχούς κυτταρικής σειράς Vero χωρίς την προσθήκη εξωγενούς θρυψίνης. Το κυτταροπαθογόνο αποτέλεσμα του NDV στα κύτταρα παρατηρήθηκε από την τρίτη δίοδο, με συνακόλουθη αύξηση της αιμοσυγκολλητικής του δύναμης και του τίτλου του. Το κυτταροπαθογόνο αποτέλεσμα χαρακτηριζόταν από στρογγυλοποίηση των κυττάρων, αποκόλλησή τους από την επιφάνεια της φιάλης και σχηματισμό συγκυτίων. Επιβεβαιώθηκε η παρουσία του προσαρμοσμένου ND ιού στα κύτταρα Vero με RT-PCR, που ανίχνευσε το γονίδιο της αιμοσυγκολλητίνης – νευραμινιδάσης στα μολυσμένα κύτταρα. Ο μέσος χρόνος των 58 ωρών που προκλήθηκε θάνατος των εμβρύων σε εμβρυοφόρα αβγά και ο δείκτης παθογενητικότητας (ενδοφλέβιας χορήγησης) 2.12 (intravenous pathogenicity index (IVPI) που εμφανίσθηκε μετά τον ενοφθαλμισμό εμβρυοφόρων SPF αβγών υποδεικνύει ότι ο προσαρμοσμένος στα κύτταρα Vero ιός διατήρησε την παθογενητικότητά του ακόμη και μετά από δέκα διόδους. Επιπλέον, τα νευρικά συμπτώματα και οι αιμορραγίες στον πρόλοβο των πτηνών, τα οποία κατάληξαν 5 ημέρες μετά τον ενοφθαλμισμό τους με P10 ιού, επιβεβαίωσαν ότι ο προσαρμοσμένος ιός στα κύτταρα Vero ήταν ένα νευροτρόπο, σπλαχνοτρόπο, ταχυγενές στέλεχος.

Λέξεις ευρετηρίασης: Ιός Ψευδοπανώλους, προσαρμογή, κύτταρα Vero

"Η απόδοση της περίληψης στα ελληνικά έγινε από την κ. ***Ξυλούρη Ε.****, DVM, MSc, PhD, Αναπλ. Καθηγήτρια ΓΠΑ"*

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1. INTRODUCTION

Virus adaptation to a host system is a pre-requisite for understanding the virus-induced pathogenesis, host-pathogen interactions, etc. (Georgieva and Jordanova, 1999). A number of viruses have been adapted to cultured mammalian cells and targets within cells on which viruses strike have been identified (Gadaleta et al., 2002; Nagaleekar et al., 2007). NDV, an avian virus belongs to the genus *Avulavirus* in the family *Paramyxoviridae* (van Regenmortel et al., 2000). It has a single stranded negative sense RNA as its genome (de Leeuw and Peeters, 1999). It causes Newcastle disease (ND) in birds of various species (Alexander, 1997). Although reports exist regarding NDV adaptation to Vero cells (Ahamed et al., 2004; Mohan et al., 2007), the titre of the virus obtained had been low. In addition, the adaptation to Vero cells was achieved with the addition of exogenous trypsin. In the current study, velogenic NDV was adapted to Vero cells without the addition of exogenous trypsin. Additionally, the strategies, which resulted in higher virus titre, have been discussed.

2. MATERIALS AND METHODS

2.1. Vero cell line, culture media, virus

Vero cells were grown in Glasgow's Modified Eagle's Medium (GMEM) supplemented with 10% fetal bovine serum, penicillin 100U/ml, streptomycin 100μg/ml and HEPES buffer (10mM final concentration) (Duchefa Biocheme, The Netherlands). The Chicken embryo fibroblasts (CEF)-adapted velogenic NDV was used for infecting the Vero cells.

2.2. Virus infection to Vero cells

In the present study, CEF-adapted virus, that has the MDT of 58 h, ICPI of 1.65, IVPI of 2.15, GenBank accession No.AJ249529, was used. The titre of the virus was $10^{6.5}$ tissue culture infective doses ($TCID_{50}$)/ml. Vero cells, grown to 80% confluence, were infected with the above virus and incubated at 37°C. Ten subsequent passages were carried out in Vero cells using previously passaged virus as source of inoculum for the next passage. At every passage the virus inoculum was diluted in maintenance medium sequentially, 1:2, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200, 1:500, 1:1.000, and 1:2.000, for up to 10 passages to avoid the defective viral particles. The culture fluids of each passage were assayed for HA activity.

2.3. Virus titration

Serial 10-fold dilutions of the virus were made in maintenance medium. Each dilution was used to infect Vero cells grown in 96 well tissue culture plates. Plate was sealed and incubated at 37°C. Cells were observed daily for characteristic cytopathic effect (CPE). The $TCID_{50}$/ml was calculated at 96 h p.i. as per the method described by Karber (1931).

2.4. Haemagglutination (HA) and haemagglutination inhibition (HI) assays

The HA and HI tests were performed as per the standard protocol recommended by the Office International des Epizooties (OIE, 2004). Briefly, for HA test, 2-fold dilutions of the virus in phosphate buffered saline (PBS, pH 7.2) was incubated with 1 % chicken erythrocytes for 30 minutes (min) at room temperature (RT). The reciprocal of the highest dilution of the virus showing HA was used to calculate 4-HA units of the virus. On the other hand, for HI, serial 2-fold dilutions of serum were made in PBS and 4-HA units of NDV were added to each dilution. Each dilution was incubated for 30 min at RT. An equal volume of 1% chicken erythrocytes diluted in PBS was added as a test indicator. The HI end-point was read as the highest dilution of the serum showing the complete inhibition of HA activity.

2.5. The mean death time (MDT) and intravenous pathogenicity index (IVPI)

The MDT and IVPI tests were performed as per the protocol described by OIE (2004) (http://www.oie.int/eng/normes/mmanual/A_00038.ht). For MDT, P10 virus was diluted in sterile saline to give ten fold serial dilutions up to 10^{-9}. 0.1 ml of each dilution was injected into 10 day old embryonated chicken eggs by allontoic route. Eggs were incubated at 37°C. Eggs were examined everyday for 7 days. The minimum lethal dose is the highest dilution of the virus that causes the death of all the embryos. The mean death time is the mean time in hours for the minimum lethal dose to kill all embryos. For IVPI, P10 virus was diluted 1/10 in sterile saline. 0.1ml of the diluted virus was injected to 10 six-week old SPF chicken by intravenous route. They were examined everyday for up to 10 days. Birds were scored 0 if normal, 1 if sick, 2 if paralyzed and 3 if dead. The IVPI is the mean score per bird per observation over the 10-day period. The details of IVPI are given in the following table.

Birds	Time interval (h)									
	24	48	72	96	120	144	168	192	216	240
1	0	0	1	2	3	3	3	3	3	3
2	0	0	1	3	3	3	3	3	3	3
3	0	0	1	3	3	3	3	3	3	3
4	0	0	1	3	3	3	3	3	3	3
5	0	0	0	2	3	3	3	3	3	3
6	0	0	1	3	3	3	3	3	3	3
7	0	0	1	2	3	3	3	3	3	3
8	0	0	0	2	3	3	3	3	3	3
9	0	0	1	2	3	3	3	3	3	3
10	0	0	1	2	3	3	3	3	3	3

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

To confirm the Vero-adapted NDV at molecular level, multiplex RT-PCR was carried out using primers specific to the 'HN' (forward primer 5' GGG GGA TAG GCA AAG AAC TCA TT 3' specifying 401-423 nucleotide position and the reverse primer 5' GTA TTG GCC GTC GAA CCC TAA C 3' specifying 843-822 nucleotide position) and β-actin (forward primer 5' GAG AAG CTG TGC TAC GTC GC 3' and the reverse primer 5' CCA GAC AGC ACT GTG TTG GC 3') genes. The cycling conditions were 35 cycles of 94°C for 30 sec, 54°C for 45 sec and 72°C for 1 min. The amplification of 443 bp specific for the HN and 275 bp for the β-actin were examined. The cDNA from the mock-infected Vero and NDV-infected CEF was used as negative and positive controls, respectively.

3. RESULTS

NDV-infected Vero cells were observed daily for cytopathic changes. Up to passage 2 (P 2), cell monolayer was intact with no visible morphological changes from that of mock-infected cells even at 96 h p.i. However, at P 3, CPE began to appear after 48 h p.i. and was complete at 96 h p.i. The CPE was characterized by rounding of cells, their detachment from the surface and syncytia formation (figure 1A). When examined by transmission electron microscopy, majority of cells were bi-nucleated, a characteristic feature of cell fusion (figure 1B). Subsequent passages elicited the consistent CPE after 35-40 h p.i. The intensity of CPE was increased with time unlike that in mock-infected cells, in which monolayer was still intact for a particular incubation period.

To examine if Vero-adapted virus was NDV, HA analysis of each passaged virus harvest was done. The HA titre was increased with concomitant increase in the passage number. At P 10, the HA titre was 1: 64 and was effectively inhibited by NDV specific hyper-immune serum at the highest dilution of 1:1024. To examine if the virus titre had increased with increase in the passage number, the virus titration was carried out in 96 well flat bottomed tissue culture plates. $TCID_{50}$/ml were calculated at 96 h p.i. as per the method described by Karber (1931). At P 5, virus titre was $10^{5.5}TCID_{50}$/ml, $10^{6.8}TCID_{50}$/ml at P 8, while at P 10, the titre was increased to $10^{7.7}TCID_{50}$/ml. This finding suggested that the infectivity of the virus was increased as it adapted to the microenvironment of the Vero cell. To confirm the Vero-adapted NDV at molecular level, multiplex RT-PCR was carried out using primers specific to the 'HN'. As expected, PCR product of 443 bp specific for the HN and 275 bp for the β-actin were amplified from virus-infected cells confirming the presence of NDV in Vero cells (figure 2A).

Since velogenic NDV was used for infecting the Vero cells at P 1, P 10 virus harvest was inoculated to embryonated chicken eggs to verify the retention of velogenicity. At 60 h post inoculation, all embryos were found dead. The MDT was found to be 58 h. The highest dilution of the virus inoculum that caused the death of the embryos was 1: 10^6. In addition, the IVPI of the adapted virus following inoculation to 6-week old SPF chicken was 2.12. This suggested that the velogenicity of NDV remained unaltered even after ten passages in Vero cells. Further, P10 virus harvest was inoculated to six birds (white leghorn, *Gallus gallus*, six weeks old) intramuscularly at the dose of 10^6TCID_{50}/bird. Birds were observed daily for signs of the ND. At day 4 post inoculation, birds began to show

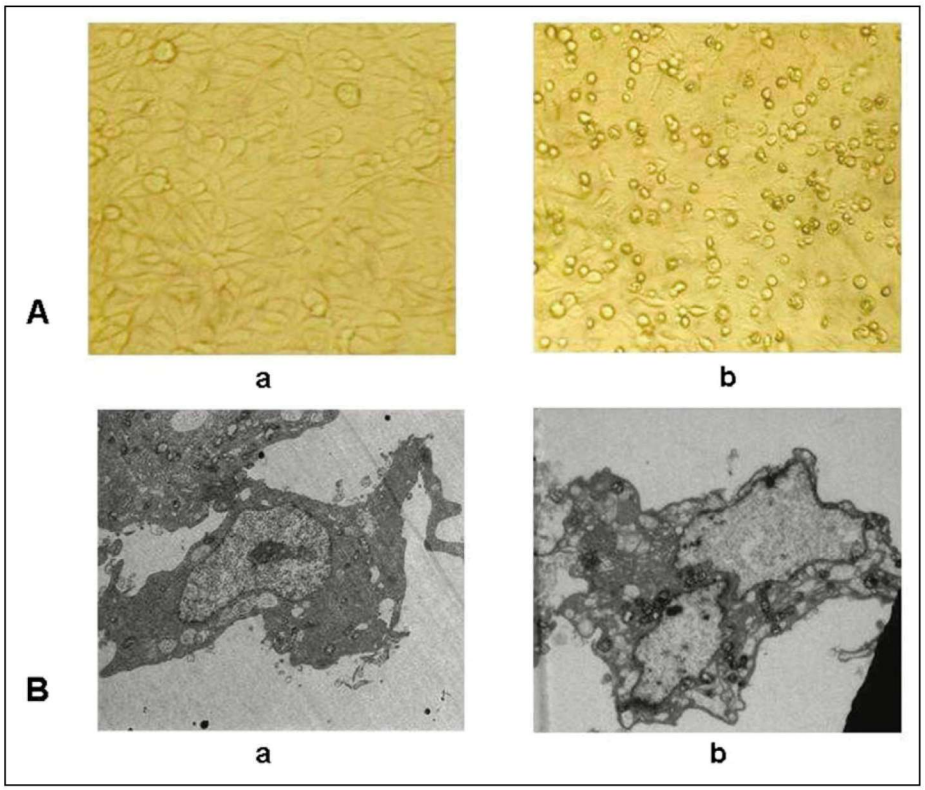

Figure 1A. Photomicrograph of Vero cells showing the cellular changes at 48 h p.i.
(a) Mock-infected Vero cells with intact monolayer.
(b) Vero adapted NDV-infected cells showing the characteristic CPE.

Figure 1B. Electron micrographs of Vero cells showing the ultra-structural changes at 48 h p.i.
(a) Mock-infected Vero cell with a prominent nucleus, nucleolus and a normal fibroblastic appearance.
(b) Vero-adapted NDV-infected cell showing the fused nuclei.

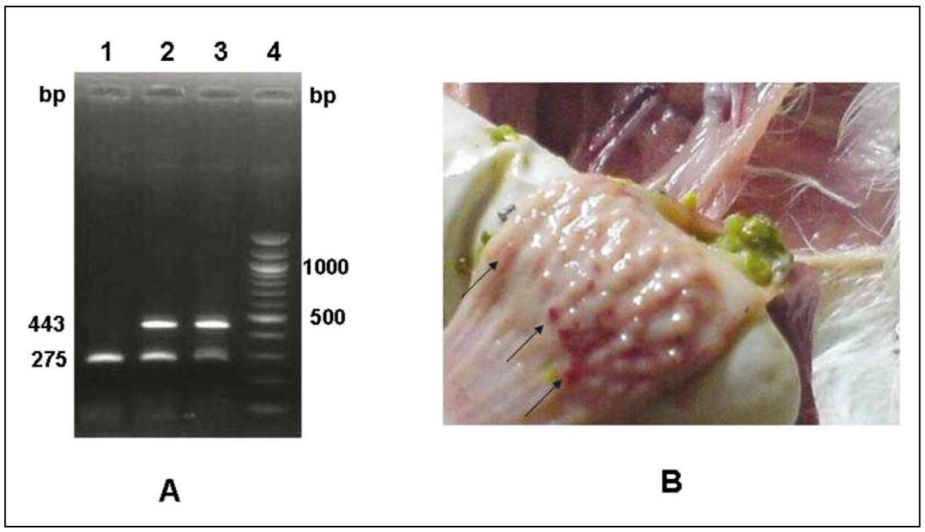

Figure 2A. Detection of NDV in infected Vero cells by RT-PCR.
Lanes 1: The PCR product from mock-infected Vero cells (negative control). **2:** The HN amplification (443 bp) from NDV-infected CEF cells (positive control). **3:** The HN amplification (443 bp) from NDV-infected Vero cells (Test).
4: 100 bp DNA ladder. Note that the β-actin amplification (275 bp) was used as an internal control.

Figure 2B. Confirmation of velogenicity of Vero adapted NDV. The **Arrow** in photograph shows haemorrhages in the pro-ventriculus of chicken.

dullness, tremors, discharge from the nose, greenish diarrhoea, wing paralysis, lateral recumbency and finally died on the next day. Postmortem examination revealed the presence of haemorrhages in the pro-ventriculus (figure 2B), intestine and caecal tonsils. Trachea was also blocked with serous fluid. Based on the postmortem lesions together with above signs, it was concluded that Vero-adapted NDV was a neurotropic viscerotropic velogenic strain.

4. DISCUSSION

In the present study, CEF-adapted velogenic NDV was adapted to Vero cells. HA, HI, RT-PCR and inoculation to experimental chicks were performed to confirm the Vero adapted NDV. A variety of viruses have been adapted to heterologous host systems (Govorkova et al., 1996; Eash et al., 2004; Nagaleekar et al., 2007). Although Ahamed et al. (2004) were successful in adapting the NDV to Vero cells, the titre of the virus at

P 5 was found to be $10^{3.9}TCID_{50}$/ml. In the present study, the virus titre was $10^{5.5}TCID_{50}$/ml at P 5 and was increased to $10^{7.7}TCID_{50}$/ml at P 10. During the first two passages, NDV did not cause CPE, however CPE was induced from P 3 onwards. This observation was similar to the observation noted by Ahamed et al. (2004), who also recorded the appearance of CPE from P 3 onwards in NDV-infected Vero cells. In the present experiment, we diluted the virus inoculum at every passage. This avoided the defective viral particles interfering virus infection to cells. This was evident from our earlier experiments that CPE was not produced even at 96 h p.i., when undiluted virus inoculum was used for infecting the Vero cells. However, when cells were infected with the diluted virus inoculum, the onset of CPE as well as the titre of the virus was gradually increased with the subsequent passages indicating the replication of NDV in Vero cells.

The addition of exogenous trypsin had been shown to promote the cleavage of pro-form of NDV fusion (F_0) protein to its active forms (F_1 and F_2), thus promoting the virus entry into cells (Nagai et al., 1976; Peeters et al., 1999). This particular strategy had been used for the adaptation of NDV to a heterologous host system (King, 1993). Since F proteins of velogenic NDV are cleaved in cultured cells, the addition of exogenous protease, such as trypsin, is not required. In the current study, velogenic NDV was adapted to Vero cells without the addition of exogenous trypsin. The other factors, such as quick freezing and thawing twice, use of healthy cells and maintenance of cold chain, were also found to influence the adaptation of NDV to Vero cells. The present approaches were similar to the strategies followed for the adaptation of influenza virus to Vero cells (Govorkova et al., 1996). Having adapted to a particular host system the infectivity of the virus for that system increases, while the virulence might decrease (Ahamed et al., 2004; Mohan et al., 2007). However, in the present study, though the infectivity of NDV to Vero cells had increased (as indicated by the onset of CPE, increase in HA and virus titres as passage number increased), the velogenicity was unaltered even after ten passages in Vero cells, thus suggesting that virus adaptation to heterologous host depends up on the virus strain, type of the host etc. The Vero adapted NDV was used for further studies for elucidating the disease pathogenesis, designing the antiviral strategies, etc.

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