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MULTIPLICATION OF HVT FC-126 (HERPESVIRUS TURKEY) VIRUS IN THE KIDNEY CELL LINES OF NO AVIAN ORIGIN

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The presented experiments were aimed to cultivate and multiplicate HVT FC-126 in the PLA (Adult pig kidney) and GL-4 (Gerbil kidney) cell lines. Two different HVT FC-126 vaccine strains were used: Marikal SPF (Veterina d.o.o., Croatia) and Lyomarex (Merial, USA). They were adapted to the PLA and GL-4 cell lines. After adaptation, they were titrated on PLA ($TCID_{50}$ $2^{4.23}$) and GL-4 ($TCID_{50}$ $2^{4.96}$). On both cell lines they show similar CPE (cytophatic effect). The difference between them was detected using Real Time PCR, which was also positive by agarose gel analysis for the virus contained in Lyomarex, but not in the Marikal SPF. It can be concluded that both cell lines are sensitive to HVT FC-126 and the virus can be multiplied in high titers though much lower than in the calf intestinal epithelial cell line (CIEB) cells ($TCID_{50}$ $2^{7.93}$).

Key words: adult pig kidney cell line, HVT FC-126, Gerbil kidney cell line, Marek's disease

INTRODUCTION

Marek's disease (MD), a lymphoproliferative disease of chickens, is of great concern for the poultry industry. In the absence of control measures, MD is capable of causing devastating losses in commercial poultry flocks. MD is caused by an alpha-herpesvirus (MDV), first isolated in 1968 (Churchill, 1968). The unique features of the MDV differentiate it from other alpha-herpesviruses. It is strictly cell associated, establishes latency in lymphocytes, includes an oncogene (meq) in its genome, and is able to induce lymphomas (Buckmaster *et al*., 1988). However, its molecular structure and genomic organization are very similar to herpes simplex virus (HSV), hence its classification within the subfamily alpha-herpesviridae. MDV includes three serotypes that have major differences not only in the genome but also in their biological features. Serotype 1 MDV includes all the oncogenic strains and their attenuated forms; serotype 2 are non-oncogenic viruses isolated in chickens, and serotype 3 are non-oncogenic viruses isolated

from turkeys, generally known as herpesvirus of turkey (HVT) (Afonso *et al*., 2001). Because of theirs close relationship, the three serotypes have been placed in the taxonomic genus Mardivirus. It has been proposed that the three different MDV serotypes represent three distinct, individual species that have undergone parallel evolution and that only serotype 1 should retain the name MDV (Witter and Schatt, 2003).

MDV is of extreme economic impact due to its severity for poultry. Among the MDV vaccine strains HVT FC 126 (Witter *et al*., 1970) was used as a vaccine in the prevention of Marek's disease. Multiplication of this virus takes place not only in chicken or quail fibroblasts, but also in their intestinal epithelial cells, like CIEB (Calf Intestinal Epithelial cells), WISH (Human Amniotic cells) and a human macrophage cell line. HVT FC 126 can be adapted to them because of its cultivation in the presence of SR-2.0552P (Serum replacement based on the porcine ocular fluid) instead of FCS (Fetal Calf Serum) (Filipič *et al*., 2002; Filipič *et al*., 2007). It was also found that MDV, as well as HVT, can be adapted to the VERO (African Green Monkey kidney) cells (Jaikumar *et al*., 2001). Production of vaccine viruses of MD in avian cell cultures or chicken embryos in the past and in the recent history proved to be the source of vaccine contamination. Well known is the episode of Egg Drop Syndrome '76 (EDS '76) that was introduced to chicken flocks (commercial layers and breeders) by virus contaminated vaccines against MD produced in duck embryo fibroblasts (Baxendale, 1978). This was confirmed by Calnek (1978) who detected specific hemmaglutination-inhibition antibodies against virus 127 (McFerran *et al*., 1976; McFerran and Adair, 1977). Among viral diseases of poultry that are vertically transmitted though being capable of contaminating avian tissue cultures known is the chicken anemia virus (Hoop, 1992; Yuasa *et al*., 1983) and viruses causing avian leucosis complex (Cotral *et al*., 1954; Rubin *et al*., 1961). Special attention should be paid to latent infection with reticuloendotheliosis virus (REV) of chickens (Motha and Egherton, 1987), turkeys (McDougall *et al*., 1980) and in very high rate (87%) in ducks (Motha, 1984) that could contaminate vaccine viruses cultivated in embryos or embryonic tissues. All adenoviruses are readily transmitted vertically (McFerran and Adair, 1977), and beside the EDS virus (Baxendale, 1978) of significant importance is inclusion body hepatitis virus (McFerran *et al*., 1976). Of no less importance is the contamination of tissue cultures or avian embryos with different avian mycoplasmas that are not easy to detect in contaminated tissues (Mycoplasma synoviae, M. iowa) (Kleven, 2008).

The presented experiments were aimed to introduce PLA and GL-4 cell lines for the multiplication of HVT FC-126 virus. They are much less expensive than SPF chicken embryos cells, and much safer in the sense of avoiding transmission of avian viruses.

MATERIAL AND METHODS

Cells: PLA and GL-4 cells (Institute of Microbiology and Immunology, Ljubljana, Slovenia) were cultivated in the Eagle's medium with antibiotics

(Penicillin, Streptomycin, Gentamicine) supplemented with 8% of SR-2.0552 P (Serum replacement based on porcine ocular fluid).

Viruses: Two HVT FC-126 vaccine strains where used e.g. Marikal SPF (Veterina d.d., Croatia) and Lyomarex (Merial, USA). DNA samples were isolated from the above mentioned cell cultures inoculated with vaccine strains of HVT FC-126 virus e.g. Marikal SPF (Veterina d.o.o., Croatia) and Lyomarex (Merial, USA). Adaptation of HVT FC-126 on the PLA and GL-4 cells: both HVT FC-126 viruses were adapted for the replication in PLA and GL-4 cells as it was described (Filipič *et al*., 2007).

Determination of the TCID$_{50}$: $TCID_{50}$ (Tissue culture infective dose) of both HVT FC-126 vaccine strain viruses i.e. Marikal SPF (Veterina d.d., Croatia) and Lyomarex (Merial, USA) on the PLA and GL-4 cells was determined according to Reed and Muench (1938). Cells were cultivated in 96-well micortiter plates in Eagle's medium supplemented with 8% SR-2.0552P on 37°C for 18 hours. On the next day, the medium was replaced with the medium containing 2% SR-2.0552P and 100 μL of virus suspension that was serially diluted from 1:2 to 1:1024, and further incubated for 72 hours at 37°C in 5% CO_2 atmosphere. Afterward, microscopically the ratio of infected/uninfected cells was determined.

DNA isolation: DNA samples were isolated from the control cell and/or inoculated cell cultures using the commercial kit DNeasy® Tissue Kit (Qiagen, USA). Isolated DNA samples were stored at -20°C until analyzed.

Real Time PCR: Real Time PCR analyses were performed using primers previously described by Islam *et al.* (2004) specifically designed for SORF1 region of HVT FC 126 (Baigent *et al*., 2006). Specific products were detected using SYBR green Brilliant® SYBR® Green QPCR Master Mix (Stratagene, USA) kit on Mx3005P (Stratagene, USA) and results (amplification and dissociation curve) were analyzed with MxPro (Stratagene, USA) software (Gottstein *et al*., 2007). After amplification, 15 μL of PCR product was analyzed by electrophoresis on 1.5 % agarose gel to confirm the Real Time PCR results.

RESULTS

During the experiments, two different HVT FC-126 vaccine strain viruses (Marikal SPF and Lyomarex) were used. They were successfully adapted to PLA and GL-4 cells. Similarly as in case of CIEB cells, 2–3 adaptation cycles were needed to get the full CPE. After adaptation, the $TCID_{50}$ was determined. Both of them showed similar values for $TCID_{50}$ (Table 1), even much lower than obtained on CIEB cells ($TCID_{50}$ $2^{7.93}$).

Table 1. $TCID_{50}$ of HVT FC 126 in the cells of nonavian origin

HVT FC-126 vaccine	Cells of nonavian origin		
	PLA (Adult pig kidney cell line)	GL-4 (Gerbille kidney cell line)	CIEB (Calf intestinal epithelial cell line)
Marikal SPF	$2^{4.23}$	$2^{4.96}$	$2^{7.93}$
Lyomarex	$2^{4.30}$	$2^{4.72}$	Not tested

On both kidney cell lines (PLA and GL-4) they showed the CPE, which is: big "plaques" at PLA and small plaques in GL-4. There was no visible difference in the form of plaques after 5 days of incubation (Figures 1, 2).

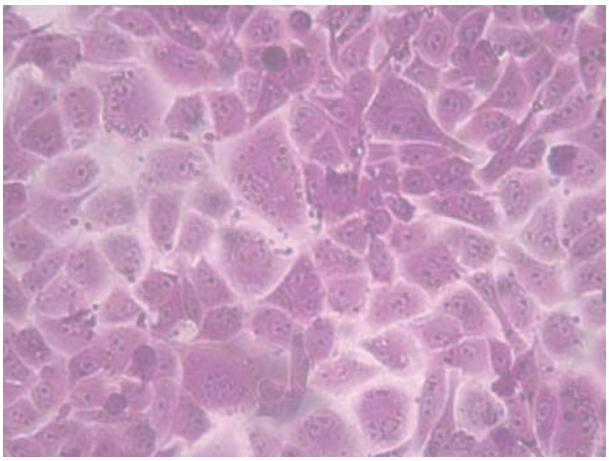

Figure 1. PLA cells – noninfected control (x 20). Cells were fixed with 3% solution of glutarldehyde containing 1% of glucose and stained with the 0.1% crystal violet in 20% of ethanol

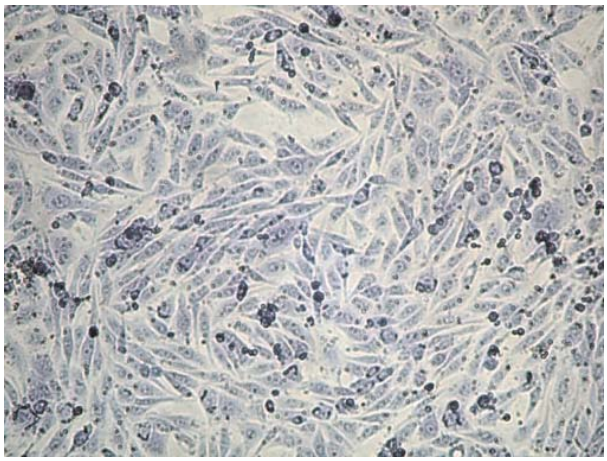

Figure 2. HVT FC-126 infected PLA cells. Visible CPE (x 10 magnification). Cell fixation and staining method is the same as in Figure 1

The main difference between viruses contained in Marikal SPF and Lyomarex can be seen after the Real Time PCR analysis: amplification and dissociation curve (Figures 3, 4). It was found that HVT FC-126 contained in

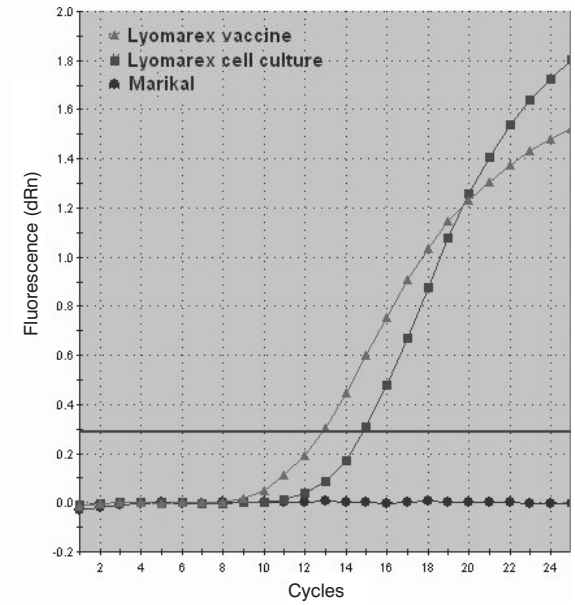

Figure 3. Amplification curves of vaccine viruses (Lyomarex , Merial, USA and Marikal SPF, Veterina d.o.o., Croatia) after 5 days of incubation

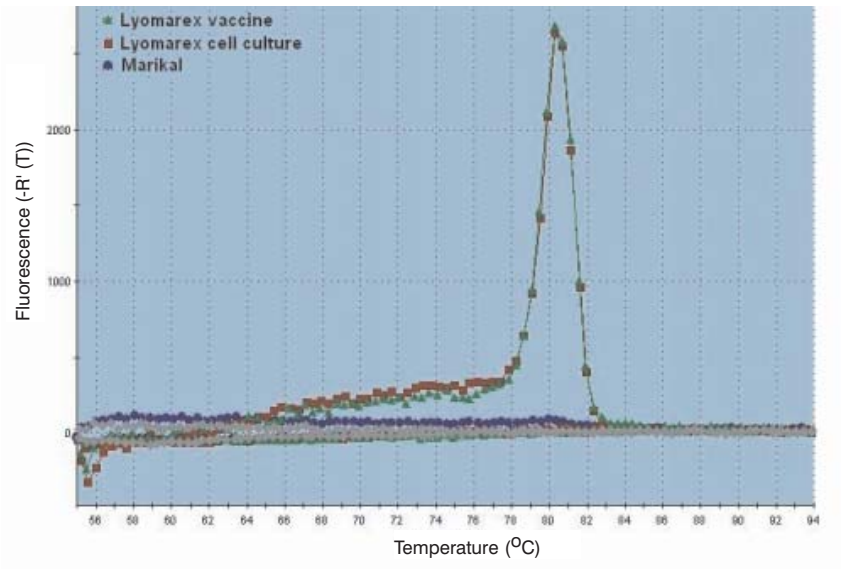

Figure 4. Dissociation curve for vaccine viruses (Lyomarex , Merial, USA and Marikal SPF, Veterina d.d., Croatia) after 5 days of incubation

Lyomarex can be detected in the culture (PLA), but not the one in Marikal SPF. Even both of them were HVT FC 126, by the use of the same primers it turns out that there could be HVT FC-126 of differences in their genomes patterns. It is probable that a specific region, SORF1 (Islam and Walkden, 2007) used for primer design could be different in few base pairs which are important for primer annealing between these vaccine strains, what could cause no amplification of Marikal SPF vaccine strain.

DISCUSSION

Virus replication for purpose of production of vaccines against different viral disease of poultry is usually performed in cells of avian origin, e.g. embryonic tissues. Direct risks of contamination of these tissues represent those viruses that are vertically transmitted from infected dames, but do not produce visible clinical signs. Known are different viruses of leucosis complex (Cotral *et al*., 1954; Rubin *et al*., 1961) including reticuloendotheliosis virus (Motha and Egherton, 1987) besides hens also latently infect turkeys (McDougall *et al.,* 1980), and in very high rate ducks (Motha, 1984). The genome of this virus seems to be a long time ago introduced to avian poxvirus (Kim and Tripathy, 2001) causing spread the disease. In Croatia, the genome of REV was demonstrated contaminating poxviruses isolated from infected hens and turkeys, but not from pigeons, indicating contamination of used vaccines (Prukner-Radovčić *et al.*, 2006). Also vertically is transmitted the virus of chicken infectious anemia (Hoop, 1992; Yuasa *et al*., 1983). Nevertheless for vaccines production only SPF embryos are used (hens, ducks and turkeys) the problem represents those viruses not known previously. The episode with Egg Drop Syndrome (EDS '76) confirms this best. To reduce the cost of MD vaccine production duck embryos were used, because ducks are not infected with most of diseases hens are.

Efforts are made to avoid avian embryo tissues in the production of vaccines for poultry, and of significant interest is the possible use of mammal tissues, especially line cell cultures. Bedigian and Sevoian (1972) and Purchase *et al*. (1971) demonstrated the susceptibility of hamster cell culture to HVT with development of specific CPE. The experiments to adapt the HVT FC-126 to the baby hamster kidney cell line (BHK_{21}) were not successful. Further experiments with VERO cell lines (Jaikumar *et al*., 2001) have shown that HVT FC-126 and Marek's disease virus Serotype 1 can be adapted for growth and multiplication. The adaptation period for HVT FC-126 in VERO cells was between 5 to 10 cycles, compared to the shorter adaptation period of HVT FC-126 in the PLA and GL-4 (2 to 3 cycles). It is also important to note, that in VERO cells the limited CPE consist of rounded cells which became spindle-shaped after further incubation. HVT FC-126 never makes plaques in VERO cells and they are present in PLA and GL-4 cells.

In our investigation the adaptation and susceptibility of PLA and GL-4 cell to HVT contained in Marikal SPF or Lyomarex vaccines could be demonstrated. Compared to $TCID_{50}$ achieved by the two viruses in PLA ($2^{4.23}$ and $2^{4.30}$) or in GL-4 ($2^{4.96}$ and $2^{4.72}$), the $TCID_{50}$ on calf intestinal epithelial cell line (CIEB) cells was

significantly higher reaching the value of $2^{7.93}$. Lyomarex vaccine was not tested on this cell line. According to present knowledge, none of the cell cultures originating from mammals could be infected with agents known to infect poultry.

Characteristic cytopatic effects on PLA and GL-4 were developed by both vaccine viruses (Figure 2). Nevertheless using Real Time PCR the difference between the two investigated viruses was demonstrated. It was positive for the HVT contained in vaccine Lyomarex but not in Marikal SPF. We can only speculate that the differences in genome of the two viruses are related to SORF1 region which is not recognized by the use of standard primers (Baigent *et al*., 2006) in Marikal SPF vaccine.

The differences among the strains of HVT FC 126 are indicated by Yachida *et al.* (1986) who established a variant type of HVT which released in large quantities cell-free viruses into the culture medium. The time needed for CPE development in chicken fibroblasts after infection with HVT FC 126 virus in Marikal SPF is very short in comparison to other HVT vaccines, and therefore titration of the virus should be performed 48 hours after cultivation (Čajavec, 1995. personal communication) which is in contrast to other strains that produce primary plaques after 5 days (Villegas, 2002).

Further experiments by additional multiplication of HVT FC-126 to determine the virus yield on PLA and GL-4 cell lines in comparison to VERO cells will show if they can be used for vaccine production. In this respect further efficacy and safety tests should be performed (Geerligs *et al*., 2008).

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UMNOŽAVANJE HVT FC-126 VIRUSA NA LINIJI ĆELIJA BUBREGA KOJI NISU POREKLOM OD PTICA

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SADRŽAJ

Ova ispitivanja su bila izvedena sa ciljem da se izvrši kultivacija i multiplikacija virusa HVT FC-126 na ćelijama PLA (bubreg odrasle svinje) i ćelijama GL-4 (bubreg gerbila). Korišćene su dva različita vakcinalna soja virusa HVT FC-126: Marikal (Veterina d.o.o., Hrvatska) i Lyomarex (Merial, USA). Ovi sojevi su adaptirani za navedene ćelijske linije. Nakon adaptacije izvršena je titracija virusa na PLA ($TCID_{50}2^{4,23}$) i GLA-4 ($TCID_{50}2^{4,96}$) ćelijama. Citopatogeni efekat je bio sličan kod obe ćelijske linije. Razlike su uočene primenom PCR - real time metode kada je analiza na gelu agaroze bila pozitivna za virus koji se nalazio u vakcini Lyomarex ali ne i za onaj sadržan u vakcini Merikal. Zaključeno je da su obe ćelijske linije osetljive na HVT FC-126 virus koji se u njima može umnožavati u visokom titru, ali ipak nižem nego na CIEB liniji epitelnih ćelija creva ($TCID_{50}2^{7,93}$).