Short Communication

Growth and isolation of Human cytomegalovirus on a new human fetal foreskin fibroblast-derived cell line in Iran†

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Abstract
Cell culture technique has been used for detection and confirmation of many different viruses in clinical samples. Although, new diagnostic methods have been developed for viral infections, traditional cell culture technique is still regarded as the "gold standard" for several infectious agents such as human cytomegalovirus (HCMV). In the present study, a new human fetal foreskin fibroblast (HFFF)-derived cell line was obtained from a normal Iranian male fetus at the end of first trimester at National Cell Bank of Iran (NCBI) and its ability for HCMV growth was investigated. Thirteen treated urine samples from renal transplant recipients were inoculated onto HFFF monolayer. HCMV growth was monitored and confirmed by typical CMV cytopathic effect (CPE), histological staining, immunological staining and polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) method and sequencing. All of the above-mentioned detection methods were confirmed that new HFFF-derived cell line is a sensitive line for growth of HCMV. This cell line has been named HFFF-PI6 and is available with accession number NCBI C170 at NCBI of Pasteur Institute of Iran.

Keywords: *Human fetal foreskin fibroblast (HFFF); Human cytomegalovirus (HCMV); Cell Culture technique, Molecular detection.*

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The history of cell culture dates from the turn of the twentieth century, since cell culture technique were developed and served for viral isolation and detection. For the first time, Smith in 1956, isolated *human cytomegalovirus* (HCMV) in human fibroblast culture from the sub-maxillary gland of a 7-month-old infant. Cytopathic changes were observed 1-2 weeks after inoculation of culture. These changes consisted of a few small, round or oval foci containing enlarged cells that were refractile, in contrast to normal fibroblast.

CMV grows readily in human diploid fibroblast cell lines and virus isolations are most often-accomplished in MRC-5 (human embryonic lung fibroblast), human foreskin fibroblast (HFF), human embryonic lung (HEL) or WI-38 (human fetal lung fibroblast) cell lines. In the cell culture technique, CMV exhibits a typical cytopathic effect (CPE) characterized by foci of flat, swollen cell named cytomegalia, which may include intranuclear inclusion bodies and giant cells. The time required for the development of the CMV CPE is directly related to the titer of CMV in the sample. Typically, the mean time for CPE to be visible is eight days, but it can range from 3 to 21 days (Wiedbrauk and Johnston, 1993; Edvard and Mocarski, 2001).

In recent years, the shell vial assay, which uses

immunologic detection of immediate-early antigens of virus in cell cultures, has provided rapid laboratory diagnosis of CMV infection (Paya *et al.*, 1987; Reina *et al.*, 1997). Alternatively, detection of CMV DNA from clinical specimens such as cerebrospinal fluid (CSF), tissue and blood using polymerase chain reaction (PCR) has been introduced (Brytting *et al.*, 1992; Sanchez and Storch,2002). The pp65 antigenemia assay is based on the direct detection of CMV antigen in circulating leukocytes through the use of fluorescent antibody or immunoperoxidase staining is being used in laboratory settings (The *et al.*, 1990; Bein *et al.*, 1991). But, traditional method for diagnosis of CMV, which accomplished by recognition of CPE in conventional cell culture is regarded as the "gold standard" of CMV detection, so far (Wiedbrauk and Johnston, 1993; Storch, 2000; Stagno *et al.*, 1989; Edvard and Mocarski, 2001).

In the present study, a new derived-cell line from a normal Iranian male fetus that named human fetal foreskin fibroblast (HFFF) was established and growth of CMV in the new derived-cell line via the standard cell culture technique, histological staining, immunological staining and molecular techniques was investigated.

HFFF cell line has been established in National Cell Bank of Iran (NCBI) at Pasteur Institute of Iran (IPI) and registered as HFFF-PI6. Briefly, this cell line was derived after primary culture of sections of foreskin tissue obtained from an Iranian 16-weeks-old normal male fetus in RPMI 1640 medium (Gibco™, Paisley, UK) pH 7.0 supplemented with 10% fetal bovine serum (FBS) (Gibco™, Paisley, UK). The cell line was characterized with respect to its karyology, which, indicated a typic karyotype of 46 chromosomes and thus classified as a diploid cell line. The fibroblastic cell line HFFF-PI6 was established as a continuously growing cell line two months later. It had undergone five passages before being used in this study.

Midstream urine of thirteen renal transplant recipients was collected simple randomized in sterile collection tubes. Urine samples transported within bag to virology laboratory, immediately. Five ml of urine was centrifuged at 4500 g for 15 minutes at 4°C. Supernatant urine pH was adjusted to pH 7.0-7.2 by sterile 1 M bicarbonate sodium and then filtered through 0.45 µm syringe filters (Sartorius, Germany). Two conditions of inoculation were performed; First, 0.2 ml of treated urines directly and other, 0.1 milliliter of treated urines diluted with 0.1 milliliter of RPMI 1640 maintenance medium, pH 7.0 were inoculated onto duplicate 110×16 mm (NUNC:A/S, Roskilde, Denmark) HFFF monolayer culture tubes. The inoculums remained on the monolayer for one hour at 37°C and then, the monolayers were washed with maintenance medium three times. At least, 1.5 ml of medium with 10% heat inactivated fetal calf serum (FCS) (Gibco™, Paisley, UK) that, supplemented with 100 IU/ml penicillin, 50 µg/ml gentamicin and 2.5 µg/ml fungizone, was added. The inoculated HFFF cell culture tubes were incubated at 37ºC, 95% humidity and 5% CO_2 and then monitored daily to found typical CMV CPE for 30 days; maintenance medium was changed weekly. Negative control consisted of treated urine of healthy persons who had never had HCMV infection. Uninoculated culture tubes served as cell controls. First appearance of CMV CPE was observed in five of thirteen samples after seven to ten days in HFFF cell lines. CMV CPE completed to a typical CMV CPE (70-80% confluent) after fourteen days. Identification of CMV CPE was based upon the presence of slowly developing focal areas of large, rounded, swollen and refractile cells that named foci in an otherwise undisturbed cell sheet (Fig. 1).

A simple and rapid staining technique was used for determination of typical CMV CPE. Cell monolayer tubes were stained using giemsa-staining solution and typical CMV CPE were photographed using an inverted microscope. HFFF stained preparations showed cell enlargement and intranuclear inclusion bodies. Their shape usually corresponded closely to that of a nucleus. In some monolayers, multinucleated cell (giant cell) could be seen with the inclusion-bearing nuclei arranged concentrically around the large inclusion. A clear, distinct zone separated the inclusion from nuclear membrane, named owl's eye changed nucleus to kidney-bean shape like (Fig. 2). This simple staining confirmed typically CMV CPE in infected HFFF monolayer.

An indirect immunofluorescent staining procedure was used for determination of CMV pp65 antigen in infected HFFF cells. Briefly, after preparation of cell slides, they were incubated with 1:5 dilution of first monoclonal mouse anti-cytomegalovirus (Clone AAC10, DAKO A/S, Glostrup, Denmark) for 45 minutes at 37ºC. After washing, a 1:20 dilution of FITC-conjugated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin was used as a secondary antibody (DAKO A/S, Glostrup, Denmark) and the cells were incubated at 37ºC for 30 minutes. Positive and negative cells were photographed using an immunofluorescent microscope (Axiophot, Zeiss, Germany). The

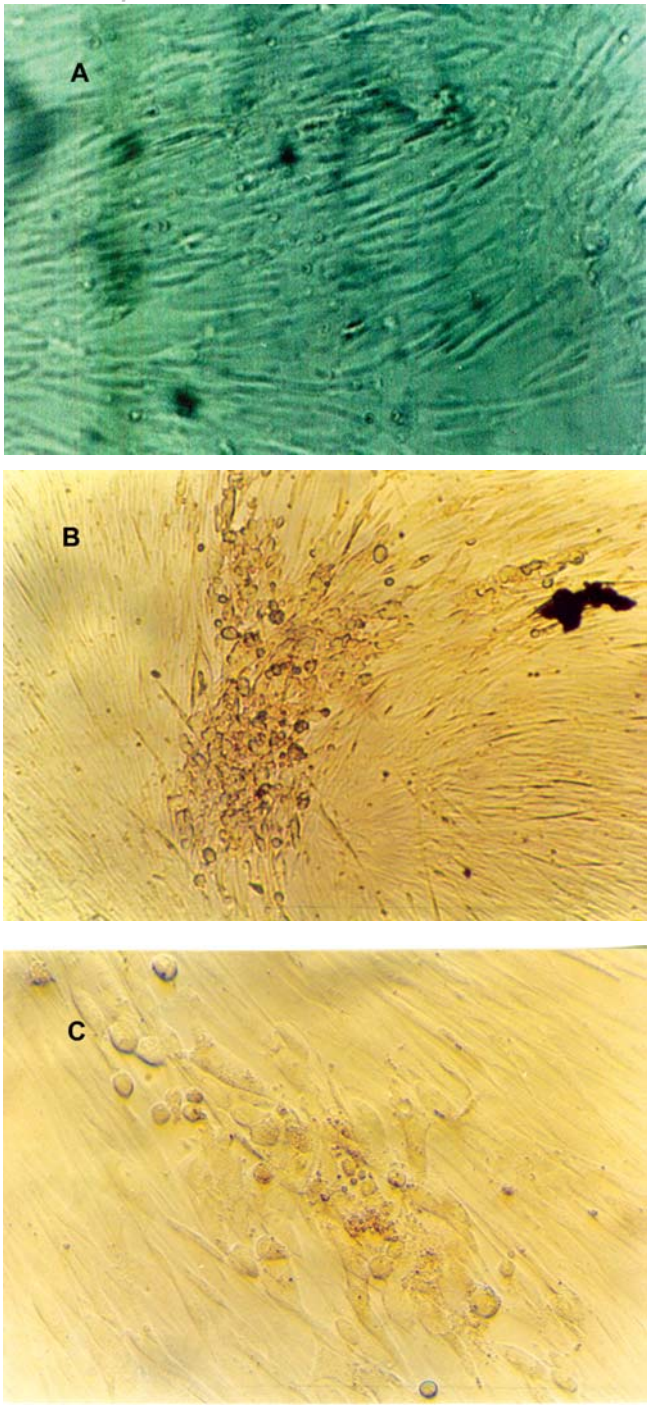

Figure 1. Un-infected and infected HFFF cell line. **(A)** Uninoculated HFFF cell line. **(B)** Focal CMV CPE in HFFF cell line after 10 days. **(C)** CMV characteristically exhibits CPE, appearing as foci of flat and swollen cells in an otherwise undisturbed cell sheet.

presence of CMV is indicated by apple-green fluorescence that covers the entire cell. Indirect immunofluorescent method determined CMV pp65 antigen and confirmed presence of CMV growth in HFFF cell line. Negative controls were stained and did not show specific apple-green fluorescence.

For molecular determination of virus, DNA of positive, negative CMV CPE monolayers and cell control monolayers were extracted using a viral DNA extraction kit (NucleoSpin® Blood, Macherey-Nagel, Duren, Germany) following instructions by the manufacturer. CMV DNA detection was performed using two primers that described previously (Drouet *et al.*, 1993). In-house PCR optimization and then HCMV PCR on the above mentioned extracted DNA was performed which described elsewhere (Amini *et al.*, 2003). A 406 bp fragment was successfully amplified from positive CMV CPE monolayer using specific primers, which located within a highly conserved region. The amplified 406 bp was digested using different restriction enzymes that confirmed 406 bp CMV amplicon in all of positive samples (Fig. 3). Cleaned-up PCR-product was inserted into pTZ57R plasmid using a T/A cloning kit (InsT/Aclone™, Fermentas AB, Vilnius, Lithuania) following instructions from the manufacturer and named pTZCMV. The pTZCMV was sequenced using the forward and reverse M13 universal primers. The sequencing results were analyzed and compared with the reference sequences and confirmed that the genotype was correct. This sequence can be accessed in GenBank with accession number of AY327403.

Recently, to shorten the HCMV diagnostic procedures, shell vial assay, antigenemia assay and PCR-based methods have been developed; however, HCMV isolation from body fluids in fibroblast cultures is still regarded as the “gold standard” method. This study determined the ability of new HFFF-derived cell line for HCMV growth. HFFF cells are thin and long with a nucleus in center of the cells. In this study, HFFF cell line was compared to commercial MRC-5 cell line (NCBI C125) for support of some properties such as ability of going under successful several passages and handling. Results showed that HFFF cell line could be used up to 15-20 passages and was easier to handle in comparison with commercial MRC-5 cell line (which had less passage, 3-5 times). Meanwhile, HFFF cell line had good resistance to non-optimal culture conditions whereas, MRC-5 cell was shown to be more sensitive to environmental changes which, may be due to non-suitable transportation or storage of cells and/or performing high passages before receiving cell line from aboard; whereas, HFFF cells which prepared in NCBI had only 5-6 passages before using. Therefore, this study demonstrated that the new HFFF-derived

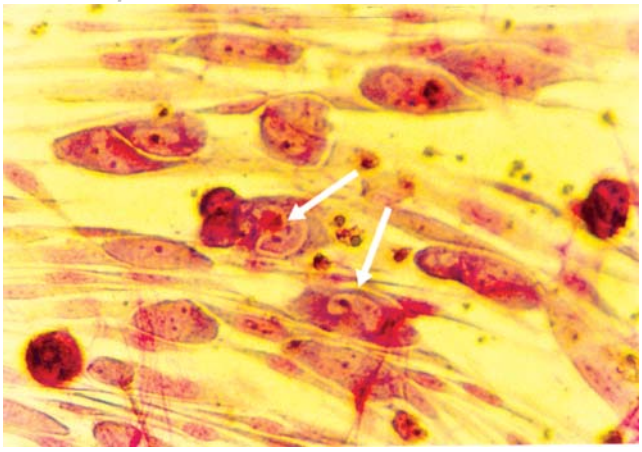

Figure 2. HCMV CPE result of histological staining. In histological staining, CMV CPE in HFFF monolayer appearing cytomegalic inclusion bodies and arrow show characteristic intranuclear owl's eye appearance within kidney-bean shape like nucleus, which indicated *cytomegalovirus* growth.

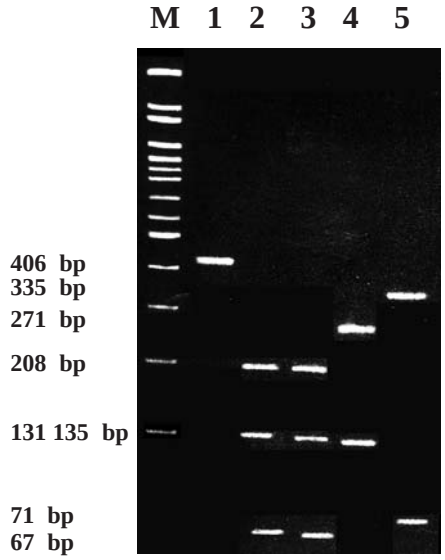

Figure 3. Result of PCR-RFLP for confirmation of CMV PCR amplicon on ethidium bromide stained polyacrylamide gel. Lane1: Undigested CMV PCR product (A 406 bp fragment); Lane2: *Sau3A-I* digestion pattern (208 bp, 131 bp and 67 bp fragments); Lane3: *Mbo-I* digestion pattern (208 bp, 131 bp and 67 bp fragments); Lane4: *Cfo-I* digestion pattern (271 bp and 135 bp fragments); Lane5: *BstX-I* digestion pattern (335 bp and 71 bp fragment); M: DNA size marker (100 bp DNA ladder plus, Fermentas AB, VilniusLithuania).

cell line, which prepared from an Iranian male-fetus, could be a sensitive line for viruses, which grow on human fibroblast cell line, such as CMV. In other study in Iran (Shahgasempour *et al.*, 2001), HSV was also grown onto HFFF cell line and the effect of it on up-regulation on integrins $\alpha 2\beta 1$ and $\alpha 3\beta 1$ expression in HFFF cell were investigated and results suggested that HSV-1 is able of induce functional changes in HFFF cells.

CMV could readily grow on HFFF cell line and first CPEs could be seen after seven days. In this study, typical CMV CPE in HFFF cell line was developed as reported in other cell lines (Stagno *et al.*, 1989; Edward and Mocarski 2001). CPEs were confirmed using a rapid, low-cost and simple histological staining, showing intranuclear inclusion typical CMV CPE. In some infected cells, nucleus changed to kidney-bean shape (owl's eye) marked by arrow in Figure 2. Since CMV is cell associated and spreads slowly from cell-to-cell, therefore the foci containing enlarged cells increased in number and size slowly, their centers become necrotic and density pigmented. All of the above conditions confirmed that HFFF cell line has suitable ability for CMV growth.

A highly sensitive PCR reaction was used as confirmatory test to confirm CMV DNA in infected monolayers. Previously, this technique was often used for detection of CMV DNA in high-risk patients (Shinkai *et al.*, 1997; Nolte *et al.*, 1995; Sanchez and srtoch , 2002). In this study, the specificity of primers and PCR was tested using other DNA viruses as *Human herpes virus type 1* (HSV-1), *Human herpes virus type* 2 (HSV-2), *Varicella zoster virus* (VZV) and *Hepatitis B virus* (HBV) DNAs, with no apparent band seen for these viruses. The 406 bp amplicon were confirmed with RFLP technique using *Cfo-I*, *Mbo-I*, *Sau3A-I* and *BstX-I* restriction enzymes. Recently, PCR-RFLP method was also served for phenotyping and genotyping of viruses and could become a valid method in molecular diagnostic (Madhavan *et al.*, 2003; Prosch *et al.*, 1994). The expected patterns of fragments, analyzed by GeneRunner software were shown completely compatible with digested fragment which, patterns on ethidium bromide stained polyacrylamide gel, which confirmed CMV DNA. In this study, sequencing of pTZCMV was also confirmed HCMV genotype. Sequence contains seven nucleotide variations in 406 bp CMV amplicon. Nucleotide variations in positions 17, 18, 142, 145 and 175 located at a non-coding region between *HCMVUS7* and *HCMVUS8* genes. Two nucleotide variations in positions 331 (A⟶C) and 333(A⟶C) located at *HCMVUS8* gene cause changes of cysteine (UGU) to arginine (CGG) (Cys⟶Arg).

In conclusion, our finding indicated established HFFF cell line is sensitive for CMV growth from patient's specimens and may also be used as a source

of virus for later studies such as genotyping.

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