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Cytopathogenicity of buffalopox and camelpox virus in buffalo fibroblast cells

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ABSTRACT

The primary cultures of fibroblast cells were established from the ear marginal tissue of a young calf of buffalo (*Bubalus bubalis*). Subsequent passages of primary fibroblast cells were carried out upon 80–90% confluency. The cells were found to be chromosomally stable and free of *Mycoplasma* contamination. Housekeeping genes namely-GAPDH and β-Actin were amplified from fibroblast cells. To check the utility of developed fibroblast cells in adaptation of virus, cell monolayers were infected with buffalopox virus (BPXV) and camelpox virus (CMLV) isolates, which showed typical virus-specific CPE. The infection was confirmed by PCR amplification of BPXV and CMLV-specific region of C18L gene. The cell line thus developed could be of immense potential in propagation of viruses adopting skin route of infection and their immuno-modulatory associations.

Key words: Buffalopox, Camelpox, Fibroblast

Buffalopox (BPXV) and camelpox viruses (CMLV) are the members of the genus *orthopoxvirus,* family *poxviridae* (Murphy 1999, Venkatesan 2010). These viruses cause pustular pock-like lesions on skin and mucous membranes of affected animals. Several outbreaks of BPXV (Venkatesan 2010, Damle 2011) and CMLV were reported from India and abroad (Al-Zi'abi 2007, Falluji 1979, Bera 2012). Under laboratory conditions, CMLV is propagated in different cell cultures (Davies 1975, Tantawi 1974, Bera 2011); BPXV virus is largely cultivated in vero cells (Singh 2006, Bera 2012). The host cell type is of great significance as the cell susceptibility and virus titre may vary depending on cell type or due to species barrier (Sugioka *et al.* 2005, Carroll and Moss 1997). Further, host-specific cell line may be useful in study of virus genetics as there would be less adaptive mutations in comparison to other cell types. However, there have been no reports of propagation of these viruses either in skin derived primary cultures or in species-specific cell lines. There are several examples of propagation of viruses in host specific cell line in several species such as duck hepatitis virus type 1 (DHV-1) in a duck embryo fibroblast (DEF) derived cell line (Fu 2012), Autographa californica nuclear polyhedrosis virus (AcNPV) in neonate larvae derived cell line (Zhang 2012), Singapore grouper iridovirus (SGIV) in *Epinephelus akaara* cell line (Gong 2011), nodavirus in cell lines from *Etroplus suratensis* (Sarath Babu 2012), and capripoxvirus in ovine cell line (Babiuk 2007). The attempt was made to find the susceptibility of the primary cultures of skin fibroblast of buffalo origin for propagation of BPXV (similar species model virus) and CMLV (cross-species model virus) and also to check whether these cells can be an alternative for isolation and characterization of pathogenic viruses primarily infecting skin of the animals.

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MATERIALS AND METHODS

To develop the primary culture, the ear marginal tissue sample was collected from 1-month old buffalo calf in DPBS containing 500 IU/ml penicillin + streptomycin 0.1 mg/ml + 2.5µg/ml amphotericin B. The tissue piece was washed and minced into small pieces and seeded individually in gelatin coated tissue culture flasks containing DMEM supplemented with 10% fetal calf serum (FCS). The tissue culture flasks were incubated at 37ºC under 5% CO_2. The half of media was replaced every other day. For subsequent passaging, primary fibroblast cells were harvested by trypsinization upon 80–90% confluency and reseeded at 1:3 split ratios. For cryogenic preservation, the media of actively growing culture was replaced 24 h prior to harvest. After trypsinisation, cells were enumerated with hemocytometer and their viability was checked using trypan blue staining. The cells were centrifuged at 1,000×g for 5 min to form a pellet, the supernatant removed and cell pellet resuspended in freezing

media (70% DMEM + 20% FBS + 10% DMSO) to reach a final concentration of 3×10^5 viable cells/ml. Aliquot of cell suspension (1.5 ml) were transferred to cryogenic vials and the properly labeled, sealed vials were frozen at 4°C for 30 min, –20°C for 30 min, –70°C overnight, and next day transferred into liquid nitrogen. The growth curve estimation was done by following the method as described by Gu *et al.* (2006). Briefly, 24-well tissue culture plates were seeded with cells at a concentration of 2.5×10^4 cells /ml and cultured for 7 consecutive days. Cell concentration was calculated from 3 wells/day until plateau was reached. The cell growth curve was plotted and population doubling time was calculated. DNA fluorescent staining was used to identify contamination of *Mycoplasma* (Guan *et al.* 2005). For genetic analysis, chromosomes were prepared, fixed and stained following standard methods (Hirofumi *et al.* 2006). After Giemsa staining, the chromosome numbers per spread were counted for 100 spreads under an oil immersion objective.

Cellular RNA was extracted from derived fibroblast cells using as per the manufacturer's protocol and RNA was eluted in 20µl RNase free water. The purified RNA was subjected to synthesis of cDNA using random hexamers and H minus reverse transcriptase as per the standard protocol. The cDNA was used for PCR amplification of β-actin and GAPDH genes using reported primers (Smith 2007). The PCR reaction mix contained 0.5 µl of cDNA, 0.5µM of each primer, 200µM of dNTP and 2.5U of dreamTaq DNA polymerase. The cycling condition consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 45 sec, 60°C for 45 sec, and 72°C for 45 sec, with a final extension at 72°C for 10 min. The amplicons were visualized by electrophoresis on 1% agarose gels containing ethidium bromide (0.5µg/ml) under UV.

Buffalo fibroblast cells were grown in 25 cm^2 T flasks up to 90% confluency. The spent medium was discarded and the cells were washed with DBPS and immediately infected with buffalopox virus (BPXV) and camelpox virus (CMLV) isolates separately, at a multiplicity of infection (moi) of 0.01. Cells were incubated at 37°C for 1 h with intermittent shaking at an interval of 15 min to allow adsorption of virus. The virus inoculum was then decanted and the infected cells were washed 2 times with serum-free DMEM. Finally, the infected cells were fed with maintenance medium containing 2% FCS and incubated again at 37°C in a CO_2 incubator. Flasks were observed daily for the appearance of CPE.

The genomic DNA was extracted from 200ml poxvirus infected cell culture supernatants using DNA extraction kit as per manufacturer's protocol. The purified DNA was subjected to C18L gene - based BPXV –specific (Singh *et al.* 2008) and CMLV-specific (Balamurugan *et al.* 2009) PCRs using the reported primers and following the PCR conditions described earlier. Briefly, the reaction mixture contained 5µl DNA, 15 pmole of each primer, 200µM dNTPs and 0.5µl (3 U) of Taq DNA polymerase in a 50µl reaction volume. An aliquot of 5µl of PCR product was analyzed in a 2% agarose gel to visualize the amplicon after staining with ethidium bromide.

RESULTS AND DISCUSSION

Modern biotechnology techniques aid in rapid determination of viral infections and isolation of viruses opens new avenues for complete study related to pathogenesis and host tropism. For isolation of viruses, most of the laboratories now- a-days use continuous cell lines such as MDCK for influenza (Sidorenko and Reichl 2004), vero for many viruses like polio, rabies, smallpox, rota, orthopox and morbillivirus etc (Barrett 2009, Singh 2006, Bera 2011 and 2012). These cell lines are required to be routinely maintained in contamination-free environment (Schiff 2005). Further, procurement of these characterized cell lines is also costly affair. Also, the maintenance of contamination free cultures adds up further to the involved cost apart from the fact that a skilled technician is required for maintaining the cell cultures. In this respect, primary cell cultures have been a potential alternative. It is important to mention that primary cultures of lung, kidney, human foreskin fibroblast, lamb keratinocyte from foreskin etc. are used for cultivation of many viruses, viz. SV40, vaccinia, influenza, orf, cowpox and CMLV etc. (Nemecková *et al.* 2001, Potter 1970, Kutinová *et al.* 1995, Duraffour *et al.* 2007, Pozzo *et al.* 2005, Keith *et al.* 2003) and recently a cell line from wildlife rodents was established and used for propagation of vaccinia, cowpox, vesicular stomatitis, sindbis and other viruses under lab conditions (Essbauer *et al.* 2011). But there have been no studies elaborating the fact that the susceptibility of the different host cells may vary for the same virus. Although, the speed of virus progression and hence CPE differs as demonstrated by Sugioka (2005) who reported that human corneal endothelial cells had similar susceptibility to initial HSV-1 attachment and adsorption as with routinely used African green monkey kidney fibroblast CV-1 cells, however, maximum total virus production was more than 3–fold higher for HCEC than for CV-1 cells.

The relation with the cell type is very specific for any virus. The infection rate depends upon accessibility and amount of receptors on cell surface, the ability to internalize the virus, the metabolic state of the cell, virus encoded host-range genes responsible for protecting host-mediated defense mechanism, and genetic differences in the virus. Hence, it is prudent to use the host-specific cells for easy adaptation and propagation of viruses without many changes in genetic setup, which will aid in isolation of viruses from clinical samples and their genetic analysis. With this objective, we attempted to explore the possibility of using indigenously developed buffalo fibroblast primary cultures for propagation of poxviruses particularly buffalopox and camelpox viruses.

The primary cultures (Fig. 1A) were established within 10–12 days by tissue explant method. The cells appeared

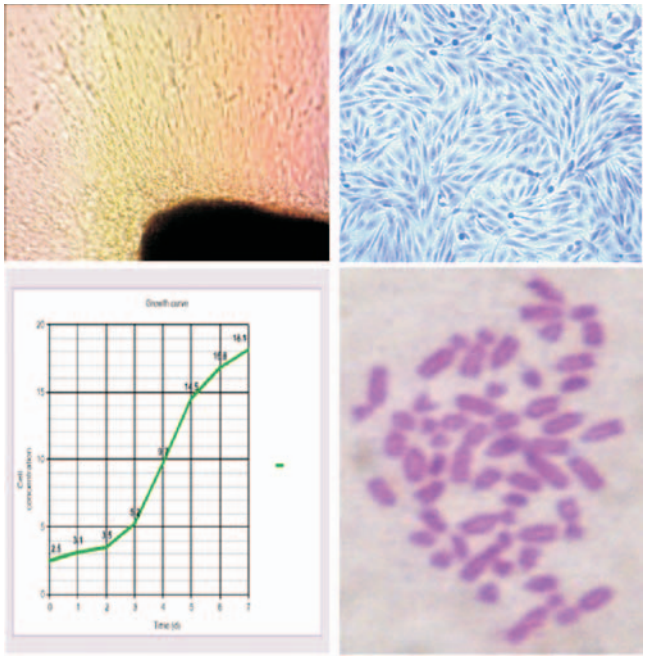

Fig. 1. (A) Primary culture of buffalo fibroblast cells originating from a tissue piece of ear pinna (100×), (B) a confluent monolayer of buffalo fibroblast cells stained with 0.4% crystal violet showing long and slender morphology of cells with tapering ends (100×), (C) growth curve of buffalo fibroblast cells (passage 6) prior cryopreservation. The cell count was estimated using hemocytometer. Each value represents the mean of 3 values, and (D) chromosomal count at metaphase in buffalo fibroblast cells 2n (50) (1,000×).

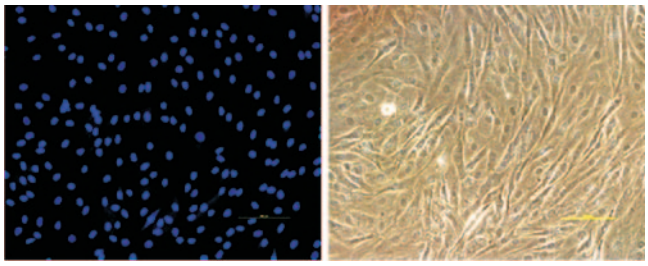

Fig. 2. Buffalo fibroblast cells (passage 6) stained with Hoechst 33258 for detection of mycoplasma contamination (A) fluorescent view, (B) view under light (400×).

heterogeneous during early passages. In respect of morphology, the cells appeared long and slender with tapering ends (Fig. 1B) initially but later upon reaching confluency, they appeared more or less flattened and polygonal type with prominent nuclei. These results are in conformity with prior studies where fibroblast cell lines were derived from Luxi cattle (Liu *et al.* 2008) and Texel sheep (Li *et al.* 2009). The buffalo fibroblast cells were successfully sub-cultured till passage no. 17 and preserved under liquid nitrogen. An S-shaped growth curve (Fig. 1C) was observed for the cells. The population doubling time as derived from growth curve was 24 h which is same as that of cattle fibroblast cell line

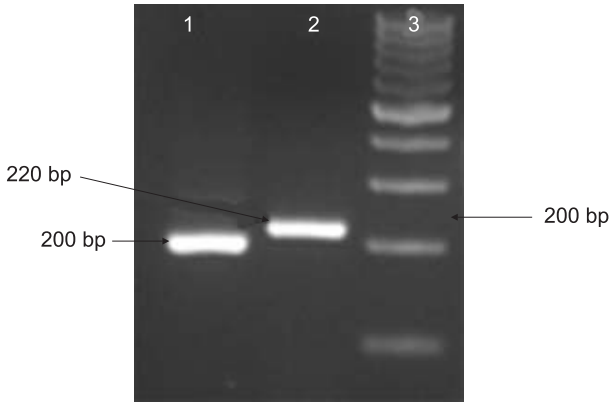

Fig. 3 Amplification of the house keeping genes by RT-PCR – lane1 (GAPDH, 200bp), lane 2 (β-Actin, 222bp), lane 3 (100bp ladder).

(Liu *et al.* 2008). The chromosomal count confirmed the origin of cell line where the cells were observed to have the 2n=50 (Fig. 1D). Staining with Hoechst 33258 showed prominent nuclei in the cells indicating freedom from *Mycoplasma* contamination (Fig. 2). Further, the cell identity was confirmed by amplification of the house keeping genes: GAPDH (222 bp) and β-actin (200 bp) (Fig. 3) using the extracted cellular RNA from derived fibroblast cells.

The infected cell-monolayers show plaque type cytopathic effect, foci of rounded cells, cell detachment and formation of syncytia in CMLV (Al-Zi'abi *et al.* 2007) and ballooning, rounding, increased refractivity and degeneration of cells in BPXV (Singh *et al.* 2006, Venkatesan *et al.* 2010). In the present study, similar kind of CPE was observed upon infection of buffalo fibroblast cell monolayers with BPXV and CMLV, 16 h post-infection (Figs 4, 5). The earliest changes consisted of cellular rounding, cell fusion, and formation of giant cells, clumping and granularity in the cell

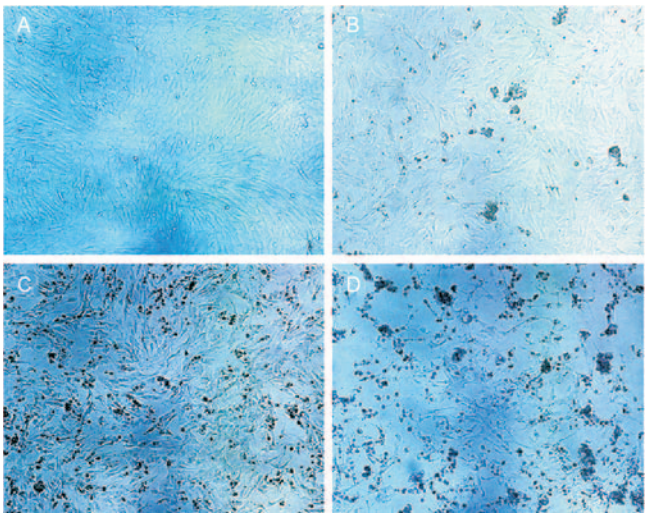

Fig. 4. Infection of buffalo fibroblast cells with serum infected with BPXV: (A) Control, (B) day 2 post infection, (C) day 3 post infection, (D) day 4 post infection.

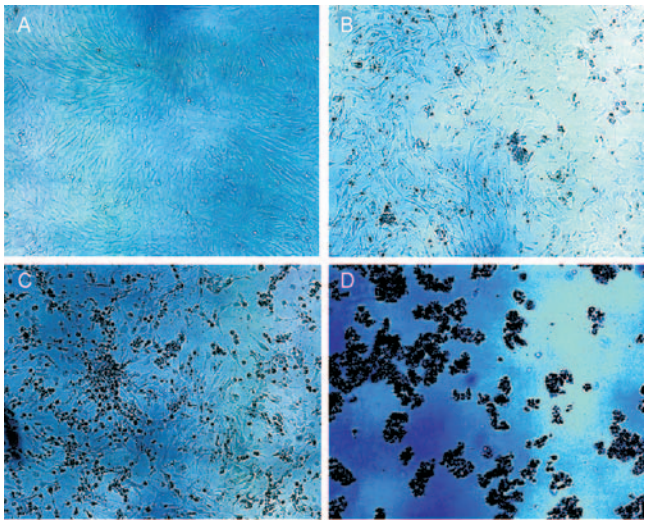

Fig. 5. Infection of buffalo fibroblast cells with serum infected with CMLV: (A) Control, (B) day 2 post infection, (C) day 3 post infection, (D) day 4 post infection.

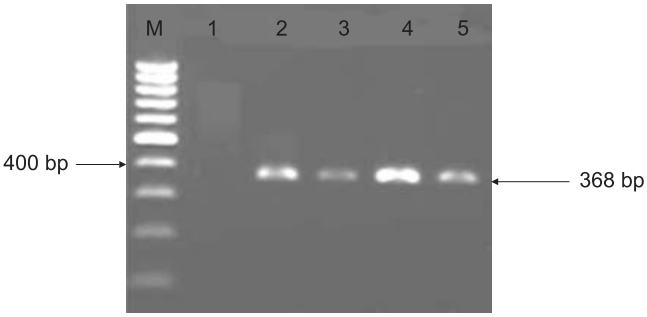

Fig. 6. PCR amplification of buffalopox virus-specific 368bp product of C18L gene. M, 100bp DNA ladder; L1, negative control; L2, 3, and 5, BPXV infected cell lysate, and L4, positive control of BPXV.

cytoplasm and tendency to form clusters. Cell contact separation and detachment from flask was noticed 3 dpi. Both the poxviruses were further confirmed in the supernatant of the infected cell culture upon PCR amplifications of 368bp and 243bp products of C18L gene specific for BPXV (Fig. 6) and CMLV (Fig. 7) as reported earlier (Singh *et al.* 2008, Balamurugan *et al.* 2009).

In conclusion, a buffalo fibroblast cell line containing biologically normal and genetically stable cells was established. The CMLV and BPXV were successfully propagated, and generation of CPE was observed in these cells. The presence of viruses was further confirmed by virus-specific PCR. The developed buffalo skin fibroblast line could be of immense potential in propagation of viruses adopting skin route of infection and to study their immuno-modulatory associations. Although the limitations of primary cultures cannot be ignored as dependence on primary cells complicates vaccine production especially in large

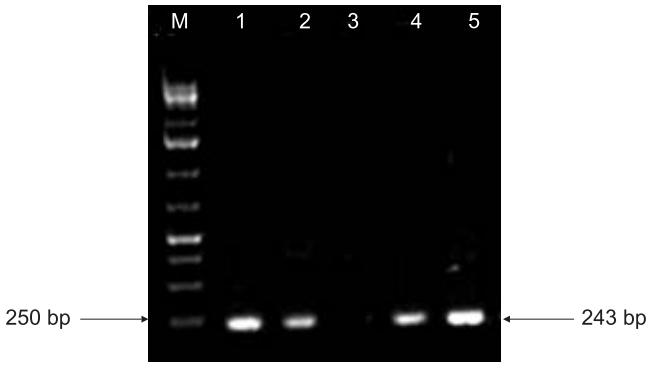

Fig.7. PCR amplification of camelpox virus-specific 243bp product of C18L gene. M, 1kb DNA ladder; L1, 2 and 4, BPXV infected cell lysate; L3, negative control; L5, positive control of CMLV.

vaccination programs. Keeping this fact in view, it would be prudent to further passage the primary culture cells to explore if we can develop a continuous cell line which can be continuously grown to homogenicity providing a continued culture system. However, the use of primary cultures in initial studies on preliminary virus isolation, detection and characterization cannot be ignored completely.

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