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Original Article

Development and Cytogenetic Characterization of a *Continuous Bovine Kidney Cell Line (IRKHBK)* and Evaluation its Susceptibility to some Viruses

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ABSTRACT

In this syudy a continuous bovine kidney cell line derived from a primary bovine kidney cells was established for the first time in Iran. The cells were originating from two-day-old normal male calf of Holstein breed. The cell cultures were continuously passaged following complete proliferation of primary cells. The specific properties or characteristics of the cell were defined using cytogenetic and tumorigenicity analysis. An increasing in cell proliferation was observed at 30th passage. Subsequently chromosomes analysis was shown the first chromosomal adhesion. In karyotyping a decrease in number of the cell′s chromosomes (n=59) was detected compared to the normal bovine cells chromosome count (2n=30). The cell obtained unlimited proliferation capacity from 70th passage and was identified as infinite cells at passage 90th. The continuous cell line named *Iran Razi Khedmati Bovine Kidney (IRKHBK)* and was deposited in National Cell Bank of Iran (NCBI), Pasteur Institute. Susceptibility of the *IRKHBK* cell line for isolation and replication processes of *bovine herpesvirus-1 (BHV-1)* and *bovine virus diarrhea-mucosal disease (BVD-MD)* were also evaluated. The results showed that this cell is more susceptible to the viruses compared to primary bovine kidney cells. According to our results, *IRKHBK* cell is recommended for routine assays of viruses as a substitution for primary bovine kidney cells.

Keywords: Cell line, *IRKHBK*, Karyotype, Chromosome

INTRODUCTION

Viruses are obligate intracellular parasites that hijack the host's cellular machinery during their replication cycle. Cultured cells, specific pathogen free eggs and laboratory animals may be used for virus isolation. Although this approach is often slow and requires considerable technical expertise, it has been regarded for decades as the "gold standard" for the laboratory diagnosis of viral disease (Leland & Ginocchio 2007). Fast replication in cell culture and high production yields are the key success factors for the broader adoption of cell culture technology for virus isolation in most laboratories (Freshney 2005). Propagation of viruses in cell cultures was first described as early as 1913 for vaccinia virus, and in the 1930s for both smallpox virus and yellow fever virus (Storch 2000, Lednicky & Wyatt 2012). Primary cell culture is useful

for virological methods purpose but preparation of primary cells from various organs of animals has several disadvantages; they must be prepared at regular intervals from a reliable supply of animal tissue which is costly and time consuming. A primary cell culture may be composed of mixtures of cell types and cannot be characterized (Pye 1989, Leland & Ginocchio 2007). Moreover there is possibility of the primary and secondary contamination of the cells to latent viruses because it cannot be readily tested for freedom from contamination prior to use (Leland & Ginocchio 2007). By the early 1970s, diagnostic virology expanded dramatically, largely because of the availability of highly of purified reagents and commercially prepared cell lines. Biological experiments are most often performed with immortalized cell lines because they are readily available and can be expanded without limitation. The cell lines do not have the above mentioned disadvantages and can be stored in liquid nitrogen until used (Pye 1989, Lednicky & Wyatt 2012). The role of virus isolation is most significant in the diagnosis of new or unexpected infection, and in yielding infectious virus for further study. Modern clinical virology relies on rapid virus detection for timely infection control and antiviral therapy (Ogilvie 2001, Storch 2000). Thus a continuous cell line, which is sensitive for virus replication with high proliferation capacity, is required. Here the development and cytogenetically characterization of a continuous cell line from a normal bovine kidney were described.

MATERIALS AND METHODS

Cell source. The standard methods were essentially followed in preparing primary bovine kidney *(BK)* cells for culture (Freshney 2005). A 2-day-old healthy male calf of Holstein breed was selected. The kidneys were removed aseptically, minced, washed with PBS two times and then dispersed 0.25% trypsin in PBS, pH 7.2 for 15 minutes at room temperature using a magnetic stirrer. The dispersed cells were centrifuged at 1500 rpm at 4 °C for 10 minutes. The supernatant was discarded and the cells were suspended to a concentration of 10^4 cells per ml in the Earle's solution supplemented with 10% new born calf serum, %0.4 per cent lactalbumin hydrolysate, and %0.08 per cent yeast extract (YLE) medium, 100 units penicillin, 0.1 mg streptomycin and 100 units mycostatin per ml. The medium was adjusted to pH 7.0 with 1% saturated $NaHCO_3$. Sterile bottles were seeded with 100 ml of the cell suspension per bottle. Cells were grown at 37ºC for 5-7 days at which time they were usually confluent and ready for passaging.

Cell passaging. The confluent culture of the primary cell culture is selected for passaging. The cell passaging involved standard procedures based on trypsin-versene solution, and fresh culture were initiated using split ratio (of 1:2-1:6) (Freshney 2005). Briefly, the medium was discarded, and the culture was rinsed with 3 ml trypsin-versene for 1-2 minutes to aid the cell liberation process, following which, the bottle was stored at room temperature for about 5 minutes. After complete cell liberation, 200 ml fresh YLE medium containing 10% serum was added to each bottle and the bottles were vigorously shaken until a homogeneous suspension was obtained and the cell suspension was divided to 2 – 6 Roux bottles. The culture was incubated at 37 °C for 5-7 days until they were ready for next passage. The morphological analyses of the cell line were carried out using microphotographs of the cultures, taken with an inverted microscope (Zeiss, Jena, Germany).

Cytogenetic. Cytogenetic analysis was carried out every ten passages of the cell line that was in an exponential growth phase, with the standard procedure (McConnell et al 1990). The cells were arrested in metaphase with 0.0016% colchicines, harvested with 0.05% trypsin, treated with hypotonic solution (0.075 KCl) for about 20 minutes at 37ºC, and fixed with 3:1 methanol/ acetic acid. Slides were prepared and submitted to standard Giemsa staining (G banding) (Lima *et al* 2004, Freshney 2005, Chauffaille *et al* 2003) and abnormalities was described.

Cryopreservation. The cell line which was named *IRKHBK* cryopreserved according to the protocol used by others with some modification (Lima *et al* 2004,

Chauffaille *et al* 2003, Freshney 2005, Simione 2009). The cells were removed in a trypsin-EDTA solution and cryopreserved in 10% dimethylsulfoxide (DMSO), 50% stoker medium, 20% fetal calf serum, 10% 0.5 M sucrose, 10% bovine albumin, aliquot in vials and the temperature is then gradually reduced to -20 ºC for 0.5 h, -70 ºC for 1 h, and the cells are then immersed in liquid N2 or stored at -70 °C. For thawing, the cells were rapidly thawed in 37 °C water after which they are placed in a rehydrating solution (10% fetal calf serum, and 25% stoker solution). After this procedure, the material is washed once or twice and then transferred to tissue culture bottles, where it is incubated and maintained at 37 °C.

Tumorigenicity test. Cells of monolayer cultures were trypsinized and counted in a haemocytometer (Freshney 2005). Then the cells suspended in Stoker medium with 2% bovine serum. Ten million cells were injected subcutaneously in the sold of mouse.

Susceptibility test. Susceptibility of different passages of *IRKHBK* cell line to viruses was examined by isolation of *bovine herpesvirus-1 (BHV-1)* and *Bovine viral diarrhea Virus (BVDV).*

RESULTS

Development of the cell line. Cultured primary bovine kidney (*BK*) cells were immortalized *in vitro* by spontaneous transformation; a biological phenomenon leads to produce the *Iran Razi Khedmati Bovine Kidney (IRKHBK)* cell line. The *BK* cells were mixture of fibroblast and epithelial like cells morphologically. As shown in figure 1 at the 70th passage the cells were established and a uniform population of cells in aspect of morphology and growth rate did all the succeeding passages. The cells were featured epithelial-like cells with cell cycle approximately 24 hours long, with maximum confluence at 36 hours.

Cytogenetic analysis. The cell incubated at 37 °C up to 6 days. Chromosome analyses were performed on 2nd, 20th, 30th, 60th, 94th, 155th and 200th passages. Karyological examination of the cell line revealed that the ploidy of the *IRKHBK* cell line evolved from diploid at 20th passage to aneuploid at the 30th passage (Figure 2). The Karyotype data are detailed in Table 1. Analysis of the different *IRKHBK* cell passages indicates the cell features at 2nd and 20th passages was normal and no differences observed between their karyotype data and primary cell culture. The first change in cell karyotype was found at 30th passage in which two chromosomes joined together from the centromer site. The translocation leads to reduction in number of chromosomes (n=59) compared to the diploid normal bovine somatic cells (n=60) (Figure 2). By continued passaging number of cell chromosome was decreased to 55, 48, 47 and 46 at 60th, 94th, 155th and 200th passages respectively (Figures 3, 4, 5, 6).

Tumorigenicity test. Tumorigenicity is defined as the ability of viable cultured cells to give rise to progressively growing tumor nodules, showing viable and mitotically active cells. In case, 20 adult mice were inoculated with the cell line and observed twice a week for appearance of tumors. After 6 months no tumors were developed at the inoculation site.

Susceptibility test. The results show that the *IRKHBK* cell line was highly sensitive for isolation of *BHV-1* and *BVD MD* compared with primary bovine kidney cell. The viruses were replicate easily in *IRKHBK* cell (Table 2) and the marked cytophatic effect due to the virus replication was observed at shorter time than primary cell culture (Figure 7).

DISCUSSION

In order to reduce the time required for the virus isolation and development of viral vaccines, host cell lines should be easily available and safe in laboratories. Because of some disadvantages associated with manufacture in primary cells, unlimited life span continuous cell lines have been developed. The cells have reduced host complexity lead to reduction in virus strain diversity and vaccine production yields. (Lednicky & Wyatt 2012, Matsuura *et al* 2011, Leland *et al* 2007, Ogilvie 2001, Pye 1989). In this study a bovine cell line, *IRKHBK*, was developed during extended *in vitro* passaging. Cytogenetic analysis forms

Table1. Characteristics of *IRKHBK* cell line at 2-200 passages levels. The numbers of Chromosomes in 50 or more cells from each passage level were counted.

Passage level	P 2	P 15	P 30	P40	P 50	P 55	P 94	P 155	p 200
Total cells	51	50	52	51	56	50	50	50	50
Normal (diploid)	46(92%)	45(90%)	44(86%)	13(26 %)	-	-	-	-	-
polyploidy	2 (4%)	3(6%)	3 (6 %)	4 (8%)	3 (6 %)	4 (8 %)	4 4 (8 %)	1 (2 %)	3 (6 %)
Hypodiploidy	1 (2%)	2 (4%)	1 (2%)	-	-	-	-	-	-
Hyperdiploids	1 (2%)	-	-	-	-	-	-	-	-
Break or Gap	1 (2%)	-	2 (4%)	2 (4%)	-	-	1 (2%)	-	-
Structural aberration %	-	-	2 (4%)	32 (64%)	56(100%)	50(100%)	50(100%)	50(100%)	50(100%)

Table 2. CPE effect of viruses on Primary *BK* cell and *IRKHBK* cell line

Viruses	Primary BK cell	*IRKHBK* cell line
BHV-1	3-4 *	1-2
PI3	4-5	2-3
RPV	5-6	3-4
BVD-MD	6-7	4-5
PPR	7-8	5-6

***** Days after inoculation of cell line with viruses that CPE seen.

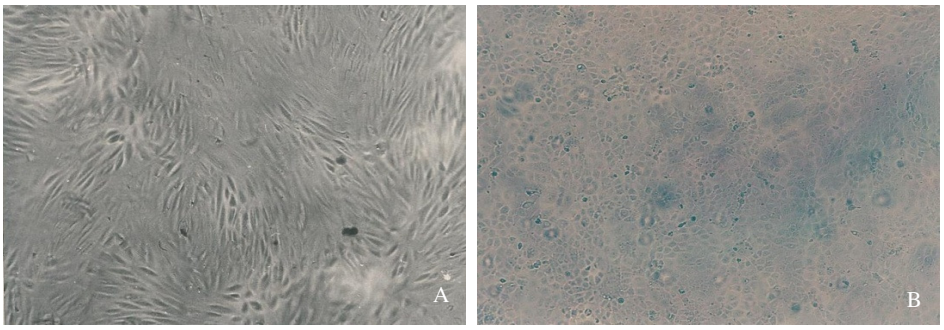

Figure 1. (a) Primary bovine kidney cells, 100X; (b) Established *IRKHBK* cell in 70th passage 100X.

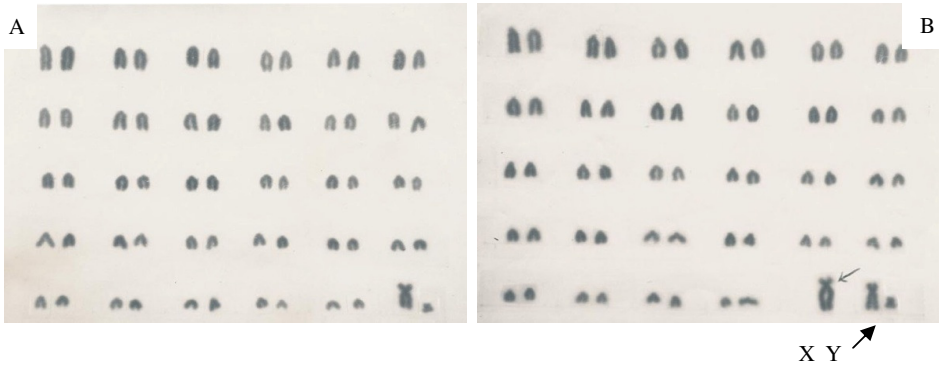

Figure 2. In this photograph the chromosomes have been stained with Giemsa (G-banding) and arranged in a standard fashion. This standard arrangement of an individual chromosome is known as a karyotype. (a) Karyotype of normal bovine somatic cells; (b) Karyotype of *IRKHBK* at 30th passage. Joining of two chromosomes from centromeric site are shown with flash (n =59).

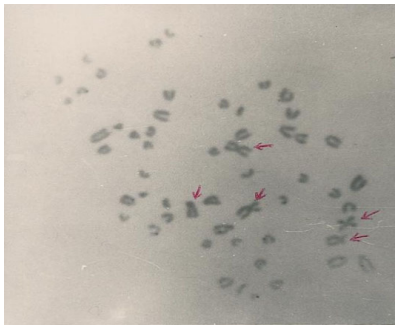

Figure 3. Karyotype of *IRKHBK* cell line at 60th passage. Numbers of chromosome of the cell is 55 (n=55). Translocated chromosomes are shown with flash.

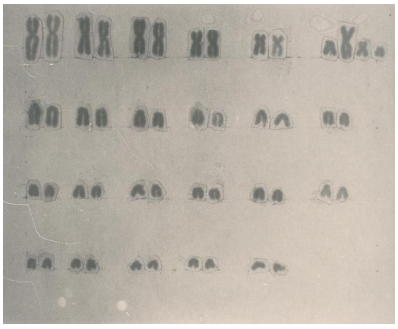

Figure 4. Karyotype of *IRKHBK* at 94th passage. Numbers of chromosome of the cell is 48 (n=48).

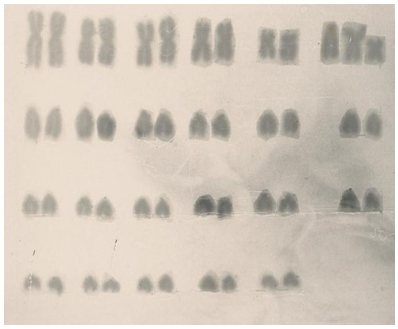

Figure 5. Karyotype of *IRKHBK* at 155th passage. Numbers of chromosome of the cell is 47 (n=47).

an essential part of characterizing and identifying cell lines. The analysis is performed on cell cultures to perform identity checks by verifying species of origin or the retention of key chromosome rearrangements in cell lines. Karyotypic changes occurred in parallel with the phenotypic changes as shown in figures 1 and 2 and immortalization occurred between passages 70 and 90. The cell was successfully passaged and until the 15th passage did not show any changes neither in growth rate nor in its morphology.

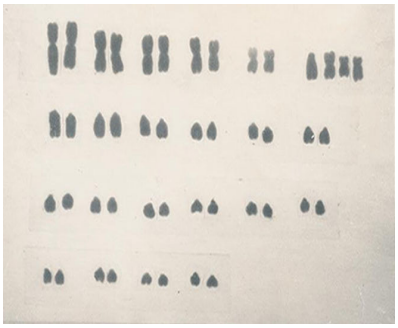

Figure 6. Karyotype of *IRKHBK* at 200th passage. Numbers of chromosome of the cell is 46(n=46).

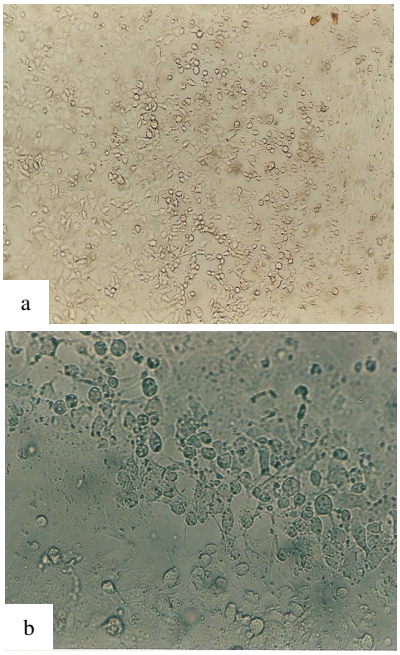

Figure 7. (a) IBR virus-infected *IRKHBK Continuous cell line,* 100X. (b) BVD-MD virus-infected *IRKHBK Continuous cell line.*

At this passage the growth rate of the cell and also the transferable cell population rate began to decrease. We had an increase in cell growth rate from the 18th passage but it is decreased severely again and we had to collect the cells of 2-4 bottle in one 2 oz till 30th passage. The first chromosomal change was observed (Figure 2) following gradually increase in growth rate of the cells at this passage and although the primary *BK* culture was a mixture of fibroblast and epithelial like

cells, the epithelial cells began to predominant from 40th passage, and at the 60th passage the fibroblast-like cells were eliminated.

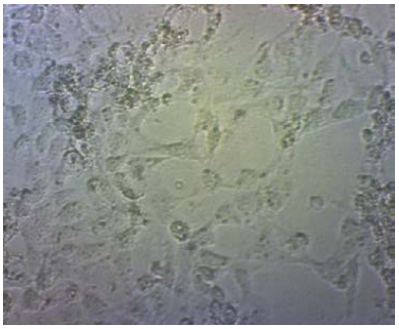

Figure 8. *IRKHBK Continuous cell line* Foot and Mouth Disease virus Infected after 72 hours (Rafiei *et al* 2014).

The growth rate of epithelial-like cells was increased in the next passages until establishment of a uniform population of cells in aspect of morphology and growth rate. From the 70th passage the cells were obtained an unlimited growth capacity in which at passage 90th identified as infinite cell. At 30th passage two chromosomes joined together revealed the first change was occurred in the cell karyotype. The number of chromosomes in somatic cells is constant and termed the diploid number or 2n (Simione 2009). Despite the normal bovine somatic diploid cell (2n=30) (Mirchamsi *et al* 1978) number of *IRKHBK* cell chromosome at 30th passage decreased indicating the ploidy of the cell was switch to aneuploid. Decrease in chromosome number was continued up to the last passage (200th passage). The *IRKHBK* cell is easy cultured and has a satisfactory growth rate with high confluences (36 hr). The continuous cell line supports the virus's replication and is even more sensitive for parasite proliferation. Several studies on impact of *IRKHBK* cell line for virus isolation indicated that the examined viruses include *BHV-1*, *equine herpes virus-1, human herpes virus-1, foot and mouth disease virus* (Figure 8), and *herpes simplex virus type-1* have been well isolated (Pishraft Sabet *et al* 2003, Rafiei *et al* 2014, Rafiei *et al* 2015, Shirvani *et al* a 2011, Shirvani *et al* b 2011, Soleiman Jahi *et al* 2004, Taghipour-Bazargani 2014). Mahmodzadeh and the co-workers (2008) have studied the proliferation of *Pneumocystis carinii* on *IRKHBK* cell line and compared its growth rate with Vero and MRC-5 cell lines. They conclude that the difference between *IRKHBK* and two other cell lines was significant ($p = 0.023$) and *IRKHBK* cell line is suitable for parasite proliferation assay. The *IRKHBK* continuous cell line also known as *Razi Bovine Kidney (RBK)* was submitted to National Cell Bank of Iran (NCBI) under C541 code.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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