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Isolation of Bovine leukemia virus from cows with persistent lymphocytosis in Iraq

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ABSTRACT

This is the first study to report on the isolation of bovine leukemia virus (BLV) from peripheral blood mononuclear cells of two cross bred cows in Iraq. The cattle were seropositive by ELISA when selected while being surveyed for the detection of BLV. Among six cows, two were cases of persistent lymphocytosis (PL). Cytopathology was characterized by the formation of multinucleated giant cells (syncytia) and cytoplasmic vacuoles. Moreover, the viruses produced clear plaques on the monolayer of the primary fetal calf kidney (FCK) cells. Inhibition of plaque formation by BLV-antisera suggested a diagnosis of BLV, which was further confirmed by PCR. Cells infected with the isolates were positive to a monoclonal antibody against the viral gp51 transmembrane glycoprotein by immunocytochemistry. Both isolates replicated and induced cytopathic effects in bovine and human cell line cultures. Phylogenetic analysis based on partial gp51 *env* gene sequences revealed that Iraqi strain highly homogenous with Turkey strain (100%) and had 1% distance value with other world strains. In conclusion, this present study found that BLV-infected cattle with PL can be a source for viral isolation, and the cytopathological features of the virus infection are arranged and differ depending on the cell type. This is the first study to report on the isolation of the EBL virus in Iraq, and it provides the basis for further studies about a BLV Iraqi strain that can help control this disease.

1. Introduction

Enzootic bovine leukosis (EBL) is characterized by life-long asymptomatic infection, persistent lymphocytosis (PL), and neoplasms, particularly B-cell lymphomas (Mirsky, Olmstead, Da, & Lewin, 1998). Bovine leukemia virus (BLV) has been isolated from cattle with EBL and shown to be associated with the disease (Frie & Coussens, 2015; Gillet et al., 2007).

While blood cells can be essential for BLV infection diagnosis, some BLV-infected animals do not produce an adequate level of the virus for advanced research; thus, virus propagation is an important method that affords a higher diagnostic value. It also produces a significant number of viruses. The efficiency of BLV to successfully infect different types of cell lines in vitro has been previously demonstrated (Donald C Graves & Ferrer, 1976; Inabe, Ikuta, & Aida, 1998). Moreover, because it is challenging to obtain detectable values for BLV directly from infected animals, viral propagation could be a useful way to further examine the biological properties of BLV in antiretroviral therapy experimental studies (Donald C Graves & Ferrer, 1976; Kohara & Yokomizo, 2007).

BLV induces chronic lymphoproliferative diseases by affecting the B-cell lineage, with the absence of chronic viremia and a long latency period. The virus encodes at least two regulatory proteins: Tax and Rex. Tax is the oncogenic protein of the virus, and it is involved in viral replication (Sagata et al., 1984). Nevertheless, a viral infection is not enough to initiate leukemogenesis; instead, gene mutations could be required (Aida, Murakami, Takahashi, & Takeshima, 2013). Tax causes malignant transformation by inhibiting DNA repair and trans-activating the disruption of cellular growth control mechanisms (Gillet et al., 2007). Recently, a research group (Buehring et al., 2014) reported the presence of BLV DNA in human breast tissue, which indicates that it has the potential to be zoonotic; however, more studies are needed to confirm that finding.

BLV is endemic in Iraq, and the country's beef and dairy industries have been significantly impacted. A previous study (Khudhair, Hasso,

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Table 1
The animals selected for BLV isolation.

Animal No.	Hematological status	ELISA[1]	PCR[2]
7	AL*	Weak +	+
16	PL**	+	+
29	AL	+	+
162	PL	+	+
279	AL	+	+
331	AL	Weak +	+

[1] = Indirect BLV-gp51-Ab ELISA test (Svanova Biotech AB, Uppsala, Sweden)
[2] = Conventional PCR targeting *pol* and *env* genes, * = Aleukemic leukemia. ** = Persistent lymphocytosis.

Yaseen, & Al-Shammari, 2016) by our team aimed to determine the percentage of BLV infection and PL in crossbreeds and local bred of Iraqi cattle that are naturally infected with BLV. However, more information on the infectious viral diseases and the characterization of the pathogens of cattle in Iraq is needed, especially those that induce malignancies. A retrospective study based the isolation of BLV in cell culture has not previously been reported, and the molecular characteristics (biologic, antigenic, and genomic) of BLV have not previously been studied. This is the first report on the isolation and characterization of BLV from cattle with EBL in Iraq.

2. Material and methods

2.1. Ethics statement

This study was approved by the scientific committee and Veterinary Medicine Review Board. Furthermore, consent was obtained from the farm animals' owners before any animal sampling occurred.

2.2. Animals

Based on three diagnostic methodologies, hematological, serological, and molecular tests, six out of 400 cows diagnosed previously as BLV infected (Khudhair, Hasso, Yaseen, & Al-Shammari, 2016), (numbered 7, 16, 29, 162, 279, and 331) were selected for the isolation study (Table 1) We did not get samples from other cows with PL because they were sold by their owners.

2.3. Cells and cell cultures

The early-passage of a fetal calve kidney (FCK) primary cell culture, established by Experimental Therapy Department, ICCMGR/ Mustansiriyah University, from a normal FCK, was minced and treated with an enzyme (0.02% trypsin with phosphate-buffered saline (PBS) for 20 min at 37 °C, and then filtered through an 80 um mesh. The filtered fluids were then centrifuged, and the precipitated cells were cultured in a tissue culture flask overnight in Roswell Park Memorial Institute medium (RPMI) (USbiological, Salem, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Capricorn-Scientific GmbH, Ebsdorfergrund, Germany), ampicillin (100 μg /ml), and streptomycin (100 μg/ml) (Capricorn-Scientific GmbH, Ebsdorfergrund, Germany). The human cervix carcinoma (Hela cell line) and human rhabdomyosarcoma (RD) cell line were supplied by the same company and maintained in RPMI medium supplemented with 10% FBS (Capricorn Scientific GmbH), ampicillin (100 μg /ml), and streptomycin (100 μg/ml) (Capricorn Scientific GmbH).

2.4. Preparation of the PBMC

The samples of the peripheral blood mononuclear cells (PBMC) were prepared by density gradient using lymphocyte separation media (Capricorn-Scientific GmbH) gradients, as described by the manufacturer's protocol. After centrifugation, the mononuclear cells were harvested from the gradient interface and washed twice with 5 ml of warmed PBS (pH 7.4). They were then mixed gently and centrifuged once at 700 X g for 15 min, and then at 400 X g for 15 min. The supernatant was then discarded. The cells were re-suspended with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 μg /ml of ampicillin per ml, and 100 μg of streptomycin/ml, and then incubated for 2 h at 37 °C in CO_2 in 50 ml universal tube (Don C Graves & Jones, 1981). After 2 h, the resting cells were counted, and the viability of the cells was assessed using trypan blue stain and 200X light microscope, and then they were adjusted to ensure a viability range of 4 $\times 10^6$ – 5 $\times 10^6$ viable cell/ml.

2.5. Isolation of BLV

For each of the selected samples, we used three viral infection and isolation methods, as described below.

2.5.1. Inoculation of PBMC into the cell cultures

The PBMC were seeded at 2 $\times 10^6$ – 3 $\times 10^6$ with the semi-confluent FCK, Hela, and RD cell cultures. The method described by (Graves, Diglio, & Ferrer, 1977) was followed. In short, after discarding the confluent flask of the FCK cell growth medium, the cells were detached using trypsin/EDTA. The cells were then resuspended, and equal amounts of the adjusted number were mixed with the PBMC in 25 ml Nunc™ EasYFlask™ Cell Culture Flasks (ThermoFisher, USA). The inoculated cultures were incubated at 37 °C and periodically passaged until the cytopathic effects (CPE) were observed.

2.5.2. Inoculation of the PBMC supernatants into the cell culture

To infect the cells with BLV, isolated PBMC were frozen and then thawed three times. They were then filtered through a Nalgene 0.45 μm-pore-size filter (Thermo Fisher Scientific, Waltham, MA USA) and used to infect the cells. Next, 1.5 $\times 10^6$ cells from the FCK primary culture and the Hela and RD cell lines were plated in 25 cm^3 falcon tissue culture flasks in duplicate. Before infection, the cells were treated with diethylamino ethyl (DEAE)-dextran (25 p g/ml) for 30 min at 37 °C and then washed. The cells were then inoculated with 1 ml of the virus-containing fluids obtained from the PBMC lysate. After being shaken slowly for 2 h at 37 °C, the virus was allowed to adsorb. The monolayers were examined daily for CPE (Graves et al., 1977).

2.5.3. Expansion of the PBMC

The method used for the expansion of the PBMC as previously described and used in isolation of the virus in several studies (Driscoll, Onuma, & Olson, 1977; Miller, Miller, Olson, & Gillette, 1969; Onuma, Watarai, Mikami, & Izawa, 1980; Rovnak, Casey, Boyd, Gonda, & Cockerell, 1991). About 2 $\times 10^6$ of the PBMC cells were cultured in duplicate in a 25 cm^3 falcon flask with 7 ml of RPMI medium supplemented with 10% FBS, 100 μg /ml of ampicillin, and 100 μg/ml of streptomycin. The sample was then incubated at 37 °C for 4 h. Later, 0.4 ml of phytohemagglutinin (PHA) was added to each falcon flask, and half of the media was exchanged on the next day and for three days later, with observation, until the development of the virus CPE.

2.5.4. Examination of the cytopathic effects

The infected cells were examined daily to determine the CPE. When the first signs of CPE were visible, the medium was discarded, and the cells were washed with warmed PBS three times, then fixed with 1% neutral formaldehyde for 10 s, before being washed with warm PBS three more times. Hematoxylin (H) stain was added for 10 s, and then the cells were washed. Next, eosin (E) stain was added, and the cells were washed with tap water, air-dried, examined under light microscope, and photographed (Mohammed, M., M., & Al-Shammari, 2019).

Table 2
The bovine leukemia virus recovery samples based on the infective stage, ELISA, PCR tests, and viral isolation.

Case no.	Clinical stat	Lymph. count	ELISA	PCR	Virus recovery
7	AL	6638	Weak +	+	–
16	PL	8271	+	+	+
29	AL	6188	+	+	–
162	PL	9675	+	+	+
279	AL	7113	+	+	–
331	AL	6910	Weak +	+	–

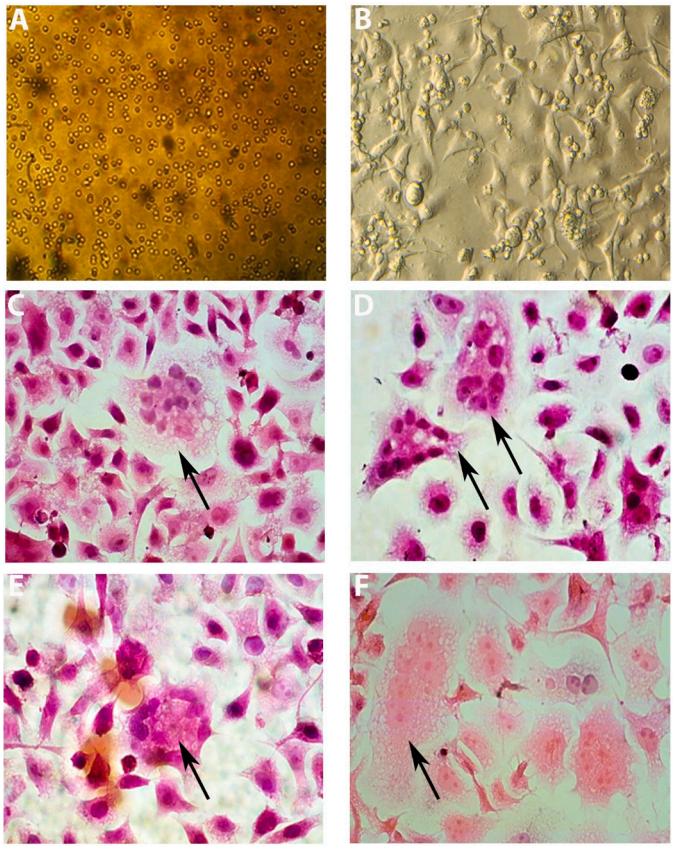

Fig. 1. Cytopathic effects of BLV infection on the PBMC cell culture; A) 2 days suspension culture of PBMC (100X); B) Phase contrast light microscope of PBMC sample 162 at day 6 of the culture (400X); C) Early syncytia in the PBMC culture; D) Multiple syncytia were observed with seven to 17 nuclei per syncytium; E and F) syncytia were show the feature arrangement of nuclei as a ring inside of syncytia of PBMC cell culture.

2.6. Syncytia induction assay and syncytia inhibition assay

The procedure that was used for the syncytia induction assay was described previously (Doménech et al., 2000; Doménech, Llames, Goyache, Suárez, & Gómez-Lucía, 1997). Briefly, the FCK cells were plated in a 12-well tissue culture plate. About 4×10^5 of the FCK cells were grown in RPMI with 10% FBS. Four wells were used for the test, four wells were used for the PBMC as the positive control, and four wells contained FCK cells as the negative control. After three to four days, the confluent monolayers were washed with PBS. Approximately 1 ml of BLV suspension was added to the test plates. For virus adsorption, the plates were shaken by hand gently every 10 min for 2 h. The monolayers were washed with PBS, and the plates were incubated for three to five days.

The syncytia inhibition assay was done in the same way as the syncytia induced assay, according to Johnson, Rommel, & Moné, (1998). The FCK recipient cells were treated with 1 ml of anti-BLV sera and incubated for 48 h, and all the plates were incubated at 37 °C for three to four days. Then, the supernatant was discarded, and the cells were air-dried and stained with hematoxylin and eosin (H&E). All of the syncytia that contained more than four nuclei were counted per 30 fields at 200X under an inverted microscope, and then the results were compared with the positive and negative controls.

2.7. Molecular test

2.7.1. Detection of BLV provirus in the expanded PBMC and infected cell cultures via PCR

Total DNA was extracted from the infected cells in order to detect if the provirus was integrated with the cell's genome using a KAPA Express Extract Kit (Kapa Biosystems Cape Town, South Africa). The extracted DNA samples were quantified and stored at −86 °C until used. Two different sets of primers were utilized, env1 set, the sense 5-CCC ACA AGG GCG CCG GTT T-3, and the antisense, 5-GCG AGG CCG CGT CCA GAG CTG G-3. The pol1 primer, the sense, 5′-CGG GAT TGA TCA CCC CGG AA-3 (546–565), and the antisense 5′-GGA CTC CGT CGG GAA GGT T-3 (Khudhair, Hasso, Yaseen, & Al-Shammari, 2016), in briefly, first set of primer target 444 bp of env gene amplified under condition 95 °C\5 min of initial denaturation followed by 35 cycles of 95 °C\20 s, 61 °C \ 20 s, 69 °C\ 1 min and 69 °C for 3 min for denaturation, annealing, extension and final extension, second primer target pol 509 bp of gene, the thermocycling conditions was, initial denaturation at 94 °C \ 3 min, followed by 35 cycles of 94 °C \ 1 min, 65 °C \20 s, 72 °C \30 s and 72 °C \5 min of denaturation, annealing, elongation and final elongation stapes. The BLV-negative cell line (uninfected FCK, Hela, and RD cell lines) was used to ensure no false-positive reaction in the negative cell lines. The amplified products were detected using electrophoresis through a 1% agarose gel containing ethidium bromide (Mohammed A. Hamad, Al-Shammari, Odisho, & Yaseen, 2016).

2.7.2. Sequence and phylogeny

The PCR results were confirmed by sequence used on DNA purified from agarose gel (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany), bidirectional sequencing used forward primer 5-CCC ACA AGG GCG CCG GTT T-3 (5099–5120), and the reverse primer 5-GCG AGG CCG CGT CCA GAG CTG G-3 (5521–5542) (Khudhair, Hasso, Yaseen, & Al-Shammari, 2016) (Macrogen Company, Seoul, Korea) Both samples show highly identity between them, one sequence nucleotides aligned at BLAST (http://blast.ncbi.nlm.nih.gov/ Blast. cgi) with global sequences in the NCBI database (http://www.ncbi.nlm.nih.gov), and submitted at GenBank under accession number MK991965.1.Phylogenetic and molecular evolutionary analyses were conducted using the neighbor-joining method with a bootstrap value of 1050 along with env gene by MEGA version 10.1.6 (Hamad, Al-Shammari, Odisho, & Yaseen, 2017).

2.8. Immunocytochemistry (ICC)

Specific monoclonal antibodies for the BLV gp51 protein were used to detect the infected cells in the cultures, the FCK cells, and the PBMC. Then, a co-culture of both FCK, and the PBMC cells was cultured to a confluent monolayer in Lab-Tek chamber slides (Thermofisher, USA), and then treated with phytohemagglutinin (PHA) (Tajima & Aida, 2005). The cells were fixed in 4% formalin for 10 min and rinsed gently with PBS. First, the cells were treated with blocking reagents for 1 h, and then they were treated with anti-BLV gp51 primary antibody overnight at 4 °C to detect the gp51 BLV antigen (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibodies were diluted 1:50 according to the manufacturer's recommendations. A secondary antibody was added, and the standard protocol for the biotin-streptavidin staining kit was applied (Santa Cruz Biotechnology, USA) (Al-Shammari, Ismaeel, Salih, & Yaseen, 2014).

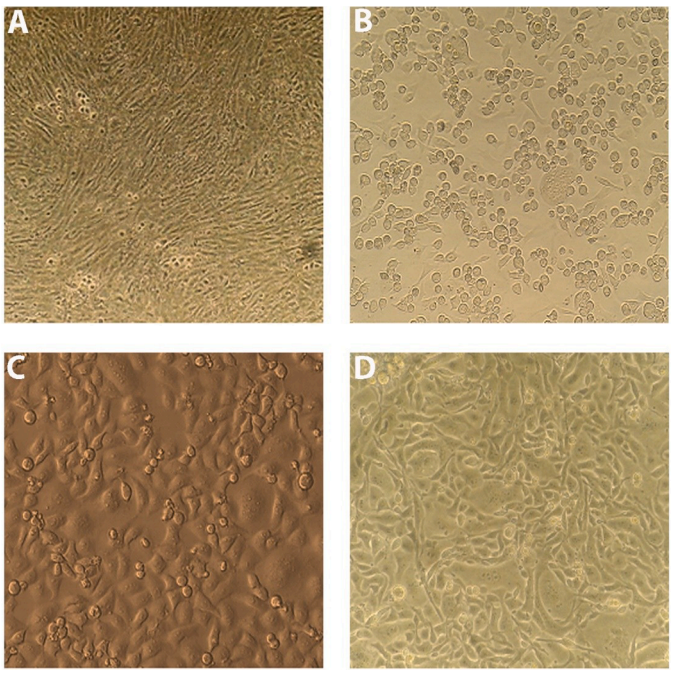

Fig. 2. CPE of BLV infection on an FCK cell culture; A) Normal FCK cells of 6 days old culture at passages number 7 (not infected) (100X); B) The FCK culture after 24 h of co-cultivation of FCK cells with PBMC from BLV-infected cattle showing a mixed culture; C) Phase contrast light microscope showing the FCK syncytia five days after the co-culture (200X); D) The FCK culture with different sized syncytia after six days of BLV infection (200X).

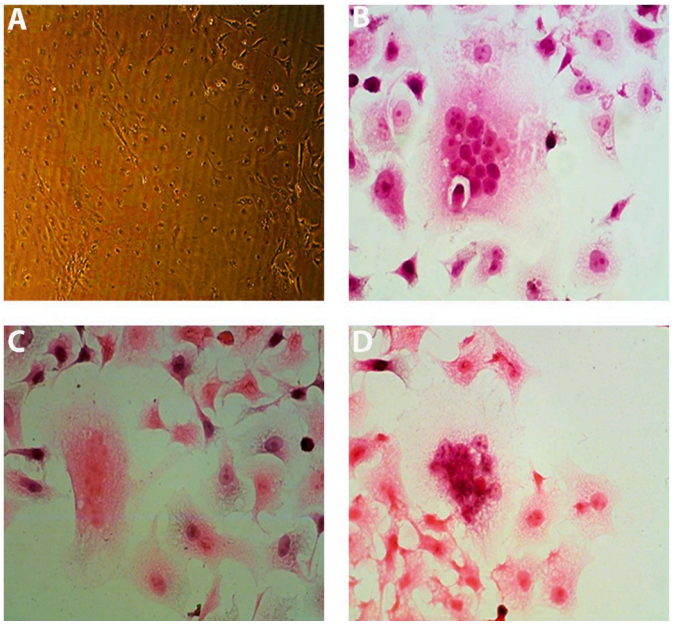

Fig. 3. Syncytia in the infected FCK cells; A) The FCK culture with very large syncytia contained more than 100 nuclei (100X) after 6 days incubation; B) The FCK culture with circular syncytia (200); C) The BLV syncytia in the FCK culture with a longitudinal arrangement of nuclei (200); D) The centrally-grouped nuclei in the syncytia of the FCK culture infected with BLV (200X).

2.9. Agar gel immunodiffusion (AGID) test

The PBMC samples were cultured to a confluent monolayer, and the cells were harvested into a 15 ml universal tube using trypsin-versine. The cells were sonicated in a water-cooled sonicator (Daihan Latech,

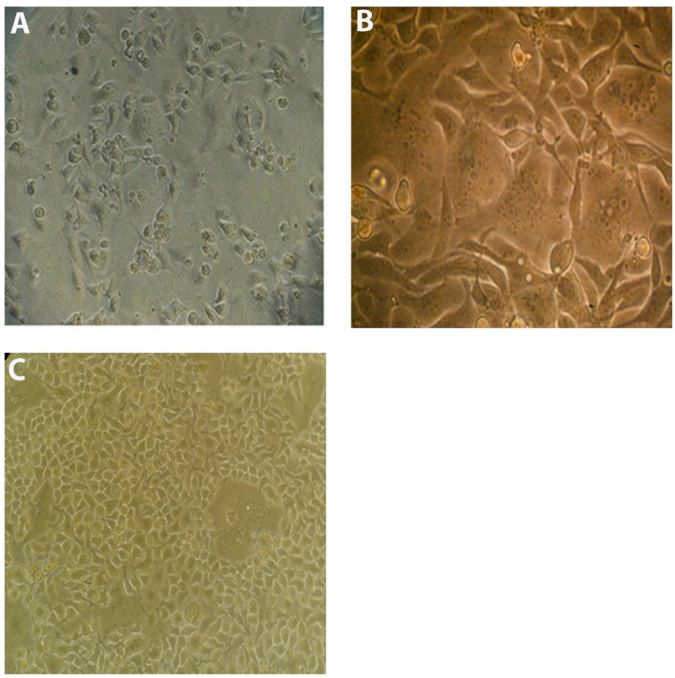

Fig. 4. A) The RD cell line co-infected with PBMC eight days after infection (200X); B) Syncytia were observed in the RD cells seven days following infection with BLV (400X); C) Several syncytia in the RD cell line were infected with BLV (100X).

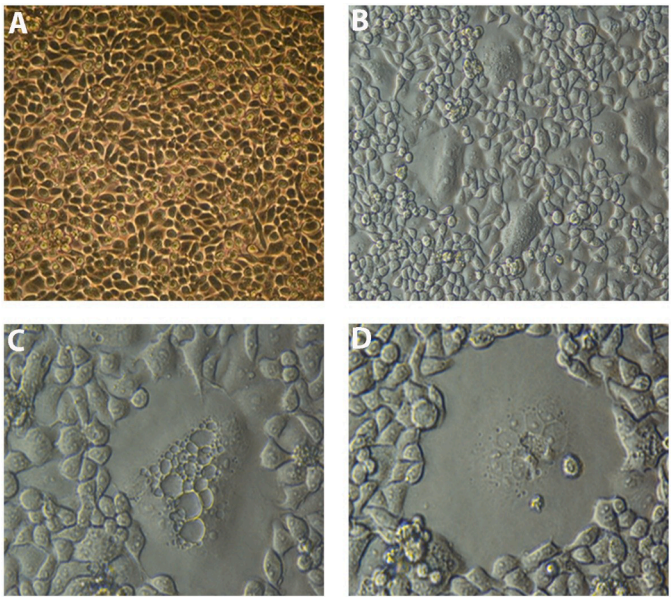

Fig. 5. A) Normal monolayer confluent in the Hela cell line of 7days old culture (200 X); B) The HeLa cell culture shows syncytia of BLV (200 X); C) Phase contrast light microscope showing large vacuoles (400 X); D) Phase contrast light microscope showing syncytia (400 X).

Table 3
Syncytia inhibition assay results for the detection of BLV.

Cell culture	PBMC	Serum
Co-infected FCK	67	19
PBMC	116	27
Control-FCK	0	0

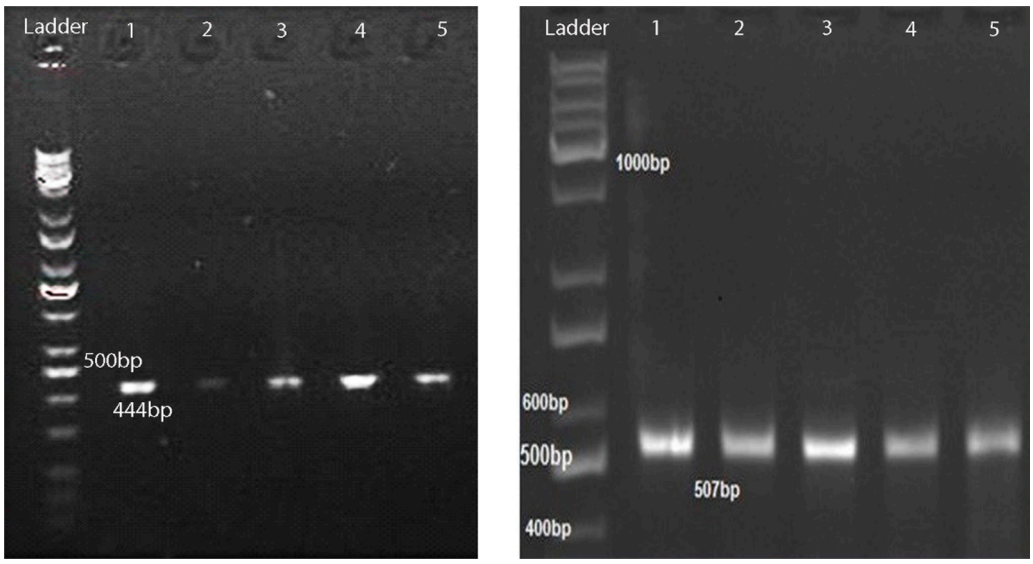

Fig. 6. PCR amplifications of BLV in the PBMC of the infected cell culture; A) Amplification of the 444 bp fragment of the env gene (1,2,3,4, and 5, are FCK, RD, Hela cell lines, PBMC16, and PBMC 162, respectively); B) The 507 bp fragment of pol gene.

LTD, Kyonggi-Do\Korea) at 15KHZ/sec at 50 min intervals. To remove the cellular debris, the homogenate was centrifuged twice at 18,000 rpm for 30 min each time. The supernatant was passed through a 0.22 um Millipore syringe filter. The agar gel immunodiffusion (AGID) tests were conducted according to the standard procedure recommended by the Office International des Epizooties (Commission & Committee, 2008). Gel diffusion plates, made of 0.8% noble agar and 8.5% NaCl, and were allowed to stand for 72 h at room temperature before the readings were taken.

3. Results

BLV was isolated from two samples (16 and 162) with the PL stage of the EBL. The samples were taken from animals with a well-defined EBL infection. The positive isolation cultures showed obvious CPE, as determined by microscopy and confirmed by highly BLV specific primers for PCR assay, BLV- gp^{51} monoclonal antibody of ICC assay, AGID, and the syncytia induction and inhibition assays; the results of the isolation are summarized in Table 2. The remaining four samples were sub-passaged 20 times for intervals of five to six days before any obvious changes were noted, and the isolation attempts were terminated.

3.1. PBMC expansion

Both the 16 and 162 PBMC samples showed positive isolation of the BLV. In sample 16, the PBMC grew as a suspension in the culture, and that growth continued for eight weeks (Fig. 1A). The culture was sub-cultured to 1:4, one to two times each week. In sample 162, the PBMC exhibited high attachment to the surface of the culture flask, and they were maintained as a monolayer. More than 40% of the cells attached during the first 24 h and over 90% of them appeared adherent only after two days (Fig. 1B).

In general, the PBMC cultures from the infected animals showed a unique CPE of BLV. Syncytia formation was prominent in the PBMC cultures, and, in sample 162, it appeared in small quantities in earlier subcultures at day 14, after which the number of PBMC was very large at 20 subcultures. H & E staining showed that the syncytia were more than 10-times their normal cell size; typically, there are five to 18 nuclei per syncytium (Fig. 1C, D). Usually, syncytia nuclei are arranged in a specific way; however, as seen in Fig. 1E, the nuclei in this present study have a unique cytopathognomic arrangement for the BLV propagated in these cells. The nuclei are arranged in a circular or ring formation at the peripheral of the syncytia (Fig. 1E). The number of syncytia produced in the PBMC cell culture increased upon further cultivation; this rapid shift was noted at high levels at 25 passages. Some of the syncytial cells detached from the flask, leaving gaps in the monolayers (Fig. 1F).

3.2. The BLV cytopathic effects (CPE) in the infected cells

Following inoculation with PBMC, and/or its supernatant, on three different cell lines, virus growth was detected first in the FCK cell culture. That growth displayed a typical BLV-CPE, with the formation of multinucleated giant cells (syncytia), cytoplasmic vacuolation, cell lysis, and blebbing of the cell membrane. Since the FCK became elongated, as seen with the light microscope (Fig. 2A), and co-cultured with round PBMC (Fig. 2B), the positive FCK culture initially appeared with vacuoles in the cytoplasm of the cells, and then the syncytia were observed after two days of co-culture (Fig. 2C). Continued culturing of FCK cells resulted in increased syncytium formation (Fig. 2D).

In the FCK culture, the syncytia contained more than 100 nuclei per syncytium (Fig. 3A). The syncytia of both isolates often formed a polymorphism pattern, but their shape is usually circular (Fig. 3B), longitudinal (Fig. 3C), or irregular (Fig. 3D). Moreover, each syncytium typically contains four to 25 nuclei, which are usually centrically or eccentrically aggregated or arranged in parallel, with the lapse of days.

The infected RD cell line (Fig. 4A) showed clear syncytia (Fig. 4B), which is characterized by a smaller size and less cytoplasmic vacuoles in comparison to the PBMC and FCK cultures (Fig. 4D). The CPE was never extensive in the RD cell line culture, and it gradually disappeared with subsequent passages. The virus-infected RD cell line developed minimal cytopathic changes with fewer cytoplasmic vacuoles. In contrast to RD, the bovine PBMC and FCK cell cultures developed overt syncytial cytopathic changes after inoculation. Multiple syncytia formation appeared in the infected Hela cell line 24–48 h after infection (Fig. 5A, B). The Hela cell line developed cytoplasmic vacuoles (Fig. 5C), and it also demonstrated syncytia formation as cytopathic changes (Fig. 5D).

3.3. Syncytia inhibition assay

The syncytia inhibition assay was used to determine the presence of BLV proteins in the plasma membrane of the infected cells. Positive cases were confirmed by specific inhibition with a BLV-positive serum; the number of syncytia was decreased when BLV-specific sera (sera obtained previously from a BLV-infected cow) were applied. The specific BLV-

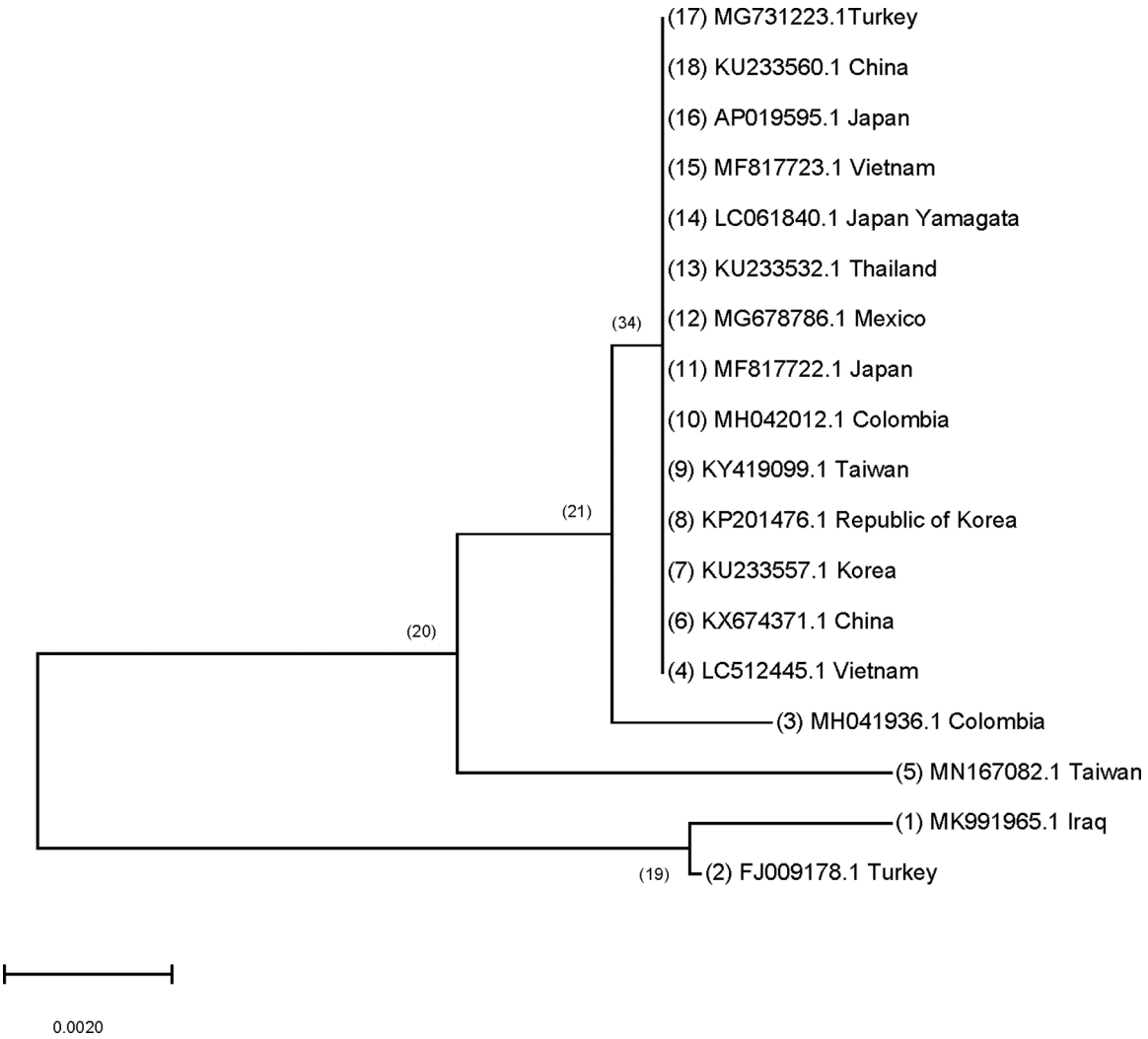

Fig. 7. Phylogenetic tree topology was analyzed by using the maximum likelihood method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 14 nucleotide partial sequences of env gene of BLV from different countries, which indicated with their accession numbers and country name. Codon positions included were 1st + 2nd + 3rd + 4th. There were a total of 389 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

syncytia evidence is indicated by the blocking of the syncytia formation in the syncytia inhibition assay in the cell cultures in comparison to the uninfected FCK cells, which were used as the negative control. The syncytia were abundant in the expended PBMC that were used as positive control (Table 3).

The ability of the PBMC and the supernatant to induce early syncytia is inhibited explicitly by antibodies in the serum of cattle infected with BLV. This indicates that the antibodies in the serum test were specifically matched with the BLV-antigens in both cell cultures.

3.4. Viral detection in cell cultures

Two BLV specific primers were used to confirm cell lines infection with BLV, an assay showed discrete positive bands, indicating the presence of BLV proviral DNA in the cellular DNA of infected cells. The two bands, 444 bp, and 507 bp, which correspond to the *pol* and *env* genes fragments based on the ladder marker, were observed using gel electrophoresis (Fig. 6A, B). Distance analysis and phylogeny with global BLV strain showed high identities with FJ009178.1 in Turkey and 0.01 with other strains Table 5, (Fig. 7).

3.5. Immunocytochemistry

Envelope glycoprotein (gp51) of the BLV could be detected in most of the infected cells after treatment with a specific mAb against viral gp51. Viral antigens were localized diffusely in the cell membrane, and they were observed in 50–60% and 80–90% of the FCK and PBMC cells, respectively. The viral gp51 glycoprotein expression in the Hela and RD cell lines was less prominent than it was in the FCK and PBMC cell cultures (Fig. 8).

3.6. Agar gel immunodiffusion test (AGID)

The AGID test was used to detect the virus and virus products in the cell cultures after exposure to the PHA mitogen, which is known to increase the cell expression of viral proteins. The viral antigen was detected in the supernatants and the lysates of the cells after they were sonicated and filtered. Both expanded PBMC gave positive results, as demonstrated by the continuous line of precipitation; this reaction was identical to the BLV-antibody reaction previously obtained from a naturally infected cow (Table 4).

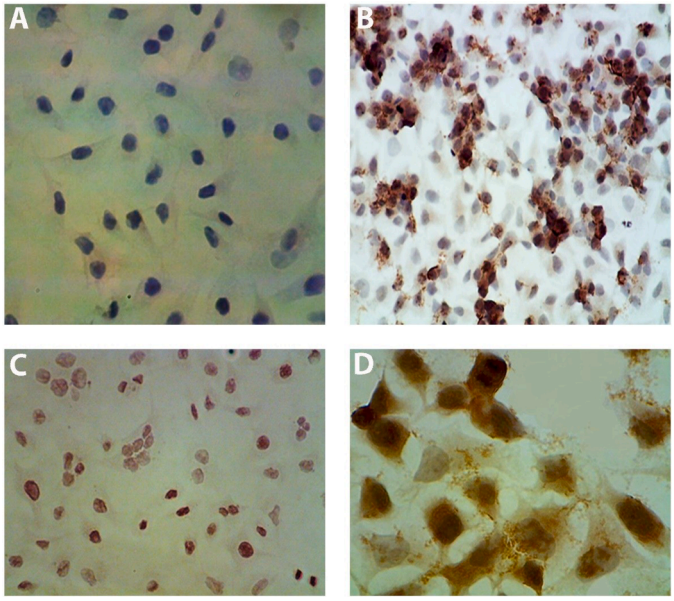

Fig. 8. The envelope glycoprotein (gp51) of BLV could be detected in most of the infected cells after treatment with a specific mAb against gp51. The same infected cells were used as a control; A) The negative PBMC control with only a secondary antibody added (200X); B) The PBMC positive cells with mAb against viral gp51reacts with most cells in the cytoplasm (200X); C) The control FCK cell stained with hematoxylin (200X); D) The infected FCK positive for viral antigens that was localized diffusely in the cell membrane and cytoplasm (400X).

Table 4
Isolation results for detection of BLV in blood samples from four cell cultures.

Cell culture	CPE	PCR		ICC	AGID
		env3	*gag*1		
PBMC	+	+	+	+	+
FCK	+	+	+	+	+
RD	+	+	+	+	–

Table 5
Distance values of sequences multiple alignment of partial *env* gene compared with global strains from different countries.

Accession N./Country	Distance
MK991965_Iraq	
AP019595.1_Japan	0.01
FJ009178.1_Turkey	0.00
KU233532.1_Thailand	0.01
KU233560.1_Korea	0.01
KX674371.1_China	0.01
KY419099.1_Taiwan	0.01
LC061840.1_Japan:Yamagata	0.01
MF817722.1_Japan	0.01
MF817723.1_Viet_Nam	0.01
MG678786.1_Mexico	0.01
MG731223.1_Turkey	0.01
MH041936.1_Colombia	0.01
MH042012.1_Colombia	0.01

4. Discussion

From the data presented in this paper, PBMC from different cattle have varying abilities to propagate BLV. Only blood cells derived from PL cattle allowed for the recovery of BLV in the cell cultures. Previously, (Forbes & Leong, 2012) reported that the isolation of BLV is more efficient from PL cattle than from non-lymphocytotic cattle, and this likely reflects the relative number and viability of the cells carrying the virus in PL cattle and non- lymphocytotic cattle (Ferrer, Abt, Bhatt, & Marshak, 1974). On the other hand, BLV-infected cattle with PL have a steadily higher provirus load than non-PL cattle, which have a significantly low provirus load (Tajima, Ikawa, & Aida, 1998). As mentioned above, the viral recovery for the sample with weak reactivity to ELISA failed (Table 1). The titer of antibodies can reflect the degree of BLV infection, as the titer of leukemic cattle is usually higher than the titer of aleukemic cattle (Ferrer et al., 1974).

Our results indicate that BLV can be isolated and serially propagated from PBMC in FCK cell cultures and human-derived cell lines. These results are similar to those of Adachi and colleagues (Adachi et al., 1986), who reported on BLV propagation by co-cultivation of the PBMC of infected cows using a fetal lamb kidney (FLK) cell culture. Thus, the primary FCK cells seem to be the most permissive for the isolation and propagation of BLV. The propagation of BLV in an FCK continuous cell line was demonstrated by (Rakowicz-Szulczynska, McIntosh, & Smith, 1999). In the Hela and RD cell lines, the infectious virus produced and expressed its antigens, and it produced low levels of the infectious virus. These data agree with the fact that BLV has been found to infect a wide variety of cells in vitro, such as FLK, feline fibroblast (CC81), Madin-Darby bovine kidney (MDBK), baby hamster kidney (BHK), and human epithelial cervical cancer (Hela) cells (Altaner, Altaneroyá, Bán, Niwa, & Yokoro, 1989; Camargos et al., 2014; Ferrer, Cabradilla, & Gupta, 1981; Ferrer et al., 1974; Sagata et al., 1984). Other studies have used bovine, ovine, and human-origin cell lines (Parfanovich et al., 1977). It seems that BLV broadly infects many cell types and it has heterocytotropism capacity (Kettmann et al., 1994). (Derse & Martarano, 1990) demonstrated that human cells seem to be sensitive to BLV infection in vitro. The BLV produced by cultured PBMC from cows with EBL is highly infectious, and it can be readily transmitted to fresh FCK cells.

BLV Iraqi isolates can induce large syncytia, but they are formed after several days, and the syncytia formation occurs immediately adjacent to the foci of the infected FCK cells. The retroviral cytopathogenicity system provides a useful indicator for viral detection (Benton, Soria, & Gilden, 1978). In this present study, the results of the CPE were compatible with many of the CPE state feature results mentioned by (Vantsis, Barlow, Fraser, Rennie, & Mould, 1976), who used FLK cells. Moreover, in our study, the CPE of the isolated BLV resembled those of spumaviruses and HIV reported by (Ivanov, 1979).

The ability of BLV to produce CPE in cell cultures has been previously reported (Onuma et al., 1980). Therefore, syncytium-inducing BLV has been clinically significant in the pathogenesis of BLV infection, and the size of the syncytia in primary FCK cell cultures has been found to vary, containing 5 – 100 nuclei per syncytium; these results are similar to the results of (Sagata et al., 1984), which revealed that, on average, the FCK culture contained 13.5 nuclei per syncytia and the Hela cell line contained 8.5 nuclei per syncytia.

Moreover, the syncytia observed in the co-cultivation of cow and sheep embryo kidney cells co-cultured with peripheral blood lymphocytes from leukemic cattle, occurred six to eight days after co-cultivation (Ivanov, 1979), and BLV-producing transfectants were established in the COS-1 and 23CLN cell lines, which did not form syncytia upon expression of BLV (Inabe et al., 1998). The PBMC seems to be unique in terms of their responsiveness to BLV propagation; this present study shows that BLV replicates well as a long-term culture. Moreover, (Dequiedt et al., 1999) observed that PBMC from PL BLV-infected cattle were highly susceptible for in vitro cultivation in comparison to cells from aleukaemic animals or uninfected animals. In this present study, the PBMC cells were similar at early passages to the monocyte/ macrophage cell line that was established by (Ferrer & Cabradilla, 1978; Popovic, Sarngadharan, Read, & Gallo, 1984). (Ivanov, 1979) reported that 22 out of 24 cultured lymphocytes of leukemic animals were capable of inducing syncytia. The marked CPE of BLV was found to induce syncytia formation in bovine indicator cells (Ivanov, 1979). The virus enters the cell, and the genome is expressed; then, viral RNA replication occurs to

produce BLV infection in a FCK primary cell culture, which continues to grow with clear CPE (Forbes & Leong, 2012). The ability of these cells to express and elaborate whole viral and viral proteins has generated the idea that cultures could provide a useful source of material for large-scale virus concentration and purification.

One CPE of BLV in FCK cultures is the arrangement of multiple nuclei in a characteristic ring formation in giant cells in the infected PBMC. These structures can be used as an indicator to detect BLV in the cultured samples. This system was similar to the structures of nuclei arrangements of the syncytia of human T-cell lymphotropic virus (HTLV) in lymphocyte cultures from patients with HTLV or AIDS; in fact, (Popovic et al., 1984) mentioned that it can be used as evidence to detect HTLV in clinical specimens. This system opens the way to the routine detection of retroviruses and related cytopathic variants in samples, and it provides a large amount of a virus for detailed molecular and immunological analyses.

Using the AGID test, the BLV antigen produced by sonicated infected monolayers cultures, virus neutralization, and absorption was obtained from all the cell cultures. These results agree with the findings for other BLV-infected cell lines reported by (Ferrer & Cabradilla, 1978). Furthermore, (Doménech et al., 1997) showed that only the serum from BLV-infected animals, which had the antibody to viral gp51, could inhibit the release of the virus from infected cells**.** The results reported by (Doménech et al., 1997) suggest that the syncytia induction assay and the syncytia inhibition assay may be more sensitive than the Western blot and AGID assay systems for determining the infection status of cattle (Johnson et al., 1998). According to identity values, BLV Iraqi strain may belong to genotype 1 due to the highly identity of Iraqi strain with Turkish strain that was genotype 1 (Alkan, Oğuzoğlu, Timurkan, & Karapınar, 2011).

5. Conclusion

This is the first study to report on the isolation of the EBL virus in Iraq, and this present study found that the virus could be isolated from BLV-infected cattle with PL. and the cytopathological features of the virus infection are arranged and differ depending on the cell type., and it provides the basis for further studies about a BLV Iraqi strain that can help control this disease.

6. Compliance with ethical standards

6.1. Funding

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Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

6.2. Consent to publish

We hereby confirming that we have obtained consent to publish from the owners of the animals.

Declaration of Competing Interest

Authors declares that there is no conflict of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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