Original Article

The Characteristics of an Ovine Lymphoid Cell-Line sensitive to Vaccinal Infectious Bursal Disease Virus Strain

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ABSTRACT

Infectious bursal disease (IBD), also known as Gumboro disease, is a globally well-known disease with a significant socio-economic effect. For control of IBD, several commercial egg- and cell-based vaccines are prepared. The cell-based IBD vaccines are significantly cost-effective; however, it is essential to confirm their safety and efficacy. The main cell line used to product the cell-based IBD vaccines, is a primary chicken embryo fibroblast (CEF). Nevertheless, manipulation of CEF is extremely challenging and time-consuming. This study aimed to characterize a sensitive suspension cell culture from ovine lymphoid, according to WHO technical report series; No. 978, Annex III. This authentication covered the growth curves, sensitivity, stability, karyotyping and identifying the adventitious agents. This cell line passed all defined tests and was considered as a suitable one for IBD vaccine preparation in a large scale.
Keywords: Infectious bursal disease, Gumboro disease, Chicken embryo fibroblast, Ovine lymphoid origin cell line, Cell characterization

Détermination des caractéristiques d'une lignée cellulaire d'origine lymphoïde de moutons sensibles à la souche vaccinale d'un virus de la bursite infectieuse des volailles

Résumé: La bursite infectieuse (maladie de Gumboro) a été rapportée de partout dans le monde et l'importance socio-économique de cette maladie est considérable au niveau international. Plusieurs types de vaccins commerciaux ont été introduits pour contrôler cette maladie. Ces vaccins sont produits par culture cellulaire ou dans des œufs embryonnés. Si les vaccins à base de cellules contre la maladie de Gumboro s'avèrent sans danger et efficaces, ils seront beaucoup plus abordables pour le traitement de la maladie. Les fibroblastes embryonnaires de poussin (CEF) sont les cellules les plus populaires pour la production du vaccin contre la Bursite infectieuse par culture cellulaire. Le procès de préparation et d'utilisation des cellules CEF est très long et difficile. Dans

cette études, l'attention est portée pour la première fois sur la référence Annex3 TRS 978, WHO, une cellule en suspension de moutons d'origine lymphoïde (H1 Cell Line) ayant montré une sensibilité au virus vaccinal de Gumboro. Cette cellule a passé avec succès tous les tests et a été adaptée à la réplication du virus de la Bursite infectieuse.

Mots clés: Cellule embryonnaire fibroblastique de poussin, Bursite infectieuse des volailles, Caractérisation des cellules, Lignée cellulaire lymphoïde de mouton, La maladie de Gumboro

INTRODUCTION

Infectious bursal disease (IBD) is a highly contagious disease of young chickens caused by infectious bursal disease virus (IBDV), which initially invades mainly an important lymphoid organ in birds, which is called as bursa of Fabricius (the bursa which is present in cloaca of the birds) (OIE Terrestrial Manual, 2012; Eterradossi and Saif, 2013). The IBD was described by Cosgrove in 1962 for the first time. IBDV is the sole member of the genus *Avibirnavirus* within the family *Birnaviridae* (Berg et al., 2000; Mahgoub et al., 2012). The economic importance of this disease is two-fold: firstly, some virus strains may cause up to 60% of mortality in chickens at three weeks of age and older; secondly, this virus causes a severe prolonged immunosuppressive disease which can inflict the chickens at early age and makes them more susceptible for the other diseases. Protection of young chicks against the early infection is of paramount importance. This is usually accomplished by a combination of passive (transfer of maternal antibodies) and active immunization of the newly hatched chicks (Eterradossi and Saif, 2013). The vaccines play an important role in the protection of the chicks against the IBD. The commercial IBDV vaccines are prepared by two methods of egg- and cell-based. It is worth to mention that the cell-based method is commonly preferred (Butt et al., 2015). The cell-based IBDV vaccines are prepared on the primary CEF culture. Given several limitations, such as heterogeneity and the risk of microbial-contamination by e.g. endogenous viruses and their various abilities to replicate (it happens at each preparation batch for the primary cell culture), several studies conducted to introduce some sensitive cell lines (Rasool and Hussain, 2006; Terasaki et al., 2008). The preparation and manipulation of the cell lines which grow in adherent cultures, is challenging and time-consuming. The introduced rare suspension cell lines, which are sensitive to the IBDV, are tumoral. In an experimental study, it was demonstrated that the ovine lymphoid suspension cell line was sensitive to the vaccinal strain of IBDV which was developed in Razi institute. This study aimed to characterize the aforementioned suspension cell line.

MATERIALS AND METHODS

Cell cultures. The primary cultures of CEFs were prepared from 9- to 11-day-old specific pathogen free (SPF) chicken embryos by standard sampling methods. The Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% and 2% fetal calf serum (FCS) were used as the growth and maintenance mediums, respectively. The solution of 0.25% Trypsin-EDTA was used for cell subculturing. Secondary culture of CEF was used in the tests. The established ovine lymphoid suspension cell line (H1) was obtained from Razi Institute, Iran, and was frozen in liquid nitrogen.

Virus. The egg-based vaccinal strain of IBDV was used after obtaining the permission of Razi Institute. The IBDV was inoculated to the healthy CEF cell culture flask. The flask was incubated at 37 °C by providing a 5% CO_2 incubator (Memmert, Germany). The culture fluid was harvested each time by three freezing and thawing cycles and clarified by centrifugation (5000 rpm for 5 min). Adaptation was achieved through serial embryo passages. The virus was named due to the characteristic cytopathogenic effects (CPEs) in CEF cells. The virus started propagating on CEF after the adaptation. The cells were harvested by centrifugation and the aliquots were frozen in the temperature of -20 °C.

Virus yield assessment. Virus yields from CEF and suspension cell line were determined in the CEF culture. The serial 10-fold dilutions of each cell cultures-virus supernatants were prepared in growth medium and 50 µl of each dilution were transferred to each of the four wells of a 96-wells microplate which contained the same volume of fresh CEF suspensions (3.0×10^5 cells/ml). The plates were incubated at 37 °C in the atmosphere of 5% CO_2 for seven days; and the virus titer was calculated using the Reed and Muench method (Reed and Muench, 1938).

Virus Adaptation to Suspension Cell Line. To adapt the IBDV on suspension cell line, the infected CEF cells (with 0.1 MOI of IBDV) were cultured in 25 cm^2 flask containing 1.5×10^6 cell/ml in the maintenance medium. The flask was incubated in the temperature of 37°C with the presence of 5% CO_2. Then the viable and nonviable cells from suspension cell line were daily counted. The flask was frozen in the temperature of -20 ℃ when the point of viability was less than 20%. Following the freezing and thawing twice, the liquid was centrifuged in 3000 rpm for 10 minutes. The unpassaged virus stock (the supernatant of co-transferred CEFs) was used for virus titration and adaptation on CEF cells. The IBDVs were serially passaged for three times.

Dilution cloning of lymphoid cell line. Based on the literature, the cells which are diluted below a certain density form the discrete colonies. The selected clones were propagated in the flask and frozen in the liquid nitrogen (Freshney, 2011). The third passage after thawing was applied to compare their sensitivity to the IBDV.

Growth Curves. Growth curves were carried out in a constant volume of 10 ml per flask with initial inoculums of 2.5×10^5 cell/ml at the beginning of the study for the secondary CEF cell culture and the selected suspension cell line. The cell count was determined daily for five days using a hemocytometer. This process was repeated three times and the doubling time was measured at each time point. The trypan blue dye exclusion test (0.4% in PBS) was used to determine the viability of the suspension cell line on the hemocytometer (Gibco, USA) (Freshney, 2011).

Sensitivity Stability of Suspension Cell Line to IBDV. Suspension cell line was passaged consecutively 20 times and at an interval between the passages, three 25 cm^2 flasks were cultured. These three groups of flasks were considered for inoculation of IBDV (0.05 MOI), the negative control, and cell passaging. While, the first flask was frozen when the viable cell count was less than 20%.

Culture of Suspension Cell Line in Cellspin. The suspension cell line containing 2×10^5 cell/ml was cultured in 500 ml of DMEM with 10% of FCS and was maintained in the special glass bottle of cellspin system with one litter capacity and incubated in a CO_2 incubator (IBS, UK). The cellspin was turned on with 70 rpm for four days and the cell count was calculated daily .

Karyotyping the Suspension Cell Line. Karyotyping was carried out according to the recommendations of Freshney in 2010 with making some modifications. Briefly, a final concentration of 0.1 µM of Colcemid was added to the 25 cm^2 flask containing the suspension cell line carrying the virus in logarithmic phase. After four hours of incubation in the temperature of 37 °C, the flask contents were triturated gently with a pipette and centrifuged at 1000 rpm for 10 minutes. Following the twenty minutes of incubation, the cells were resuspended in distilled water (by 0.076 M KCl). The same volume of cold acetic methanol was added to the mentioned content, mixed, and centrifuged at 1000 rpm for 5 minutes at the temperature of 37 °C. The pellet was resuspended by acetic methanol solution and one drop of the suspension cell line and then it was tossed into a cold slide. Finally, Giemsa staining was performed on the obtained cells. The Chromosomes of each cell were counted using a microscope. Digital photographs were taken from the chromosome spreads and sorted by size and shape using Adobe Photoshop software version 25.

Suspension Cell Species Identification. The previously published primers were used to amplify the

267 bp of cytochrome C oxidase subunit I (COI) (Cooper et al., 2007). For this purpose, 1 µl of 10 µM selected primer mixtures were used (Table 1). The primers were synthesized in CinnaGen and the DNA was extracted from all 10^6 cells by using the DNA extraction kit (CinnaGen, Iran), based on the manufacture's recommended protocol. The CEF and lamb kidney (LK) primary cell cultures were used as the negative and positive controls, respectively. To perform polymerase chain reaction (PCR), 1 µl of DNA was used as a template. The PCR buffer was consisted of 12.5 µl of master mix (CinnaGen, Iran) containing Taq DNA Polymerase, $MgCl_2$, dNTPs, 1 µl of mixed primer, 1 µl of DNA template, and up to 25 µl of molecular biology-grade water. The thermocycling conditions for the PCR entailed the temperature of 95 ºC for 5 min, 30 cycles of the temperature of 94 °C for 50 sec, 53 ºC for 50 sec, 72 ºC for 60 sec, anone cycle of 72 ºC for 10 min, and indefinite hold at 4 °C. The PCR products were visualized on 2% of agarose gel with SYBR safe (Fermentase, Spain).

Adventitious agents. The Human (H1) cell line lysate (by two times of freezing and thawing) was cultured on the marker cells including Razi bovine kidney cell line (RBK), LK, and vero, according to the WHO Technical Report Series (TRS), No. 978, and Annex III. These cells were checked in terms of any CPE and hemadsorption. In addition, the enzyme-linked immunosorbent assay test (ELISA) was used for antigen detection of bovine viral diarrhea virus (BVDV) (Bio-X Diagnostics, Belgium).

Sterility Test. For detection of any bacteria, mycoplasma, and fungi contaminant, the sterility test was applied as a result of EP 2005.

Tumorigenicity Testing. Tumorigenicity testing was conducted in animal facility of Hospital-Based Cancer Registry Institute, Imam Khomeini Medical Center. Briefly, H1 cells were centrifuged and resuspended in phosphate-buffered saline (PBS) with the concentration of 5×10^7 cell/ml. The human diploid fetal lung fibroblasts (MRC5) and HeLa cell lines were prepared with the same method as negative and positive

controls, respectively. Afterward, 0.2 ml of these suspensions were injected subcutaneously to a single site of two nude mice. The tumor growth was monitored in nude mice for at least 21 days after the injection. All mice were euthanized and the samples obtained from the injection site, tumors, lymph node, spleen, liver, and lung tissues were sent to the pathology laboratory for microscopic examination.

Statistical analysis. Data analysis performed using SPSS software version 22. Runs Test was applied to determine the ploidy stability of H1 cells during the serial passages. All the measurements were carried out in three different replicates. In all the measurements, P-value less than 0.05 was considered statistically significant.

RESULTS

Lymphoblastoid like H1 cell lines were grown in suspension culture with cells clumped in loose aggregates. These aggregates could be dissociated by gently agitating the culture or by gentle trituration with a pipette. The H1 cell line should be subcultured for 48 hours interval. For seeding cells during this process, each flask was enough for 5 to 10 new flasks with similar volume. In the other words, the concentration of 1.5×10^5 to 2×10^5 cell/ml was required for the cell passaging. Due to adaptation of IBDV on H1 cell line, on the primary CEF culture, three harvests had 6.7 log10, 6.9 log10, and 8.5 log10 TCID50/ml, respectively. A significant rise of IBDV count in third passage indicated a good adaptation; these frozen aliquots were used in the next step. After dilution cloning of H1 cells, four colonies with better morphology and growth were selected. The stock cells after the third passage were used for comparing the sensitivities after thawing (data are not shown). Finally, H1H9 clone was selected for further evaluations due to the highest sensitivity to IBDV rather than the others. The species of H1 cells were identified based on the species-specific PCR primers. Therefore, using the cattle and goat specific primers demonstrated the purity of H1 cell line, at least in contrast with two above

species (Figure 1). The growth curve of H1 suspension cell line in static (flask) and stirred was compared with primary CEF culture (Figure 2). The duration of lag phase was considerable only in the CEF. Saturation density, which means the cell density at plateau phase, for CEF and H1 in static and spinning situation, were 3.1×10^5, 1.7×10^6, and 3.2×10^6 cell/ml, respectively (Table 2). As a result, the doubling time of H1 cells was fewer than CEF, especially in cellspin culture method. There was a significant difference between doubling time of CEF and H1 according to the Friedmans analysis method ($P<0.05$). So is for terminal cell density. The H1 cell line karyotyping was performed in 12 sequential passages with one interval, in the other word, a total of 285 cells in the metaphase stage were counted by passaging fifty cells in each time. The ploidy was 20 to 35 chromosomes per cell with the modal of 32 (Figure 3). While the ploidy of the normal lamb cells is 54, in this study, the most frequent ploidy was 30 to 33. The morphology of the chromosomes was completely different in comparison to the normal cells. In this study, the chromosomes of 81.2% of H1 cell line were submetacentric and metacentric, whereas about 89% of the normal chromosomes of lamb cells are reported to be acrocentric. In sex chromosomes, single X and Y were observed; the X chromosome was metacentric unlike to the normal one which was acrocentric. Perhaps the Robertsonian translocation occurred in H1 cell line during the establishment (Arslan and Zima, 2011). Analysis of the chromosome number during the cell passaging by using runs test demonstrated that the changes in ploidy did not follow a certain principle ($P<0.05$). In the other words, chromosome number in certain passages was stable. The sensitivity and stability testing of H1 cell line determined that the 8.3 log10 TCID50/ml was the most repeated titer. According to the results, the sensitivity of H1 cell line was stable in about 20 passages. Based on the results of tumorigenicity testing, the mice which were inoculated the H1 and MRC5 diploid cell lines showed no microscopic lesions in tissue sections, while the

positive control group which were injected HeLa cell line demonstrated tumoral cells with macronucleus and hyperchromasia (Figure 4). For detection of any biological contaminant, all of the tests were considered to be negative in contrary to the positive control group.

**Table 1.** Oligonucleotide primer sequences (Cooper et al., 2007)

Size (bp)	Primer name	Nucleotide sequences
267	Ovis aris (sheep)	**Oa-F** CGA TAC ACG GGC TTA CTT CAC G **Oa-R** AAA TAC AGC TCC TAT TGA TAA T
117	Capra hircus (goat)	**Ch-F** ATA TCA ATC GGG TTT CTA GGA TTT ATT **Ch-R** AGT TGG GAT AGC GAT AAT TAT GGT AGT
102	Bos taurus (cow)	**Bt-F** GCTATTCC AAC CGG GGT AAA AGT C **Bt-R** GAAAAT AAA GCC TAG GGC TCA C

**Table 2.** Cell culture growth specificity of CEF and H1 cell lines

| Cell specify	CEF	H1 (flask)	H1 (cellspin)
Doubling time (h)	32	21	14
Average specific growth rate (μ)	0.02	0.03	0.05
Terminal cell density cell/ml	310000	1800000	3200000
Lag phase duration (h)	>48	<24	<10

DISCUSSION

The IBDV is very contagious and highly resistant to inactivation. Despite strict hygiene measures, vaccination is inevitable under high virulence and mandatory to protect chickens against infection during the first weeks after hatch (Muller et al., 2003). Growth and propagation of IBDV in chicken embryos is very challenging and time-consuming (Hitchner, 1970). Briefly, maybe the sensitivity of embryonating eggs to the IBDV was unstable due to their flocks history, high risk for contamination to adventitious agents, routs of egg inoculation is effective in final virus titer. D) Injection of the virus into 10 days old embryonating eggs results in embryo mortality from days 3-5 PI, in the other word, for virus propagation cycles needs to at least 15 day (Ingrao et al., 2013). Several alternative IBDV-sensitive cell cultures are introduced. It is confirmed that the primary CEF cell culture is more
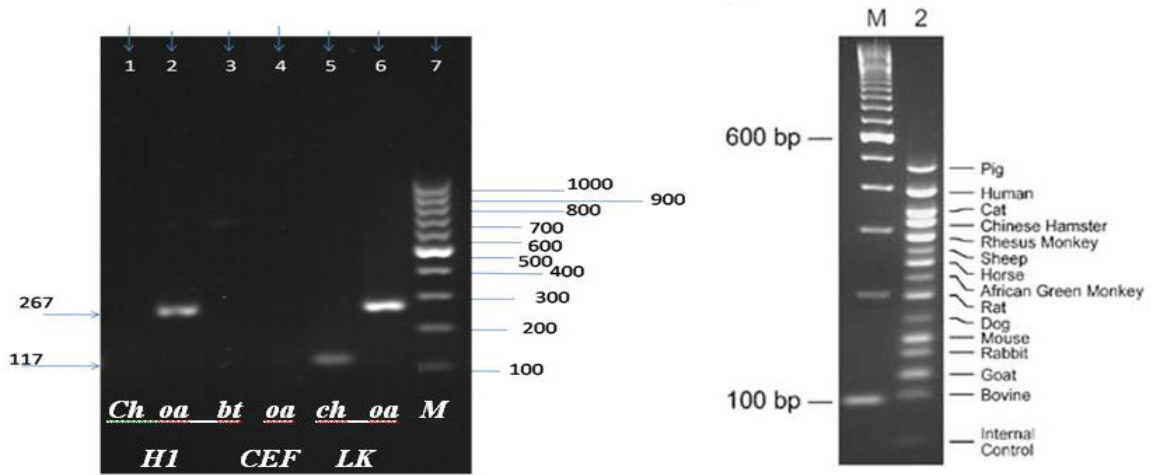
Figure 1. Detection of H1 cell line species by PCR . Left: Spectral signature bands for all species are seen descending by their size (Cooper et al., 2007). Right: H1 cell line origin assay, lines 1, 2 and 3 H1 DNA template with ch (goat), oa (sheep) and bt (bovine) primers for detecting any cross contamination; line 4: the CEF DNA template with oa (sheep) primer as the negative control; line 5 and 6: Lk cell as a positive control DNA template with ch (goat) and oa (Sheep); line 7: 100 bp Ladder (M)
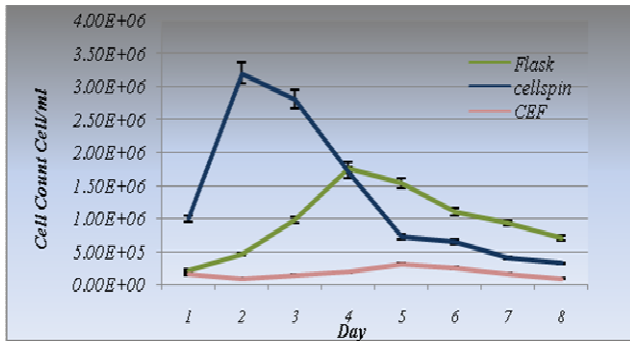
Figure 2. Growth curve comparison between H1 cell line (in static and stirred situation) and primary CEF cell culture.
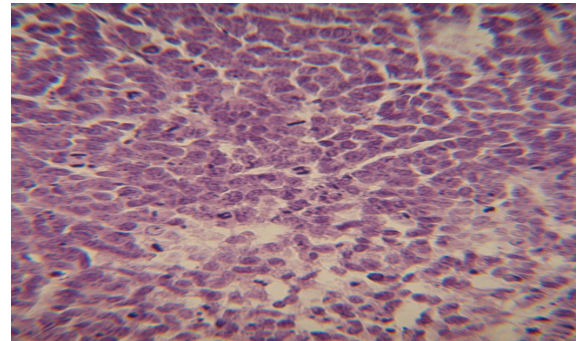
Figure 4. Phase contrast image of tumoral cells with macronucleus and hyperchromasia and pseudoglandular structutre subsequent of Hela cell line injected to the nude mice as a positive control in tumorigenicity testing, stained with **Haemotoxylin and Eosin** ,40X
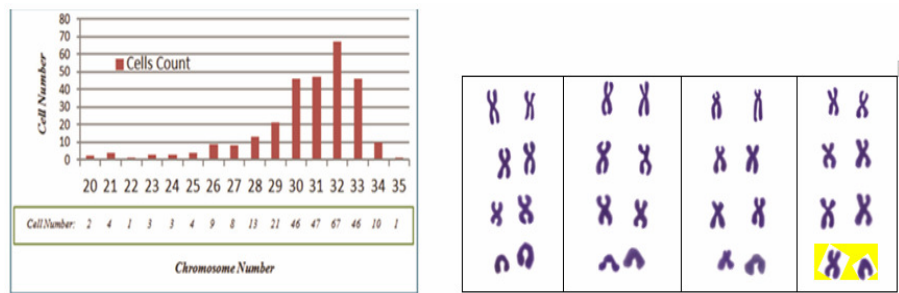
Figure 3. Karyotyping of H1 cell line, Left: Giemsa stained metaphase chromosomes, modal chromosome number is 32, Right: Distribution of chromosome number in total metaphase plate. sex chromosomes are yellow.

sensitive than the chicken embryonating eggs (Eterradossi and Saif, 2013). Various continuous cell line obtained from avian and mammalian were reported to be suitable for the IBDV propagation (Rasool and Hussain, 2006; Terasaki et al., 2008; Eterradossi and Saif, 2013). Currently, the commercial IBDV vaccines are prepared by two methods of egg- and cell-based (Muller et al., 2012). The most commonly used cell line for preparing the cell-based IBDV vaccines are CEFs (Hossain et al., 2006). Preparation of CEF culture is difficult and time-consuming as well as the other primary cell cultures (Freshney, 2011). Additionally, the CEF is an adherent culture with several handling limitations especially in large scale. In this study, a suspension cell line from ovine lymphoid cells, which is highly sensitive to IBDV (About 8.3 log10 TCID50/ml) was identified based on the WHO TRS, No. 978, and Annex III, 2013. This suspension cell line is easy handling due to the suspension form. Given the results, the H1 cell line was sensitive and suitable for using as a vaccinal strain of IBDV in Razi institute. After passing the safety and efficacy tests, the IBDV vaccines which were prepared based on the H1 cell line, might be introduced as cost-effective vaccines and comparable to the primary CEF cell-based vaccines.

Ethics

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

Conflict of Interest

The authors declare that they have no conflict of interest.

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