A cell line resistant to avian leukosis virus subgroup B infection

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ABSTRACT The expression of env proteins that bind to viral cell receptors on avian leukosis virus (ALV)-susceptible cells can block ALV infection. In this study, we constructed a cell line (DF-1/B) by expressing the ALV-B env protein in DF-1 cells. PCR, immune fluorescence assay, Western blot, and immune electron microscopy results showed that the env gene can be stably expressed in DF-1 cells and the env protein could be detected on the DF-1 cell membrane. An antiviral experiment concluded that the DF-1/B cell line could be resistant to $1 \times 10^4$ TCID$_{50}$ ALV-B virus infection, but had no inhibitory effect on other subgroup ALV. This means that the DF-1/B cell line is specifically resistant to ALV-B and can be used as a tool for ALV-B diagnosis.

Key words: ALV-B, env protein, cell line

INTRODUCTION

Avian leukosis virus (ALV) belongs to the α retrovirus genus and can cause a variety of poultry diseases. Clinically, the main manifestations of ALV infection are immunosuppression, growth retardation, tumors in multiple organ tissues, and other characteristics (Hang et al., 2013). Since the discovery of the virus, it has caused enormous economic losses to the poultry industry. ALV are divided into 11 subgroups (A to K) (Payne et al., 1992; Wang et al., 2012) based on their host range, viral envelope glycoprotein antigenic structure, and receptor interference, of which subgroups A–E, J, and K infect chickens. Subgroups A, B, and J are the main pathogenic exogenous viruses and are the 3 most prevalent exogenous virus subgroups that can trigger tumors (Zhao et al., 2010; Wang et al., 2011), posing a significant threat to the poultry industry. Subgroups C and D also have pathogenic viruses, but rare in the flock. ALV-K is a novel AVL, and more and more ALV-K strains have been isolated in recent years (Wang et al., 2012; Cui et al., 2014; Li et al., 2016; Shao et al., 2017); subgroup E is an endogenous virus. In the mid-1980s, an international breeder company was successful in eradicating subgroup A and B ALV infection in chickens, but because of ALV purification, it could not be fully launched in China at that time, so the Chinese flocks, especially Chinese native chickens, still carry ALV-A, ALV-B, and ALV-J infection (Zhao et al., 2010). Over the past decade, ALV-B co-infection with ALV-A, a recombinant virus of ALV-B and ALV-A, and a recombinant virus of ALV-B and ALV-J have been isolated (Lupiani et al., 2006; Li et al., 2013; Wang et al., 2017). ALV-B is still a threat to the Chinese poultry industry and is part of a list of diseases necessary to be prevented and eradicated.

So far, there have been no effective vaccines and drugs specifically available for the prevention of ALV. The only option left for the prevention of ALV infection is the elimination of infected chickens. At present, the methods employed for the detection of exogenous ALV mainly include virus isolation, ELISA, PCR, and immune fluorescence assay (IFA) (Spencer and Gilka, 1982; Payne et al., 1993), and also some new detection methods have been recently established, such as RT-PCR, QC-RT-PCR, LAMP, and colloidal gold test strips (Kim and Brown, 2004; Zhang et al., 2010; He et al, 2013; Dai et al., 2015).

According to the ALV env protein, a glycoprotein encoded by the virus envelope (env) gene can be recognized by the specific viral receptors on the host cell membrane to block the same subgroup ALV infection of susceptible cells (Hunt et al., 1999; Holmen and Federspiel, 2000). A genetically engineered cell line (DF-1/J) resistant to ALV-J infection was developed and applied to screen large numbers of ALV-J field samples (Hunt et al., 1999). A cell line DF-1/A and a cell line DF-1/K resistant to ALV-A and novel
subgroup ALV-K infection, respectively, have also been constructed and applied (Mingzhang et al., 2017). In order to establish a specific method for the detection of ALV-B, the env protein of ALV-B was required to be stably expressed on DF-1 cells to obtain an immortal cell line that can be exclusively used to resist ALV-B infection. This cell line would be further applied to evaluate clinical plasma samples and investigate the prevalence of ALV-B infection in Chinese native chickens. This is the first report of a cell line specifically resistant to infection by ALV-B.

**MATERIALS AND METHODS**

**Viruses and Cell Lines**

ALV-B (CD08) and ALV-J (CHN06) (GenBank accession number HQ900844) were isolated and maintained in our laboratory (Dai et al., 2015). The titers of CD08 and CHN06 strains were determined by ELISA (IDEXX, Inc., Westbrook, MA) and were presented as TCID_{50} mL^{-1} calculated using the Reed–Muench method. A DF-1 cell was grown in Dulbecco’s modified Eagle medium (DMEM; GIBCO, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, New Zealand) and maintained in DMEM supplemented with 1% FBS at 37°C with 5% CO2.

**Plasmid Construction**

The ALV-B env gene was amplified by PCR using the gene-specific primers (Table 1) and subcloned into the pMD-18T vector (TaKaRa, Dalian, China). It was then cloned into the eukaryotic expression vector pcDNA3.1 using the BamHI and NotI sites. The pcDNA-env-B vector was sequenced and found to have the predicted nucleotide sequence. The pcDNA-env-B-EGFP vector, which contains an EGFP tag, was constructed by the PCR amplification of EGFP fragment and ligated with the env gene, and the fusion fragment was cloned into the pcDNA3.1 vector.

**Screening Cells With Zeocin**

The DF-1 cells were transfected with the pcDNA-env-B plasmid, pcDNA-env-B-EGFP plasmid, and pcDNA3.1/Zeo(+) plasmid. After 48 h, the transfected DF-1 cells grew to monolayer, and cells in one of the 6-well cell culture plates were digested with 0.25% trypsin (GIBCO). Then, the cells with a medium containing DMEM, 15% FBS, and 200 µg/mL zeocin were seeded into 500 µL/well 24-well tissue culture plates. The transfected DF-1 cells were selected for resistance to zeocin. Thereafter, cells were treated with a 500 µL/well medium containing DMEM + 15% FBS + 200 µg/mL zeocin, and then this medium was replaced every 3 D. The zeocin-resistant cells were passaged through 60 generations and then frozen. After 3 mo, these cells were refreshed and cultured in a medium free of zeocin.

**PCR Assay**

To demonstrate whether the DF-1/B cell line was constructed successfully, routine PCR tests were carried out with genomic DNA extracted from the ALV-B-resistant cell line, designated as DF-1/B cells. The DF-1 cells served as a negative control. The specific primers were as described in Table 1. Total cellular RNA was extracted from DF-1/B cells and DF-1 cells using the RNAfast200 kit (Fastagen, Shanghai, China), followed by cDNA synthesis using the RevertAid First strand cDNA synthesis kit (Fermentas, Burlington, Canada) according to the manufacturer’s instructions. The cDNA was then used for routine PCR and real-time PCR amplification. Real-time reverse transcription (RT)-PCR was done with primers designed for the envelope gene and gene-specific primers (Table 2) synthesized by TaKaRa Company (Dalian, China). DNA sequences were determined by Invitrogen (Shanghai, China). For all reactions, PCR amplification and DNA sequencing were carried out at least twice independently to avoid PCR errors. Real-time PCR was performed on an ABI 7500 Real-time PCR System (Applied Bio systems) using Premix ExTaq (Probe qPCR) reagents (TaKaRa, Dalian, China) according to the manufacturer’s specifications. Fluorescent signals were recorded during the elongation step. The β-actin gene

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**Table 1.** Primers used for amplifying the ALV-B env gene and EGFP fragment in this study.

| Primer | Sequence (5′–3′) | Product size (bp) |
|--------|------------------|-------------------|
| B-env-F | CGGGGTACCCAGCCTATGGAAGCGCTATAAGATGAGGCGA | 1845 |
| B-env-R | AAGGAAAAAGCGGCCGCTATACTGCTCTTTCCGGGCTTIA | |
| EGFP-F | TTCCGGCGCCCTGTTGAGCCTAAGGGCAGGA | 720 |
| EGFP-R | GCTCTAGATTACCTGTACAGCTCGTCCA | |

1F and R represent upstream primer and downstream primer, respectively. Italic for restriction sites, the underlined represents the Kozak sequence.

**Table 2.** Primers for real-time reverse transcription for amplifying the ALV-B env gene.

| Primer | Sequence (5′–3′) | Product size (bp) |
|--------|------------------|-------------------|
| q-B-env-F | ATAAGATCGGCGTGGACAAC | 1845 |
| q-B-env-R | TGGAATTTCCTGCATTCCTC | |

1F and R represent upstream primer and downstream primer, respectively.
served as a reference gene. The relative expression level of the env gene was normalized by β-actin. Finally, real-time quantitative PCR analysis was carried out using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

**Indirect IFA**

DF-1/B and DF-1 cells were washed with pre-cold PBS once, fixed with cold paraformaldehyde for 20 min at −20°C, then washed with PBS 3 times and allowed to air-dry. The cells were then incubated with monoclonal antibodies for ALV-B (provided by Dr Ji-aqian Cai, Shandong Agricultural University) at 37°C for 1 h, washed 3 times with PBS, and further incubated with goat antimouse IgG conjugated with FITC (Sigma, Mannheim, Germany) at 37°C for 1 h. After 3 washes with PBS, the cells were observed using fluorescence microscopy (LEICA, Germany).

**Western Blot Analysis**

DF-1/B cells and DF-1 cells were once washed in 100-mm dishes with cold PBS and harvested by a cell-scraping device, then homogenized with NP-40 lysis buffer containing 1 × protease inhibitor cocktail (Roche) and incubated on ice for 30 min. Lysates were centrifuged at 12,000 × g for 5 min at 4°C. The supernatants were analyzed for total protein content using the BCA protein assay kit (Fermentas, Life Technologies). Total protein (20 μg) was resolved by 12% SDS-PAGE and transferred onto nitrocellulose membranes (Whatman, Maidstone, UK). The membranes were blocked with 5% (w/v) skimmed milk for 1 h at 37°C, and then incubated overnight at 4°C with monoclonal antibodies for ALV-B and β-actin (Santa Cruz, sc-1616-R). β-Actin served as a reference. After 3 washes with PBS Tween20 (PBST) buffer, the membranes were incubated at 37°C for
Cell Line Screening

To establish single zeocin-resistant DF-1 clones, single cells from DF-1 cells transfected with the pcDNA-env-B plasmid and pcDNA-env-B-EGFP plasmid were

1 h with IRDye 800-conjugated anti-mouse IgG secondary antibody (1:10,000; Rockland Immunochemicals, Limerick, PA) diluted in PBS. Membranes were washed 3 times with PBST, then visualized and analyzed with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

**Immune Electron Microscopy**

The DF-1 and DF-1/B cells were repaired and sliced with a slice thickness of 75 nm according to the preparation method of ordinary electron microscopy samples. The ultrathin slices were then transferred to a nickel mesh using a Fang Hua film (to ensure slice continuity) and then incubated with goat anti-mouse EGFP antibodies (Sigma) and monoclonal antibodies for ALV-B for 1 h at 37°C. After washing the sections with PBSA (PBS with 5% BSA) solution 6 times, they were incubated with 10 nm colloidal gold-labeled goat anti-mouse IgG (Sigma) for 1 h at 37°C. The sections were washed with ultrapure water and dried at room temperature. Finally, the samples were examined under a JEM-2010HR transmission electron microscope (JEPL, Japan).

**Antiviral Experiment**

ALV-B (CD08) and ALV-J (CHN06) were diluted from $10^5$ TCID$_{50}$ to $10^1$ TCID$_{50}$ for 5 gradients and then 100 µL per well of those cells were seeded in a 24-well plate containing 1 mL DF-1/B cells at $1.7 \times 10^5$ cells/well. Three wells on each plate served as negative controls. Each dilution of virus was performed in triplicate. After 2 h of incubation, supernatants were removed and a maintenance medium containing DMEM with 1% FBS was added, and the plates were incubated at 37°C and 5% CO$_2$ for another 6 D. The supernatant fluid was then harvested for ALV p27 antigen using the ELISA detection ALV-p27 Ag Test kit (IDEXX, Inc., Westbrook, MA). A mock-infected DF-1 cell group was established in parallel as a control.

**RESULTS**

**Cell Line Screening**

To establish single zeocin-resistant DF-1 clones, single cells from DF-1 cells transfected with the pcDNA-env-B plasmid and pcDNA-env-B-EGFP plasmid were
Figure 4. Detection of ALV-B env protein in DF-1/B cells by IFA. (A) No green fluorescence was observed in DF-1 cells, which served as negative controls. (B) Green fluorescence was observed in DF-1/B cells. Magnification is × 100 for (A) and (B).

Figure 5. Western blot analysis of env protein expression in DF-1/B cells. (DF-1/B): DF-1/B cell lysate; (DF-1): DF-1 cell lysate was used as a negative control. ALV-B env protein in the cell lysates were detected with monoclonal antibodies for ALV-B at a dilution of 1:200. β-actin cell lysates was also detected using actin antibodies for control for equal loading. A IRDye 800-conjugated anti-mouse IgG (1:10,000; Rockland Immunochemicals, Limerick, PA) diluted in PBS was used as the secondary antibody.

picked, respectively. The picked single cells became actively proliferated (Figures 1A and 1B), and this single-cell colony appeared to increase in size over the next 6 to 10 D (Figures 1B and 1C). The cells grew to near completion by approximately 21 D in culture (Figure 1D). For 3 to 4 wk, the cells were digested with 0.25% trypsin (GIBCO), and then plated in 6-well cell culture plates at 37°C and 5% CO2 until they grew to monolayer. The zeocin-resistant cells were cultured in the medium containing zeocin, passaged continuously for 60 generations, and then frozen. After 3 mo, these cells were refreshed and cultured in a medium free of zeocin, and the env gene or env protein in the transfected DF-1 cell was detected.

PCR Detection of the env Gene in DF-1/B Cells
The ALV-B env gene in the first generation of DF-1/B cells was detected by the PCR method (Figure 2A) and the ALV-B env gene was still stable in the genome after 20 to 60 passages (Figure 2B). The ALV-B env fragment of 1845 bp in length was amplified from all DF-1/B DNA samples. This PCR product was purified, and sequencing analysis verified that the fragment corresponds to ALV-B env (Figure 2B). The results showed that the ALV-B env fragment was still able to inherit stably in DF-1/B cells during passages.

Analysis of env Gene Transcription
The viral envelope gene transcription level in DF-1/B cells was tested by conventional PCR and real-time RT-PCR, and the results are shown in Figure 3A. The ALV-B env gene fragment of 1,845 bp in length was amplified from RNA extracted from DF-1/B cells. Compared to the DF-1 cell negative control, the ALV-B env gene mRNA was highly expressed in DF-1/B cells (Figure 3B).

Indirect IFA Testing of ALV-B env Gene Expression
In the presence of env protein expression in the DF-1/B cells, the expression of env genes can be detected by specific monoclonal antibodies for ALV-B via IFA (Figure 4). The green fluorescence signal was bright in DF-1/B cells, but no green fluorescence in the cytoplasm could be observed in DF-1 cells. This indicates that the exogenous ALV-B env gene is successfully expressed in DF-1/B cells.

Western Blot Analysis of env Protein Expression in DF-1/B Cells
A Western blot experiment was performed to confirm the envelope protein expression in DF-1/B cells. The results of Western blot along with the lysate obtained from the DF-1/B cells transfected with the
Antiviral Experiment

The antiviral effect of the DF-1 and DF-1/B cells was determined using representative strains of ALV-B (CD08) and ALV-J (CHN06). Both ALV-B and ALV-J are capable of infecting and replicating in DF-1 cells. In contrast, only ALV-J was able to infect DF-1/B cells, whereas ALV-B was effectively blocked from infecting DF-1/B cells. As shown in Figures 6A and 6B, DF-1/B cells inhibited the replication of ALV-B but not of ALV-J based on ELISA measurements of the viral p27 protein. To further validate the clinical utility of DF-1/B, ALV of clinically isolated unidentified subgroups were inoculated in DF-1 and DF-1/B cells. One of them was significantly blocked from infecting DF-1/B cells, but can infect DF-1 cells (Figure 6C); the virus was identified as ALV-B by PCR. The antivirus assay showed that DF-1/B cells can have resistance to infection at a viral dose of $1 \times 10^4$ TCID$_{50}$ ALV-B, and at lower doses, infection was completely blocked. When the ALV-B infection dose reached $1 \times 10^5$ TCID$_{50}$, the ability of ALV-B to infect DF-1/B cells was still strongly inhibited (Figure 6A).

**Immune Electron Microscopy**

A colloidal gold immune electron microscopy examination revealed that the ALV-B $env$ fusion protein is found in the DF-1/B cell membrane (Figure 7). The immune electron microscopy ALV-B fusion protein was detected with goat antimouse EGFP antibodies and monoclonal antibodies for ALV-B individually. As the secondary antibody, 10 nm of colloidal gold-labeled goat antimouse IgG was used. As a negative control, TBS (pH 7.4) was used in place of the primary antibody of control group. After uranyl acetate and lead citrate staining, images of the samples were obtained with a JEM-2000EX transmission electron microscope. Immunogold particles were observed in the cell membrane. In the controls, there were no immunogold particles in the cell membrane area.

**DISCUSSION**

ALV is a retrovirus, which can be divided into 11 subgroups (subgroups A to K) according to their host range, patterns of receptor interference, and neutralization reaction (Payne et al., 1992; Wang et al., 2012). They have spread all over the world and caused enormous economic losses in the international poultry industry. In the mid-1980s, an international large-scale breeder company was successful in eradicating subgroup A and B ALV infection in chickens, but because of ALV purification, their drug could not be fully launched in China, so the Chinese chicken flocks, especially Chinese native chickens, still carry ALV-A (Zhang et al., 2010) and ALV-B (Zhao et al., 2010) infection. In recent years, ALV-B has been detected not only in chickens, but also in wild birds (Li et al., 2013) and has demonstrated the tendency of a recombinant virus (Lupiani et al., 2006; Wang et al., 2017). This indicates that the host range of ALV-B is changing. Traditional detection methods such as ELISA, PCR, real-time PCR, QC-RT-PCR, IFA, LAMP (Spencer and Gilka, 1982; Payne et al., 1993; Kim and Brown, 2004; Dai et al., 2015), and other methods have some
Figure 7. Electron microscopy image of DF-1/B cells that were pre-incubated with TBS (pH 7.4) and subsequently incubated with 10 nm colloidal gold-labeled goat anti-mouse IgG. These samples served as negative controls. (A) TEM × 25,000, (B) TEM × 100,000. (C) Electron microscopy image of DF-1/B cells that were pre-incubated with goat anti-mouse EGFP antibodies and monoclonal antibodies for ALV-B individually and subsequently incubated with 10 nm colloidal gold-labeled goat anti-mouse IgG. TEM × 250,000. The arrows point to gold particles.

limitations. The DF-1/B cell line described in this report has been genetically engineered to be selectively resistant to ALV-B through receptor interference. Whether it is the co-infection or single infection of the clinical sample, ALV-B can be definitively identified by inoculating the DF-1/B cell line and inoculating DF-1 as a control. The established immortal DF-1/B cell line, specifically resistant to ALV-B infection, not only enriches the diagnostic toolbox that distinguishes ALV subgroups, but also provides a rapid and reliable method to screen large numbers of samples.

In order to generate this cell line, which is genetically resistant to ALV-B, the env gene of ALV-B isolates, CD08, was cloned and expressed in the DF-1 cell line. The expressed env protein occupies the viral cell receptor binding sites and selectively interferes with the ALV-B infection cell line. To demonstrate whether the DF-1/B cell line was constructed successfully, PCR, IFA, Western blot, immune electron microscopy, and antivirus assay were used to detect the expression of env and the resistance to ALV-B infection. The results showed that DF-1/B cells could stably express the ALV-B env gene, and the env fusion protein is localized in the cell membrane area of DF-1/B cells. The antivirus assay showed that DF-1/B cells are resistant to $1 \times 10^4$ TCID$_{50}$ ALV-B infection, but not to ALV-J. To further verify this, we also tested with clinical samples. The result showed that ALV-B in field samples can be definitively identified. This means that the cell line is specifically resistant to ALV-B and can be used as a tool for ALV-B diagnosis.

Thus, the DF-1/B cell line has enriched the identification toolbox for identifying different subgroup ALV, provided a tool for identifying the molecular mechanism of ALV-B env protein interaction with host cells and the isolation and identification of viral
cell surface receptors, provided a theoretical basis for the cultivation of disease-resistant chickens, and has also overcome the deficiencies of the current method of isolating and purifying ALV subgroups of mixed infection.

SUPPLEMENTARY DATA

Supplementary data are available at Poultry Science online.

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