Isolation, identification, and gp85 characterization of a subgroup A avian leukemia virus from a contaminated live Newcastle Disease virus vaccine, first report in China

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ABSTRACT To identify if any exogenous avian leukemia virus (ALV) exists in a live vaccine of poultry according to the directives of the Ministry of Agriculture of the People’s Republic of China, a live vaccine strain of the Newcastle disease virus (NDV) was neutralized using an anti-NDV antibody, and was subsequently used to inoculate DF-1 cells to investigate the presence of exogenous ALV. The DF-1 cells were cultured for 21 d and subsequently screened using an ELISA for the p27 antigen of the ALV. An exogenous ALV, designated ALV-NDVP4, was identified. The nucleotide sequence of the gp85 gene of the ALV-NDVP4 was compared with those of the various subgroups of the ALV. The amino acid sequence identities for the predicted gp85 of the ALV-NDVP4 and those of the ALV reference strains ranged from 88.2 to 99.5% for the 12 of the subgroup A strains of ALV (ALV-A) and from 82.7 to 87.4% for the B, C, D, and E subgroup strains. The amino acid sequence identities for the gp85 of the ALV-NDVP4 and those of the subgroup J reference strains ranged from 48.7 to 49.9%. The ALV-NDVP4 shared the highest level of homology with the SDAU09C3 strain of ALV-A, which was isolated in China, suggesting a common origin. This is the first report of ALV-A contamination in a live vaccine for poultry in China. Our findings highlight the need for improved monitoring methods for poultry vaccine production.

Key words: subgroup A of avian leukemia virus, gp85 gene, Newcastle disease virus, live vaccine, contamination

INTRODUCTION

Avian leukemia virus (ALV) is a common avian retrovirus associated with neoplastic diseases. Exogenous ALV in chickens are classified into A, B, C, D, and J subgroups based on their host range, cross-neutralization, and viral interference, they can induce different types of neoplastic diseases in chickens (Fadly and Nair, 2008). The ALV-J and ALV-A subgroups have been the most commonly identified ALV in chickens in China during the past decade (Cui et al., 2003; Xu et al., 2004; Li et al., 2009; Lai et al., 2011).

The ALV subgroups A and B (ALV-A and ALV-B) infect mainly egg-type chickens, usually leading to typical symptoms of lymphoid leukemia and the most common B-cell lymphoma of chickens (Saif et al., 2008). Lymphoid leukemia induced by ALV-A and ALV-B is one of the most common tumor diseases in chickens (Fadly et al., 1989). Although low mortality occurs in chickens with lymphoid leukemia, the reductions in weight gain and egg production can cause substantial economic losses (Gavora, 1987). Although eradication measures have been taken to eliminate ALV-A and -B in certain areas, lymphoid leukemia in egg-producing chickens remains widespread on a global scale (Gavora, 1986; Payne and Fadly, 1997). A previous study confirmed that ALV-A also infects meat-producing chickens (Li et al., 2009).

Previous studies have reported that certain live vaccine strains for poultry were contaminated with ALV, including Marek’s disease virus (MDV) vaccine that was found to be contaminated with ALV-A (Hussain et al., 2003; Zavala and Cheng, 2006; Barbosa et al., 2008). Large breeder farms have suffered substantial economic losses after using such contaminated vaccines.

The gp85 is the most variable of the structural proteins in the genome of ALV, which is associated with virus neutralization (Pandiri et al., 2010). As the major viral envelope protein, gp85 has exhibited high diversity since it was reported (Venugopal et al., 1998; Silva et al., 2000; Gao et al., 2010). The group-specific antigen p27, which is shared by both exogenous and endogenous ALV, is very conservative. Although the

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commercial ELISA test kit aimed to antigens is dependent on antigen p27, it is also usually the first choice for the identification of ALV in the poultry industry and a high positive rate of p27 is usually a good index to estimate the risk of ALV infection (Himly et al., 1998; Chesters et al., 2002; Zhao et al., 2012).

In our current study, an ALV-A strain was isolated from a live Newcastle disease virus (NDV) vaccine. We also determined the phylogenetic relationships between the contaminating ALV-A and various ALV reference strains, based on the gp85 gene sequence. This is the first report of an ALV-A strain isolated from a live vaccine for poultry produced in China.

**MATERIALS AND METHODS**

**Analysis of the Live NDV Vaccine**

To control the spread and dissemination of exogenous viruses, the Ministry of Agriculture of the People’s Republic of China requests that all vaccines to be tested for exogenous viruses before marketing. To identify any exogenous ALV existed in a Newcastle Disease Mild Vaccine (Clone30 Strain, live vaccine), ten 6-wk-old specific-pathogen-free (SPF) chickens were inoculated with the NDV vaccine and 10 SPF chickens were fed in other shielded cases as blank control; serum samples were collected at 6 wk postinoculation. The serum samples were analyzed for the presence of anti-ALV antibodies using the Avian Leukosis Virus Antibody Test Kit (IDEXX Laboratories, Westbrook, ME), and the presence of anti-ALV-J antibodies using the Avian Leukosis Virus Antibody Test Kit for Subgroup J (IDEXX Laboratories), according to the manufacturer’s instructions. All serum samples were analyzed in duplicate.

**Isolation and Identification of the Contaminated ALV**

The avian fibroblast cell line, DF-1 (American Type Culture Collection, Manassas, VA), was used to propagate the contaminating ALV-A strain. The DF-1 cells were cultured in Gibco Dulbecco Modified Eagle medium (Life Technologies, Carlsbad, CA) containing 100 U of penicillin/mL, 100 µg of streptomycin/mL, and 10% of fetal bovine serum at 37°C in 5% CO₂. After neutralization using anti-NDV antibodies (China Institute of Veterinary Drugs Control, Beijing, China), the live NDV vaccine was passed through a 0.22-µm filter (EMD Millipore, Billerica, MA) and used to inoculate the DF-1 cells. The cells were cultured at 37°C for 2 h, and the supernatant was replaced with fresh medium containing 1% fetal bovine serum (Qin et al., 2013). The cells were incubated for an additional 7 d, and blind passages were performed for 2 generations over a total period of 21 d.

A 100-µL aliquot of cell-culture supernatant was analyzed for the presence of the p27 antigen of ALV using the Avian Leukosis Virus Antigen Test Kit (IDEXX Laboratories). The ALV-positive supernatant samples were stored at −80°C. The ALV-positive cells were fixed in an acetone-ethanol (3:2) bath for 5 min, and analyzed using an immunofluorescence assay (IFA) with the JE9 anti-ALV-J monoclonal antibody (Qin et al., 2001) and an ALV-A/B antiserum (Li et al., 2013), as previously described. Primary antibody reactivity was detected using a fluorescein isothiocyanate-labeled anti-mouse IgG antibody (Sigma-Aldrich, Saint Louis, MO). A drop of 50% glycerol was added to the coverslip, and the cells were observed using a fluorescence microscope.

**Amplification and Sequence Analysis of the gp85 Gene of the ALV Isolate**

The primers used for the amplification of the gp85 cDNA of the ALV isolate were designed, as previously described (Zhang et al., 2010). The viral RNA was extracted from the DF-1 cells using the Viral RNA Kit (Omega Bio-Tek, Doraville, CA), and the cDNA was generated from the viral RNA using reverse transcription (RT) and PCR. The PCR products were separated by electrophoresis on a 1% agarose gel. The gp85 cDNA bands were purified from the gel using the EZNA Gel Extraction Kit (Omega), and ligated into PMD-18T plasmid (Takara Bio, Shiga, Japan). The vector was used to transform a competent DH5α Escherichia coli. The sequence of the gp85 cDNA was determined by a commercial service (Invitrogen, Shanghai, China). At least 2 independent RT-PCR experiments were performed for each sample to avoid PCR-induced errors.

The gp85 sequence of the ALV isolate was compared with those of reference strains of the various ALV subgroups (Table 1). The nucleotide sequence alignment was performed using with the Clustal application in the MegAlign program of the DNAStar, version 7.01, software suite (DNAStar, Madison, WI). The Clustal W computational tool was used for the sequence alignments in the phylogenetic analysis.

**Identification of Contamination Source and Investigation of the ALV Infection in 10 SPF Chicken Flocks in China**

To investigate the possible source of the ALV contamination in the NDV live vaccine, 6-wk-old SPF chickens were inoculated with the original seed strain of the NDV vaccine or the commercial vaccine as conducted in 2.1. Serum samples were collected at 6 to 7 wk postinoculation and analyzed for the presence of anti-ALV antibodies using the Avian Leukosis Virus Antibody Test Kit (IDEXX Laboratories). Each sample was tested in duplicate to ensure the accuracy of the results.

To estimate the risk of ALV infection in 10 SPF chicken flocks in China (Table 2), serum samples from 100 chickens from each of the SPF chicken farms were
collected and analyzed for the presence of anti-ALV and anti-ALV-J antibodies, as described above. One hundred SPF chicken embryos from each of the farms were also collected and tested for the presence of the p27 antigen of ALV, as described above. The serum and embryo samples were tested in duplicate to ensure the accuracy of results.

**RESULTS**

**Analysis of the Live NDV Vaccine**

Ten 6-wk-old SPF chickens were inoculated with the NDV vaccine, the serum samples were collected at 6 wk postinoculation and submitted to ELISA test for antibody against ALV. The serum was detected as positive to ALV-Ab antibody test, indicating that the NDV vaccine was contaminated with an exogenous ALV strain.

**Isolation and Identification of the Contaminating ALV**

The NDV vaccine samples were inoculated into a DF-1 cell and the cell was incubated for an additional 7 d; blind passages were performed for 2 generations over a total period of 21 d. The supernatant of cell culture was then assayed for ALV p27 antigen, and results indicated it was positive for p27 antigen, indicating the presence of an exogenous ALV in the NDV vaccine. Further evidence of ALV in the samples was demonstrated by the positive result in IFA test on infected DF-1 cells using ALV-A-specific mono-specific serum, whereas ALV-J was negative in the IFA test. These data indicated that an exogenous ALV designated ALV-NDVP4 was isolated and identified from this contaminated NDV live vaccine.

**Sequence Analysis of gp85 Gene of the ALV Isolate**

The amino-acid sequence of the predicted gp85 polypeptide of the ALV-NDVP4 strain was compared with those of the reference strains of the various ALV subgroups. The shared amino-acid-sequence identities for the predicted gp85 of ALV-NDVP4 and that of the ALV reference strains ranged from 87.9 to 99.1% for the 12 subgroup A strains, whereas homology with subgroups B, C, D, and E was 76.6 to 78.9, 83.7, 82.5, and 83.0 to 83.8%, respectively. The homology between the ALV-NDVP4 and the subgroup-J strains ranged from 37.8 to 39.7%.

The phylogenetic analysis of the gp85 sequence of the ALV-NDVP4 confirmed that the ALV-NDVP4 isolate was a subgroup A strain (Figure 1). The comparison of the gp85 sequence of the ALV-NDVP4 and the various ALV-A reference strains showed that a 4-amino acid deletion was found at position 202–205 in the ALV-NDVP4, MAV-1, and SDAU09C3 of the ALV-A, compared with other ALV-A strains (Figure 2).

**Source of ALV Contamination and Investigation of the ALV Infection in 10 SPF Chicken Flocks**

The serum samples from the SPF chickens inoculated with the seed strain of the NDV vaccine tested negative for anti-ALV and anti-ALV-J antibodies at 6 wk postinoculation, whereas those from SPF chickens inoculated with the NDV commercial vaccine tested positive for anti-ALV antibodies, indicating that the SPF chicken embryos were most likely the source of the ALV contamination in the live vaccine. A total of 1,004 serum samples from 10 SPF chicken farms tested negative for...
anti-ALV and anti-ALV-J antibodies, and 4 eggs tested positive for the p27 antigen of ALV out of 1,004 eggs (Table 2).

**DISCUSSION**

In the past 10 yr, avian retrovirus contaminants in live vaccines for poultry have increasingly gained the attention of the research community. The reticuloendotheliosis virus (REV) has been identified as a contaminant of fowl pox virus (FPV) vaccines in China and other countries (Awad et al., 2010; Wang et al., 2010). Considerable attention has also been given to investigating ALV contaminants in live vaccines for poultry, and multiple studies have identified ALV contamination in live MDV vaccines (Hussain et al., 2003; Zavala and Cheng, 2006; Barbosa et al., 2008). Breeder farms that used contaminated vaccines experienced overall reductions in the growth of their chickens. The ALV contamination in live vaccine make it very difficult to remove the ALV completely from primary breeder flocks even after a prolonged effort, although some effective eradication measures have been taken to eliminate ALV.

Virus isolation, ELISA, and IFA have been used for the detection of retrovirus contaminants in live vaccines for poultry. In 1997, the use of IFA-, PCR-, and ELISA-based methods were compared for the detection of REV in a live FPV vaccine (Fadly and Witter, 1997). Whereas the cytopathic effect of the FPV, NDV, and MDV in CEF or DF-1 cells after inoculation thus inhibits or reduces the replication of ALV, it can significantly influence the result of exogenous virus detection.

### Table 2. Detection of avian leukosis virus (ALV) antibody in serum or p27 antigen in eggs from 10 specific-pathogen-free (SPF) chicken flocks of China (random collection)

<table>
<thead>
<tr>
<th>Farm no.</th>
<th>Age (d)</th>
<th>Antibody positive to ALV-Ab/total (%)</th>
<th>Antibody positive to ALV-J/total (%)</th>
<th>Positive to p27 antigen in eggs/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPF01</td>
<td>260</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
</tr>
<tr>
<td>SPF02</td>
<td>160</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
</tr>
<tr>
<td>SPF03</td>
<td>138</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
</tr>
<tr>
<td>SPF04</td>
<td>160</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
</tr>
<tr>
<td>SPF05</td>
<td>230</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
<td>1/100 (1.00)</td>
</tr>
<tr>
<td>SPF06</td>
<td>200</td>
<td>0/99 (0.00)</td>
<td>0/99 (0.00)</td>
<td>0/99 (0.00)</td>
</tr>
<tr>
<td>SPF07</td>
<td>340</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
</tr>
<tr>
<td>SPF08</td>
<td>410</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
</tr>
<tr>
<td>SPF09</td>
<td>406</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
<td>0/100 (0.00)</td>
</tr>
<tr>
<td>SPF10</td>
<td>220</td>
<td>0/105 (0.00)</td>
<td>0/105 (0.00)</td>
<td>3/105 (2.86)</td>
</tr>
<tr>
<td>Total</td>
<td>0/1,004 (0.00)</td>
<td>0/1,004 (0.00)</td>
<td>4/1,004 (0.398)</td>
<td>4/1,004 (0.398)</td>
</tr>
</tbody>
</table>

**Figure 1.** Phylogenetic relationships of the isolate avian leukosis virus (ALV)-A and other strains based on the gp85 gene. Color version available in the online PDF.
Therefore, the vaccination of SPF chickens is the most reliable method of identifying ALV contaminants in live vaccines. In our current study, we initially identified the ALV contaminant using these standard methods. We isolated the exogenous ALV, ALV-NDVP4, by inoculating DF-1 cells with the NDV vaccine, and the IFA results confirmed that the cells were infected with an ALV-A. Our findings represent the first report of an ALV-A contaminant in a live vaccine for poultry in China.

In the production of attenuated live vaccines, ALV contamination may originate from various sources. The use of ALV-infected chicken embryos is likely the major source of ALV contamination. In our current study, we investigated the possible source of ALV contamination in the NDV vaccine, and our results indicated that...
the contamination most likely originated from the SPF chicken embryos used in vaccine production.

The Ministry of Agriculture of People’s Republic of China specifies that all attenuated vaccine for poultry must use SPF chicken embryos and cells as the raw materials for the production of attenuated vaccines since January 1, 2008. However, investigation shows that the quality of China’s SPF chicken embryos is not perfect. In 2012, the REV was isolated from an SPF chicken in China (Wang et al., 2012). We conducted a large-scale screening of 10 SPF chicken flocks that provide embryos to the manufacturer of the clone-30 live NDV vaccine. Unluckily, several of the SPF eggs tested positive for the p27 antigen of ALV. As reported, a high level of p27 antigen is a sensitive and practical means of identifying dams congenitally transmitting ALV for some special samples just as in light albumin of chicken embryos because there is a very low frequency of false positives caused by the p27 antigen in eggs (Crittenden and Smith, 1984; Himly et al., 1998). These results highlight the need for more extensive screening of SPF chicken embryos in China to reduce the threat of ALV contamination in live vaccines for poultry.

Considering the risk of ALV infection in some SPF chickens as investigated above, the China Institute of Veterinary Drugs Control performed a serosurvey on attenuated vaccines stored during the period from 2006 to 2010 after the ALV-NDVP4 was found from live NDV vaccine, and discovered the antibody positive for ALV-Ab after vaccination with a live vaccine of infectious bursal disease, titers with the MAV-1 and SDAU09C3 strains of ALV-A; and Smith, 1984; Himly et al., 1998). These results highlight the need for more extensive screening of SPF chicken embryos in China to reduce the threat of ALV contamination in live vaccines for poultry.

For classification of subgroups of ALV isolates, standard methods were based on phenotypes such as cross viral neutralizations in cell cultures or interference tests among different isolates and reference strains of different subgroups. However, these methods are complex and require reference strains of all the subgroups. Because subgroups of ALV are determined by their envelope protein gp85, most laboratories for ALV have started to use homologous comparisons of gp85 for identification and differentiation of their subgroups (Pan et al., 2012). To better characterize the ALV-NDVP4 at the molecular level, we compared the gp85 sequence of the ALV-NDVP4 strain with those of the ALV strains in the GenBank database (Table 1).

Comparison of gp85 amino acid sequences between ALV-NDVP4 and other ALV strains of different subgroups indicated that ALV-NDVP4 had the highest homology with 12 reference strains of ALV-A, and the phylogenic tree based on gp85 sequence indicates that the ALV-NDVP4 definitely fell into subgroup A. The phylogenetic analysis also showed that the ALV-NDVP4 shared the highest level of amino-acid-sequence identity with the MAV-1 and SDAU09C3 strains of ALV-A; phylogenetic analysis showed that they belonged to the same subbranch (Figure 1). The MAV-1 strain was isolated in France in 1993, and the SDAU09C3 strain was isolated in China in 2009. Obviously, SDAU09C3 was likely to be responsible for the ALV infection through the contaminated vaccine; future studies are warranted to further characterize the phylogenetic relationship between the ALV-NDVP4 and SDAU09C3 strains.

The vaccination of chickens with an ALV contaminated live vaccine poses significant risks to chickens. A previous study showed that infection with ALV-J significantly decreased NDV hemagglutination-inhibition antibody titers in 20-, 30-, and 40-d-old commercial broilers compared with control birds (Cui et al., 2006). In China, the Newcastle Disease Mild Vaccine (Clone30 Strain, live vaccine) was vaccinated at 7 d old through drinking water for the first time and the ALV contamination in the vaccine may lead to reduced serological response to NDV and render the birds more sensitive to NDV infection (Cui et al., 2006). Future studies are warranted to assess the pathogenicity of the ALV-NDVP4 strain in chickens to better determine its effects on contaminated NDV vaccine.

In summary, we isolated and characterized an ALV-A strain from a live NDV vaccine produced in China. The analysis of the gp85 amino acid sequence showed that the ALV isolate shared a high level of sequence identity with the SDAU09C3 strain of ALV-A, which was previously isolated from chickens in China, indicating that the 2 strains share a common origin. Our findings highlight the need for more extensive screening of live vaccines for poultry in China to reduce the threat of contamination with exogenous viruses.

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REFERENCES


