Avian Pathology

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Thuy D. Pham, J.L. Spencer, Vicki L. Traina-Dorge, David A. Mullin, Robert F. Garry & Eric S. Johnson

Published online: 17 Jun 2010.

To cite this article: Thuy D. Pham, J.L. Spencer, Vicki L. Traina-Dorge, David A. Mullin, Robert F. Garry & Eric S. Johnson (1999) Detection of exogenous and endogenous avian leukosis virus in commercial chicken eggs using reverse transcription and polymerase chain reaction assay, Avian Pathology, 28:4, 385-392, DOI: 10.1080/03079459994650

To link to this article: http://dx.doi.org/10.1080/03079459994650
Detection of exogenous and endogenous avian leukemia virus in commercial chicken eggs using reverse transcription and polymerase chain reaction assay


1Department of Biostatistics and Epidemiology, SL 18, Tulane University Medical Center, 1501 Canal Street, New Orleans, LA 70112, USA, and 2Agriculture Canada, Animal Diseases Research Institute, P.O. Box 11300, Station H, Nepean, Ont. K2H 8P9, Canada, and 3Tulane Regional Primate Research Center, Covington, LA 70433, USA, and 4Department of Cellular and Molecular Biology, Tulane University, New Orleans, LA 70118, USA, and 5Department of Microbiology, Tulane University Medical Center, New Orleans, LA 70112, USA

Avian leukemia retroviruses (ALV) cause lymphomas and other cancers in chickens. Previous studies have used enzyme-linked immunosorbent assays (ELISA) and indirect immunofluorescence assays (IFA) to detect ALV p27 group-specific antigens (GSA) in commercial chicken eggs. In the poultry industry eradication programme against exogenous ALV, ELISA assays are used to identify chickens infected with the virus. The inability of ELISA and IFA assays to discriminate between ALV GSA of endogenous or exogenous origin, and actual virus, have limited rigorous assessments of viral transmission dynamics. Here, we report the use of a newly developed reverse transcriptase-polymerase chain reaction (RT-PCR) assay, with direct sequencing of the RT-PCR product, to show endogenous and exogenous ALV in albumen from unfertilized chicken eggs. We found that 95% of 20 eggs from ALV-exposed commercial chickens and 14.2% of 240 egg samples from 20 randomly chosen New Orleans retail stores were ALV-positive by RT-PCR. In comparison, only 2.5% of the same egg samples from the retail stores were positive by ELISA. Corresponding direct sequencing of randomly chosen RT-PCR products showed that four of six egg samples contained endogenous ALV, while two of the six samples were positive for exogenous subgroup A ALV. The finding of endogenous subgroup E ALV in unfertilized chicken eggs emphasizes that the transmission of endogenous ALV is common and should be considered in the implementation of ALV eradication programmes by the poultry industry.

Introduction

Avian leukemia viruses (ALV) can infect chickens and cause various diseases, malignant tumour formations, and mortality in infected chickens. These viruses cause serious economic losses in the poultry industry worldwide since infected chickens not only develop neoplasia, but also have decreased production efficiency (Gavora et al., 1980,1982; Crittenden, 1991). ALV is categorized into subgroups A, B, C, D, E, and J according to their ability to infect chicken embryo fibroblasts (CEF), patterns of viral interference with other viral subgroups, and viral envelope antigens (Vogt & Ishizaki, 1966; Duff & Vogt, 1969; Hanafusa, 1975; Bova et al., 1986; Payne et al., 1991). The exogenous and oncogenic ALV subgroups A, B, C, and D are horizontally and vertically transmitted. Endogenous virus or ev genes, classified as subgroup E viruses, are transmitted by Mendelian inheritance (Astrin, 1978; Crittenden & Kung., 1984; Crittenden, 1991).

* To whom correspondence should be addressed. 369 Village Farm Lane, Folsom, LA 70437, USA. Tel: +1 504 796 8031. Fax: +1 504 584 1706. E-mail: tpham@altavista.NET
Received 16 November 1998. Accepted 29 March 1999.

ISSN 0307-9457 (print)/ISSN 1465-3338 (online)/99/040385-08 © 1999 Houghton Trust Ltd
As C-type retroviruses, the ALV RNA form is (5') R-U5-gag-pol-env-U3-R (3'). The env gene encodes for two glycoproteins: gp85<sup>env</sup> which resembles knob-like structures on the surface of a retrovirus particle, and gp37<sup>env</sup> which appears as highly glycosylated spikes that link the knobs to the viral lipid envelope (Bova et al., 1986, 1988; Bova & Swanstrom, 1987). Variable regions in the sequences of gp85<sup>env</sup> determine the viral envelope properties specific for ALV subgroups A, B, C, D, or E (Coffin et al., 1978, 1983; Dorner et al., 1985; Bova et al., 1986, 1988; Bova & Swanstrom, 1987).

Besides horizontal transmission of exogenous ALV from chicken to chicken, infected chickens can pass the virus congenitally at a high frequency through the egg. Congenitally infected chickens tend to have lower egg production and survivability than chicks which are horizontally infected with exogenous ALV (Fadly & Okazaki, 1982; Payne et al., 1991). ALV-infected hens that test positive for group-specific antigens (GSA) in egg albumen are called shedders. Eggs from GSA shedding hens are characteristically lower in quality, fertility, and hatchability than eggs from non-GSA shedding hens (Garwood et al., 1981; Gavora et al., 1980, 1982).

To prevent transmission of exogenous ALV, the poultry industry must effectively identify virus-infected hens and eggs, and remove them from the breeding populations. Current methods for identifying ALV infections include virus isolation in tissue culture, immunofluorescence assay (IFA), and enzyme-linked immunosorbent assay (ELISA) (ELISA) (Rubin, 1967; Sarma et al., 1969; Okazaki et al., 1975; Wisdom, 1976; Smith et al., 1979; Spencer & Gilka, 1982; Spencer et al., 1984; Spencer, 1987). Traditional virus isolation procedures and IFA identification are generally not used to identify ALV infections in commercial poultry flocks because these assays require about 2 weeks to determine viral infection. Currently, the poultry industry uses an ELISA assay to screen for ALV GSA in infected chickens (Spencer, 1984, 1987).

Since both exogenous and endogenous ALV exhibit common GSA, ELISA assays cannot be used reliably to discriminate between GSA of exogenous and endogenous origin. Reverse transcriptase-polymerase chain reaction (RT-PCR) assays have been used to detect ALV in infected materials (Hauptli et al., 1997; Smith et al., 1998). However, these RT-PCR assays were not developed to distinguish the nucleotide sequence of wild-type ALV infection in unfertilized commercial chicken eggs.

The objective of this investigation was to use a newly developed RT-PCR assay (Pham et al., 1999), with direct sequencing of the RT-PCR product, to effectively screen for ALV in eggs obtained from egg-laying commercial chickens naturally exposed to ALV infections. In addition, the same approach was used to determine the prevalence of ALV in commercial chicken eggs obtained from randomly chosen retail stores in New Orleans, Louisiana, USA.

### Materials and Methods

#### Stocks of chickens

To investigate the presence of ALV in egg albumen at the nucleic acid level, we used unfertilized eggs from three commercial egg-laying stocks of Single Comb White Leghorn chickens, referred to as stock Q, stock F, and stock N. Stock Q chickens (n = 5) were reared by a commercial company that participates in a campaign to eradicate exogenous ALV. Stock Q chickens had the following endogenous (ev) ALV genes: ev1, ev3, ev4, ev5, ev6, ev7, and ev9 (Smith, 1987; Crittenden, 1991). Stock F chickens (n = 2) were from a company without an effective ALV control programme. The stock Q and stock F chickens were kept under commercial conditions where they were naturally exposed to pathogens. Both stock Q and stock F chickens were selected for study because the poultry companies initially observed ALV infections but were unable to perform further verification or to delineate ALV subgroup identification. The laboratory flock of stock N chickens (n = 5), free of ALV and other known pathogens, was maintained in an isolation facility at the Canadian Food Inspection Agency, Ont., Canada.

#### Specimens tested for virus and viral antigen

Methods for obtaining samples of egg albumen, feather pulp, and chicken blood have been reported previously (Spencer, 1987). Avian leukosis virus stocks of subgroup A (RAV-1) (Bova et al., 1988) and subgroup B (RAV-2) (Bova et al., 1986) were obtained (US Department of Agriculture, Avian Diseases and Oncology Laboratory in East Lansing, MI), and used as positive controls in ELISA and IFA tests (Spencer, 1987).

#### Survey of eggs sold by retail stores in New Orleans, LA, USA

A telephone survey of the retail stores in the New Orleans Metropolitan Area was conducted between December 1996 and February 1997. The sampling frame was grocery stores listed in the New Orleans Yellow Pages for 1995/1996, and the respondents were individuals who either owned, managed, or ordered eggs for the store. A total of 417 grocery stores in the New Orleans area was listed in the Yellow Pages telephone directory (1995/1996). Of these, 64 (15%) stores were no longer in existence. Of the remaining 353 grocery stores, 74 retail stores did not sell eggs. Of the 279 stores, telephone surveys were completed for 275 retail stores, a participation rate of 99%. A total of 275 grocery stores sold 174,871 dozens of eggs per week or 8.4 million eggs per month in New Orleans, LA. Based on an expected 20% ALV prevalence (Okazaki et al., 1982), we used the equation \(N = Z_{0.05}(p)(1-p)/d^2\) (Rosner, 1995) to determine the sample size of 240 chicken eggs obtained from 20 randomly chosen New Orleans retail stores.

#### ELISA and IFA analysis of egg albumen, feather pulp, and blood samples

ELISA was performed on the egg albumen to detect ALV GSA, while IFA analysis was performed on C/E cells inoculated with chicken egg albumen, feather pulp, and blood specimen to detect GSA of replicating ALV. C/E cells are resistant to subgroup E endogenous ALV infections but are permissive to viral infections of exogenous ALV subgroups A, B, C, and D (Spencer, 1987). IFA assays to detect replicating ALV in CEF, and ELISA assays to detect ALV viral antigens have been described (Spencer, 1987).
**Table 1. Detection of ALV by ELISA, IFA, and RT-PCR**

| Chicken
type | ELISA | IFA | RT-PCR |
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<td>Egg albumen</td>
<td>Egg albumen</td>
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<td>5N</td>
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*Q and F, two commercial stocks of White Leghorn chickens. N flock of White leghorn chickens were negative for exogenous and endogenous ALV and other poultry pathogens.*

**Oligonucleotide primers**

A set of primers, called PA1 and PA2, was designed for ALV subgroup A (Pham et al., 1999): PA1, 5'-CTACAGCTGGTAGTCCAGT-3'; PA2, 5'-GCTATCCGCTGTCACCACTG-3'.

**RT-PCR analyses**

To reduce the risks of contamination, RNA extraction of egg albumen was performed in a laboratory separate from the RT-PCR procedure, and RNAase free reagents and pipette tips were used (Pham et al., 1999). The RNA Agents Total RNA Isolation System (Promega, USA) was used to extract total RNA from the egg albumen. Egg albumen from unfertilized eggs was used to limit false positive reactions due to avian leukaemia endogenous proviruses, potentially established in parental genes found in fertilized eggs or in maternal genes found in the vitelline membrane surrounding the egg yolk, and to limit false negative reactions due to inactivation of ALV by maternal antibodies, such as IgY, found in the egg yolk (see Kottaridis et al., 1967; Smith, 1987; Yamamoto et al., 1997).

The RT-PCR analysis (Pham et al., 1999) was performed using the Access RT-PCR system (Promega, USA) and consisted of the following reagents in the reaction tube: nuclelease-free water added to a final volume of 50 μl per reaction tube, 10 μl of AMV/Taq 5× reaction buffer with a final concentration of 1×, 1 μl of dNTP mix (10 mM) with a final concentration of 0.2 mM, 50 pM for the forward primer (PA1), 25 pM for the reverse primer (PA2), 2 μl of 25 mM MgSO₄ with a final concentration of 1 mM, 1 μl of AMV reverse transcriptase (5 U/1 μl) with a final concentration of 0.1 U/1 μl, 1 μl of Taq DNA polymerase (5 U/1 μl) with a final concentration of 0.1 U/1 μl, and 2 μl of the sample that may contain isolated viral RNA. Negative controls consisted of nuclease-free water instead of RNA templates, and isolations of RNA from egg albumen obtained from eggs laid by stock N chickens, which were negative for exogenous or endogenous ALV.

**RT-PCR amplification profiles**

Amplification of first-strand cDNA synthesis was for one cycle at 48°C for 45 min, and one cycle at 94°C for 2 min (Pham et al., 1999). The amplification profile for second-strand cDNA synthesis and PCR amplification was a total of 40 cycles with each step cycle at 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min, 68°C for 7 min, and 4°C for overnight reactions.

**Nucleotide sequencing of RT-PCR product**

Sequencing was performed by Commonwealth Biotechnologies, Inc. (Virginia, USA). The randomly chosen RT-PCR products were sequenced using sense primer (PA1) and antisense primer (PA2), which resulted in 100% similarity between the sense and antisense nucleotide sequences. Nucleotide sequences generated from the sequenced samples and the published viral subgroup A, B, C, D, and E sequences (Bova et al., 1988) were aligned using the computer program Sequencher (Gene Code Corporation, Michigan, USA). Multiple sequence alignments were produced using MacVector Clustal W(1.4) (Oxford Molecular, CA, USA).

**Results**

ELISA assays showed that four of five (80%) stock Q chickens had GSA in egg albumen, but the virus was not present in cultured C/E cells tested by IFA analysis (Table 1). The four ELISA-positive chickens had absorbance values of 0.5 or greater. One ELISA-positive stock F chicken had infectious ALV as detected in C/E cells using the IFA assay, while the other stock F chicken was negative by both ELISA and IFA analyses (Table 1). The ELISA-positive F chicken had an absorbance value of >0.5. All five stock N chickens were ELISA negative for viral GSA in the egg albumen (Table 1).

We performed RT-PCR analysis on egg albumen samples from the stock Q, F, and N chickens in a masked fashion to minimize bias. ALV was
detected in 14 of 14 (100%) egg albumen samples from all the stock Q chickens (Table 1). In the stock F chicken that was ELISA and IFA positive, all three of three (100%) egg albumen samples were positive by RT-PCR, while in the stock F chicken that was consistently negative by ELISA and IFA, two of three (67%) egg albumen samples were positive for ALV virus (Table 1). All five of the non-exposed stock N chickens, as well as the assay controls, were negative by RT-PCR (Table 1).

RT-PCR analysis of chicken eggs being sold by retail stores in New Orleans, LA, showed that 80% of the egg cartons sampled contained one or more ALV-infected eggs. Each carton of 12 eggs contained an average of 1.7 ALV-infected eggs. Overall, 14.2% of 240 eggs were infected with ALV, as determined by the RT-PCR assay, while the
ELISA assay indicated that only 2.5% of the same egg samples were infected with ALV. ELISA-positive eggs had a mean optical density (OD) of 2.1 and a range of 0.45 to 2.99 levels of GSA in egg albumen. Based on our survey, we estimated that 1.2 of 8.4 million eggs being sold per month in New Orleans are contaminated with ALV.

Six egg albumen samples, positive for ALV by RT-PCR, were randomly selected and subjected to direct nucleotide sequencing using the amplified RT-PCR products (Figure 1). Nucleotide sequences of sample 6F from stock F chickens, 7Q and 10Q from stock Q chickens, and sample 207 from New Orleans retail stores showed a ≥95% homology to each other and had a ≥95% homology to RAV-0 (Coffin et al., 1983; Dorner et al., 1985; Bova et al., 1986), the prototype for subgroup E ALV (Table 2). Both sample 2F from stock F chickens and sample 65 from New Orleans retail stores shared 95% homology to each other and had an 84% homology to RAV-1 (Bova et al., 1988), the prototype virus for subgroup A ALV (Table 2).

Discussion

The combination of using a newly developed RT-PCR assay with direct sequencing has provided valuable insights into the transmission dynamics of exogenous and endogenous ALV in commercial chicken eggs. We expected the RT-PCR to detect subgroup A ALV because the prevalence of subgroup A ALV infections in chickens is higher than subgroup B ALV, while infections of subgroup C ALV and subgroup D ALV in chickens have only been reported in Finland (Sandelin & Estola, 1973; Calnek et al., 1991). However, subgroup E avian leukosis virus was not expected in commercial chicken eggs because infections of endogenous ALV usually do not result in detectable levels of GSA in egg albumen of egg-laying stocks (Spencer & Chambers, 1992). In this investigation, nucleotide sequence analysis performed on randomly selected RT-PCR products of albumen from eggs obtained from two different sources, White Leghorn chickens and retail stores, showed four of six samples were positive for endogenous ALV, while two of six samples were positive for exogenous ALV.

Based on previous studies, ELISA absorbance values of egg albumen infected with exogenous ALV are usually 0.5 or greater, while infections of endogenous ALV do not have detectable levels of GSA in egg albumen (Spencer & Chambers, 1992). The four samples positive by RT-PCR for endogenous ALV had ELISA absorbance values of 0.15, 0.18, 0.52 and 0.54, while the two samples positive for exogenous ALV had absorbance values of 0.97 and 2.99. These findings suggest that ELISA absorbance values are not reliable indicators of the presence or absence of endogenous ALV infections. This study demonstrates that the RT-PCR assay with direct sequencing can effectively discriminate between exogenous and endogenous ALV infections in albumen of commercial chicken eggs. Furthermore, this study shows that endogenous ALV infections of eggs is more common in commercial egg-laying hens than previously expected.

Our newly developed RT-PCR assay provides a sensitive technique for detecting ALV virus in representative chicken flocks of the poultry industry and in commercial eggs from retail stores. While the RT-PCR detected ALV in 14.2% of 240 egg samples obtained from randomly chosen retail stores, the ELISA assay only detected ALV in 2.5% of the same egg samples. The poultry industry maintains an intense eradication programme that is based on detection of ALV infection by ELISA (Spencer et al., 1984; De Boer, 1986; Spencer, 1987; Spencer & Fairfoul, 1993; Chase, 1991; Payne & Howes, 1991). Our study indicates that detection by RT-PCR may be five times more sensitive than the standard ELISA assays currently
being used. Therefore, future investigations of the prevalence of ALV in chicken flocks should involve the use of the RT-PCR strategy, along with direct sequencing of the RT-PCR product, to more accurately detect viral presence and discriminate viral subgroups.

Our demonstration of wild-type endogenous ALV, with ≥ 95% homology to RAV-0, in eggs from commercial egg-laying chickens and retail stores emphasizes the common occurrence of vertical transmission. Congenital transmission, a highly efficient means of exogenous ALV viral transmission, occurs when an infected hen sheds the virus into the egg (Spencer et al., 1980). Many studies have suggested that the existence of endogenous ALV in chickens is probably due to the stable incorporation of exogenous ALV into the ancestral chicken germ line about 5000 years ago (Astrin, 1978; Crittenden & Kung, 1984; Crittenden, 1991; Boyce-Jacino et al., 1992). A few studies have shown that, in rare instances, some endogenous viral genes from unselected chickens can form infectious subgroups E viral particles (i.e. ev2, ev7, ev12, and ev21) (Smith & Crittenden, 1981; Bacon et al., 1988; Crittenden, 1991). ev2 encodes for RAV-0, which is denoted as the prototype for endogenous ALV because a high percentage of RAV-0 nucleotide sequences, are found in the genome of chickens (Vogt & Friis, 1971; Crittenden et al., 1977; Astrin et al., 1980). In experimental studies involving RAV-0 and K28 chickens that are highly viremic to endogenous ALV, Robinson & Eisenman (1984) reported that congenital transmission of RAV-0 in experimentally infected K28 chickens does not occur, since the virus was not detected in egg albumen of fertilized eggs using an RNA-directed DNA polymerase assay. In additional case studies of Smith (1987) and Ignatovic (1988), shedding of endogenous ALV did not occur in studies involving chickens that were experimentally infected with endogenous ALV. However, infection and shedding of endogenous ALV was found to occur commonly in meat-type chickens, but not in egg-laying chickens (Ignatovic, 1986; Spencer & Chambers, 1992). In contrast, we found endogenous ALV in egg-laying chickens from two different commercial chicken stocks.

In addition to using the RT-PCR assay with direct sequencing to study transmission of ALV viruses in chicken eggs, we used egg albumen from unfertilized commercial chickens instead of fertilized chicken eggs that have been used in previous studies. There are several advantages to using unfertilized rather than fertilized chicken eggs. For instance, we were able to isolate and characterize wild-type exogenous and endogenous ALV without false positive reactions arising from parental genes found in fertilized egg or maternal genes present in the vitelline membrane surrounding the egg yolk. In addition, by using egg albu-


**RÉSUMÉ**

**Détectio des virus endogènes et exogènes de la leucose aviaire à partir d’œufs provenant de troupeaux commerciaux par les réactions de transcription inverse et d’amplification en chaîne par polymérase**

Les rétrovirus de la leucose aviaire (ALV) induisent des lymphomes et d’autres cancers chez la poule. Des études antérieures ont fait appel à la technique ELISA et à l’immunofluorescence indirecte (IFA) pour
détecter l’antigène spécifique de groupe (gsa) p27 des ALV, à partir des œufs de souches commerciales. Le programme d’éradicace des ALV exogènes conduit par l’industrie avicole est réalisé à l’aide de tests ELISA pour identifier les sujets infectés. Les tests ELISA et l’IF ne permettent pas de discriminer le gsa des ALV d’origine endogène ou exogène et pour la détection du virus des évaluations rigoureuses se sont limitées aux dynamiques de transmission virale. Dans cet article, l’utilisation des nouvelles réactions de transcrption inverse, d’amplification en chaîne par polymérase (RT-PCR) et de séquençage direct des produits amplifiés est décrite pour mettre en évidence les ALV endogènes et exogènes à partir d’albumen d’œufs non embryonnés. Il a été observé que 95% des œufs sur un total de 20, provenant de troupeaux commerciaux pouvant être infectés par des ALV et que 14,2% des œufs sur un total de 240, provenant de 20 magasins de détail de la Nouvelle-Orléans se sont révélés positifs par la technique de RT-PCR. Par comparaison, seuls 2,5% des mêmes échantillons prélevés dans les magasins de détail se sont révélés positifs par la technique ELISA. Le séquençage direct des produits de RT-PCR choisis au hasard a montré que 4 œufs sur 6 contenaient de l’ALV endogène, alors que 2 sur 6 étaient positifs vis-à-vis du sous-groupe A exogène. Le fait de trouver le sous-groupe E endogène dans des œufs non embryonnés met en évidence que la transmission de l’ALV endogène est courante et devrait être prise en considération dans la mise en place des programmes d’éradicace des ALV par l’industrie avicole.

ZUSAMMENFASSUNG
Nachweis von exogenem und endogenem Geflügelleukosevirus in kommerziellen Hühnereiern mit Hilfe der Reverse-Transkriptase-Polymerase-Kettenreaktion

RESUMEN
Detección del virus de la leucosis aviar exógeno y endógeno en huevos comerciales utilizando técnicas de transcripción inversa y reacción de la polimerasa en cadena
Los retrovírus de la leucosis aviar (ALV) causan linfomas y otras neoplasias en pollos. En estudios previos, se han utilizado técnicas de ELISA e inmunofluorescencia indirecta (IFA) para la detección de antígenos p27 del ALV específicos del grupo (gsa) en huevos de pollo comerciales. En la industria avicola se utilizan técnicas de ELISA para identificar los pollos infectados con el virus en los programas de erradicación del ALV exógeno. La incapacidad de las técnicas de ELISA e IFA para diferenciar entre los gsa del ALV de origen endógeno o exógeno y el propio virus han impedido estudios rigurosos acerca de la dinámica de transmisión del virus. Presentamos la utilización de una nueva técnica de transcripción inversa y reacción de la polimerasa en cadena (RT-PCR), junto con la secuenciación directa de su producto, para demostrar la presencia de ALV endógenos y exógenos en albumen de huevos no fertilizados. Se encontró que un 95% de 20 huevos de pollos expuestos a ALV y un 14% de 240 muestras de 20 establecimientos al por menor de Nueva Orleans, elegidas al azar, eran positivos a ALV mediante la técnica de RT-PCR. En comparación, solamente un 2,5% de las mismas muestras elegidas al azar fueron positivas mediante la técnica de ELISA. La correspondiente secuenciación directa de los productos de RT-PCR de las muestras elegidas al azar demostró que 4 de 6 muestras de huevos contenían ALV endógeno, mientras que 2 de las 6 muestras fueron positivas para el ALV exógeno, subgrupo A. El hallazgo del virus ALV endógeno, subgrupo E en huevos no fertilizados enfatiza que la transmisión de ALV endógeno es frecuente y de debería considerarse en la mejora de los programas de erradicación de ALV en la industria avicola.