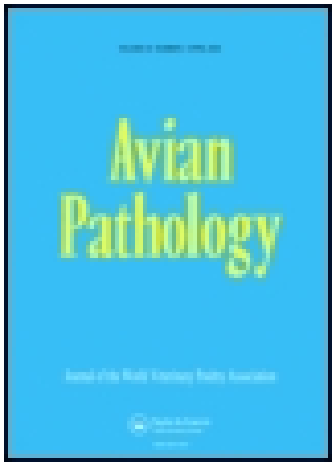

Avian Pathology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/cavp20

Detection of exogenous and endogenous avian leukosis virus in commercial chicken eggs using reverse transcription and polymerase chain reaction assay

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

Avian Pathology (1999) **28,** 385–392

Detection of exogenous and endogenous avian leukosis virus in commercial chicken eggs using reverse transcription and polymerase chain reaction assay

Thuy D. Pham[1]*, J. L. Spencer[2], Vicki L. Traina-Dorge[3], David A. Mullin[4], Robert F. Garry[5] & Eric S. Johnson[1].

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

Avian leukosis 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 leukosis 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

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: $gp85^{env}$ which resembles knob-like structures on the surface of a retrovirus particle, and $gp37^{env}$ 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 $gp85^{env}$ 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) (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: *ev*1, *ev*3, *ev*4, *ev*5, *ev*6, *ev*7, and *ev*9 (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. In addition, we also screened for ALV in commercial chicken eggs obtained from randomly chosen retail stores in New Orleans, LA, USA.

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 $N = Z^2_{0.975}\,(p)(1-p)/d^2$ (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 permissible 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[a] number and strain	ELISA[b]	IFA[c]			RT-PCR[d]
	Egg albumen	Egg albumen	Feather pulp	Blood	Egg albumen
7Q	+	−	−	−	+ (3/3)
8Q	+	−	−	−	+ (3/3)
9Q	+	−	−	−	+ (3/3)
10Q	+	−	−	−	+ (2/2)
11Q	−	−	−	−	+ (3/3)
2F	+	+	+	+	+ (3/3)
6F	−	−	−	−	+ (2/3)
1N	−	NT	NT	NT	− (0/1)
2N	−	NT	NT	NT	− (0/1)
3N	−	NT	NT	NT	− (0/1)
4N	−	NT	NT	NT	− (0/1)
5N	−	NT	NT	NT	− (0/1)

[a]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.
[b]+ or −, presence or absence of ALV GSA as detected by ELISA.
[c]Each specimen was inoculated onto C/E cells that were susceptible to exogenous ALV, but not endogenous ALV. Infection in cells (+ or −) was detected by IFA and there was consistent agreement between two replicate cultures.
[d]Primer set PA1 and PA2 (Pham *et al.*, 1999), designed to detect subgroup A ALV, was used in the RT-PCR assay; +, RT-PCR positive reaction. (Number of positive samples/total number of eggs tested).
NT, not tested.

Oligonucleotide primers

A set of primers, called PA1 and PA2, was designed for ALV subgroup A (Pham *et al.*, 1999): PA1, 5′-CTACAGCTGTTAGGTTC-CCAGT-3′; PA2, 5′-GCCTATCCGCTGTCACCACTG-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 RNAgents 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 leukosis 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: nuclease-free water added to a final volume of 50 μl per reaction tube, 10 μl of AMV/*Tfl* 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 $MgSO_4$ 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 *Tfl* 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 Clustral 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

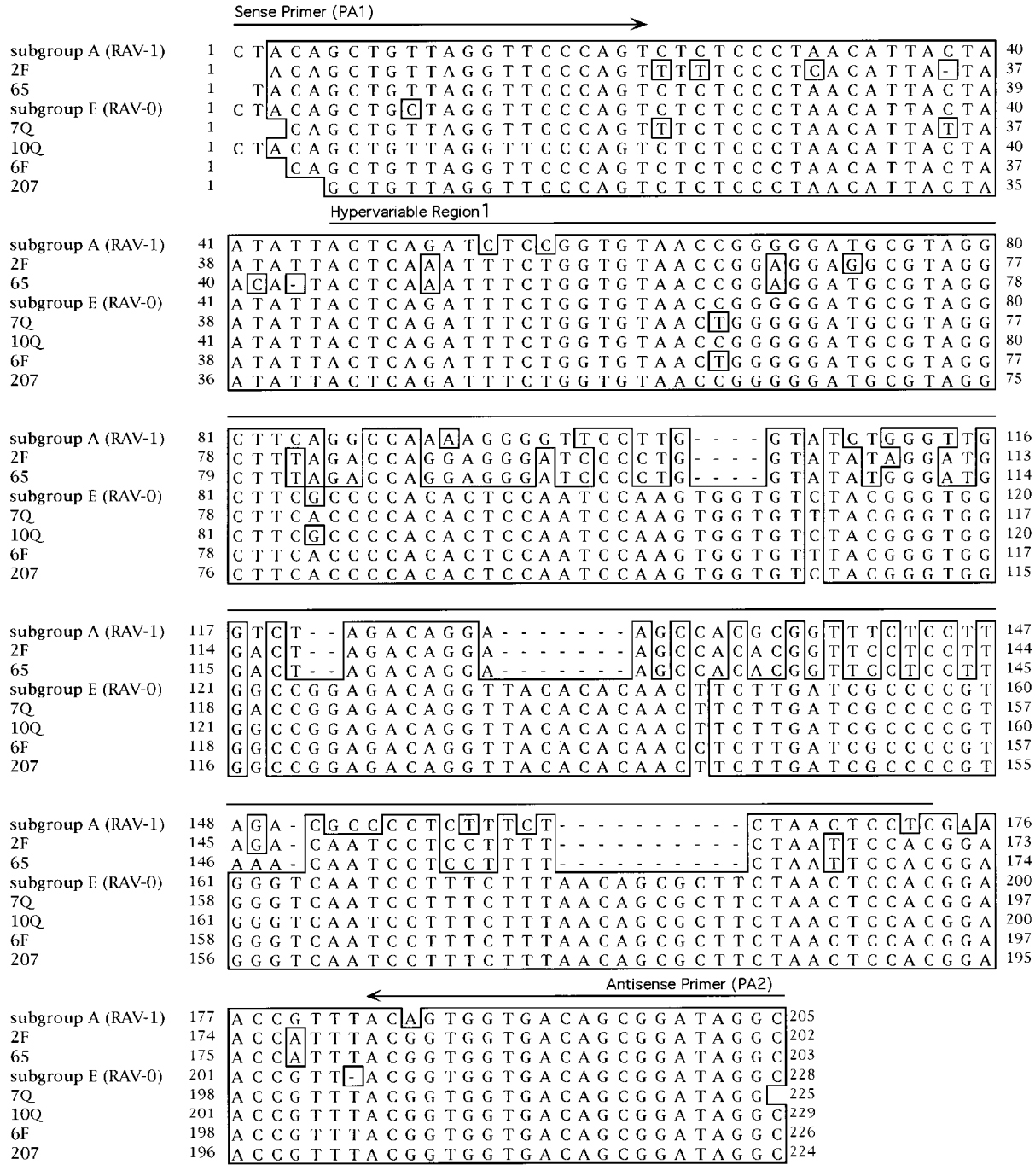

Figure 1. *Alignment of the ALV subgroup E (RAV-0) and ALV subgroup A (RAV-1) nucleotide sequences with those obtained from six RT-PCR products of egg albumen samples (2F, 6F, 7Q, 10Q, 207, and 65). Matching sequences are boxed with a thin line. The sequence comparison begins at nt5563 of Pr-C RSV and ends at nt5792 of Pr-C RSV (Schwartz* et al.*, 1983). The locations of the PA1 and PA2 primers used in the RT-PCR reaction are marked with arrows. The* gp85^{env} *hypervariable region 1 (hr 1) (Bova* et al.*, 1988) is marked with a straight line. Numbers at the left represent the position of the first nucleotide in the sequence, and numbers on the right represent the position of the last nucleotide in the sequence. Hyphens represent gaps inserted for optimum alignment.*

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

Table 2. *ALV viral subgroup classification of six sequenced RT-PCR products from egg albumen*

Origin of egg albumen	Specimen number	Analysis for ALV: GSA in albumen	Analysis for ALV: Virus isolated[a]	Analysis for ALV: RT-PCR[b]	Viral subgroup and sequence homology (%)[c]
Stock F chickens	2F	+	+	+	A (84%)
Stock F chickens	6F	–	−	+	E (95%)
Stock Q chickens	7Q	+	−	+	E (95%)
Stock Q chickens	10Q	+	−	+	E (99%)
Commercial egg	207	−	NT	+	E(96%)
Commercial egg	65	+	NT	+	A (84%)

[a]NT, not tested.
[b]Data from Table 1.
[c]Subgroup classification based on percent sequence homology compared with the published viral sequences (Bova *et al.*, 1988). See Figure 1.

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 $\geq 95\%$ homology to each other and had a $\geq 95\%$ 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 & Fairfull, 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 $\geq 95\%$ 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 subgroup E viral particles (i.e. *ev*2, *ev*7, *ev*12, and *ev*21) (Smith & Crittenden, 1981; Bacon *et al.*, 1988; Crittenden, 1991). *ev*2 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 viraemic 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 chicks 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 albumen, we circumvented the problem of false negative reactions that may occur if egg yolk were used, because a high proportion of maternal IgY is found in this region (see Yamamoto *et al.*, 1997). Therefore, our assays depicted actual wild-type avian leukosis viruses that are currently persisting in the field.

In conclusion, our investigation has three main findings. First, in comparison with the ELISA assay, the RT-PCR assay showed a fivefold higher prevalence of ALV in commercial chicken eggs obtained from retail stores in New Orleans, LA. Second, both wild-type endogenous and exogenous ALV isolated from chicken eggs were sequenced directly from RT-PCR amplified products. Third, transmission of endogenous ALV may occur more frequently in egg-laying chickens than previously suggested. The capability of genetically characterizing wild-type endogenous and exogenous ALV from chicken eggs without the need for tissue culture or cloning techniques provides an interesting means for future studies to investigate the modes of natural transmission of wild-type ALV, to determine the epidemiology and persistence of various viral subgroups, and to detect newly emerging viral variants. Therefore, the combined approach of RT-PCR with direct sequencing of the amplified product should improve the ability of the poultry industry to eradicate ALV from infected commercial chicken flocks and eggs.

Acknowledgements

We thank J. Lefante, and B. Benkel for comments, and P. Whelton, L. Weber, T. C. Pham, L. B. Pham, K. O'Donnell, and S. Gautier for assistance.

References

Astrin, S.M. (1978). Endogenous viral genes of the White Leghorn chicken: common site of residence and sites associated with specific phenotypes of viral gene expression. *Proceedings of the National Academy of Science, USA, 75,* 5941–5945.

Astrin, S.M., Crittenden, L.B. & Buss, E.G. (1980). *ev*2, a genetic locus containing structural genes for endogenous virus, codes for Rous-associated virus type 0 produced by line 7_2 chickens. *Journal of Virology, 33,* 250–255.

Bacon, L.D., Smith, E.J., Crittenden, L.B. & Havenstein, G.B. (1988). Association of the slow feathering (k) and an endogenous viral (*ev*21) gene on the Z chromosomes of chickens. *Poultry Science, 67,* 191–197.

Bova, C.A. & Swanstrom, R. (1987). Host range determinants of avian retrovirus envelope genes. In *Positive Strand RNA Viruses* (pp. 509–518). New York: Alan R. Liss, Inc.

Bova, C.A., Manfredi, J.P. & Swanstrom, R. (1986). *env* genes of avian retroviruses: nucleotide sequence and molecular recombinants define host range determinants. *Virology, 152,* 343–354.

Bova, C.A., Olsen, J.C. & Swanstrom, R. (1988). The avian retrovirus *env* gene family: Molecular analysis of host range and antigenic variants. *Journal of Virology, 62,* 75–83.

Boyce-Jacino, M.T., O'Donoghue, K. & Faras, A.J. (1992). Multiple complex families of endogenous retroviruses are highly conserved in the genus *Gallus*. *Journal of Virology, 66,* 4919–4929.

Calnek, B.W., Barnes, H.J., Beard, C.W., Reid, W.M. & Yoder, H.W. (1991). *Diseases of Poultry* 9th edn. Ames: Iowa State University Press.

Chase, W.B. (1991). Eradication of avian leukosis virus by breeder companies: results, pitfalls and cost benefit analysis. *Proceedings of the Avian Tumor Virus Symposium,* Seattle, WA, pp. 5–8.

Coffin, J.M., Champion, M. & Chabot, F. (1978). Nucleotide sequence relationships between the genomes of an endogenous and an exogenous avian tumor virus. *Journal of Virology, 28,* 972–991.

Coffin, J.M., Tsichlis P.N., Conklin, K.F., Senior, A. & Robinson, H.L. (1983). Genomes of endogenous and exogenous avian retroviruses. *Virology, 126,* 51–72.

Crittenden, L.B. (1991). Retroviral elements in the genome of the chicken: implications for poultry genetics and breeding. *Critical Reviews in Poultry Biology, 3,* 73–109.

Crittenden, L.B. & Kung, H.J. (1984). Mechanisms of induction of lymphoid leukosis and related neoplasms by avian leukosis viruses. In J.M. Goldman & J.O. Jarrett (Eds.), *Mechanisms of Viral Leukaemogenesis* (p. 64). Edinburgh: Livingstone.

Crittenden, L.B., Motta, J.V. & Smith, E.J. (1977). Genetic control of RAV-0 production in chickens. *Virology, 76,* 90–97.

De Boer, G.F. (1986). Approaches to control avian lymphoid leukosis, in: G.F. De Boer (Ed.), *Avian Leukosis* (pp. 261–286). Boston: Martinus Nijhoff Publishing.

Dorner, A.J., Stoye, J.P. & Coffin, J.M. (1985). Molecular basis of host range variation in avian retroviruses. *Journal of Virology, 53,* 32–39.

Duff, R.G. & Vogt, P.K. (1969). Characteristics of two new avian tumor virus subgroups. *Virology, 39,* 18–30.

Fadly, A.M. & Okazaki, W. (1982). Studies of avian leukosis virus infection in chickens from a commercial breeder flock. *Poultry Science, 61,* 1055–1060.

Garwood, V.A., Okazaki, W., Crittenden, L.B. & Lowe, P.C. (1981). Association of lymphoid leukosis virus and performance in a randombred lay population. *Poultry Science, 60,* 2619–2621.

Gavora, J.S., Spencer, J.L., Gowe, R.S. & Harris, D.L. (1980). Lymphoid leukosis virus infection. Effects on production and mortality and consequences in selection for high egg production. *Poultry Science, 59,* 2165–2178.

Gavora, J.S., Spencer, J.L. & Chambers, J.R. (1982). Performance of meat-type chickens test-positive and -negative for lymphoid leukosis virus infection. *Avian Pathology, 11,* 29–38.

Hanafusa, H. (1975). Etiology-viral carcinogenesis. In F.F. Becker (Ed.), *Cancer: A Comprehensive Treatise* Vol. 2 (pp. 49–90). New York: Plenum.

Hauptli, D., Bruckner, L., & Ottiger, H-P. (1997). Use of reverse transcriptase polymerase chain reaction for detection of vaccine contamination by avian leukosis virus. *Journal of Virological Methods, 66,* 71–81.

Ignatovic, J. (1986). Replication-competent endogenous avian leukosis virus in commercial lines of meat chickens. *Avian Diseases, 30,* 264–270.

Ignatovic, J. (1988). Isolation of a variant endogenous avian leukosis virus: non-productive exogenous infection with endogenous viruses containing p27 and p27(0). *Journal of General Virology, 69,* 641–649.

Kottaridis, S.D., Chomiak, T.W. & Luginbuhl, R.E. (1967). Compared antibodies for Rous sarcoma virus in chicken serum and egg yolk with the metabolism inhibition test. *Avian Diseases, 11,* 65–68.

Okazaki, W., Purchase, H.G. & Burmester, B.R. (1975). Phenotypic mixing test to detect and assay avian leukosis viruses. *Avian Diseases, 19,* 311–317.

Okazaki, W., Purchase, H.G. & Crittenden, L.B. (1982). Pathogenicity of avian leukosis viruses. *Avian Diseases, 26,* 553–559.

Payne, L.N. & Howes, K. (1991). Eradication of exogenous avian leukosis virus from commercial layer breeder lines. *The Veterinary Record, 128,* 8–11.

Payne, L.N., Brown, S.R., Bumstead, N., Howes, K.. Frazier, J.A. & Thouless, ME. (1991). A novel subgroup of exogenous avian leukosis virus in chickens. *Journal of General Virology, 72,* 801–807.

Pham, T.D., Spencer, J.L. & Johnson, E.S. (1999). Detection of avian leukosis virus in albumen of chicken eggs using reverse transcription polymerase chain reaction. *Journal of. Virological Methods, 78,* 1–11.

Robinson, H.L. & Eisenman, R.N. (1984). New findings on the congenital transmission of avian leukosis virus. *Science, 225,* 417–419.

Rosner, B. (1995). *Fundamentals of Biostatistics* 4th edn. Duxbury Press:, Wadsworth Publishing Company, U.S.A..

Rubin, H. (1967). The behavior of cells before and after virus-induced malignant transformation. *Harvey Lectures, 61,* 117–43.

Sandelin, K. & Estola, T. (1973). Chicken leukosis virus and its occurrence in Finland. *Scandinavian Journal of Clinical and Laboratory Investigation, 31(suppl 130)*, 33.

Sarma, P.S., Log, T.S., Huebner, R.J. & Turner, H.C. (1969). Studies of avian leukosis group-specific complement-fixing serum antibodies in pigeons. *Virology, 37,* 480–483.

Schwartz, D.E., Tizard, R. & Gilbert, W. (1983). Nucleotide sequence of Rous sarcoma virus. *Cell, 32,* 853–869.

Smith, E.J. (1987). Endogenous avian leukosis viruses, in G.F. De Boer (Ed.), *Avian Leukosis* (pp. 101–120). Boston: Martinus Nijhoff Publishing.

Smith, E.J. & Crittenden, L.B. (1981). Segregation of chicken endogenous viral loci *ev*-7 and *ev*-12 with the expression of infectious subgroup E avian leukosis viruses. *Virology, 112,* 370–373.

Smith, E.J., Fadly, A. & Okazaki, W. (1979). An enzyme-linked immunosorbent assay for detecting avian leukosis-sarcoma viruses. *Avian Diseases*, *23,* 698–707.

Smith, L.M., Brown, S.R., Howes, K., McLeod, S., Arshad, S.S., Barron, G.S., Venugopal, K., McKay, J.C. & Payne, L.N. (1998). Development and application of polymerase chain reaction (PCR) tests for the detection of subgroup J avian leukosis virus. *Virus Research, 54,* 87–98.

Spencer, J.L. (1984). Progress towards eradication of lymphoid leukosis viruses—a review. *Avian Pathology*, *13,* 599–619.

Spencer, J.L. (1987). Laboratory diagnostic procedures for detecting avian leukosis virus infections, in: G.F. De Boer (Ed.), *Avian Leukosis* (pp. 213–240). Boston: Martinus Nijhoff Publishing.

Spencer, J.L. & Chambers, J.R. (1992). Endogenous leukosis viral antigen in eggs from meat-type chickens on an avian leukosis virus eradication programme. *Avian Pathology*, *21*, 251–259.

Spencer, J.L. & Gilka, F. (1982). Lymphoid leukosis: detection of group specific viral antigen in chicken spleens by immunofluorescence and complement fixation. *Canadian Journal of Comparative Medicine, 46,* 370–375.

Spencer, J.L. & Fairfull, R.W. (1993). Study to eradicate avian leukosis virus from a caged White Leghorn population. *Proceedings of the Western Poultry Diseases Conference,* Sacramento, CA, pp. 65–68.

Spencer, J.L., Gavora, J.S. & Gowe, R.S. (1980). Lymphoid leukosis virus: natural transmission and nonneoplastic effects. In M. Essex, G. Todaro & H. Zur Hausen (Eds.), *Viruses in Naturally Occurring Cancers (Book A)* (p. 533). New York: Cold Spring Harbor Laboratory.

Spencer, J.L., Gilka, F., Gavora, J.S. & Wright, P.F. (1984). Distribution of lymphoid leukosis virus and p27 group-specific antigen in tissues from laying hens. *Avian Diseases, 28,* 358–373.

Vogt, P.K. & Ishizaki, R. (1966). In W.J. Burdett (Ed.), *Viruses Inducing Cancer* (pp. 71–90). Salt Lake City, University of Utah Press.

Vogt, P.K. & Friis, R.R. (1971). An avian leukosis virus related to RSV(0): properties and evidence for helper activity. *Virology, 43,* 223–234.

Wisdom, G.B. (1976). Enzyme-immunoassay. *Clinical Chemistry, 22,* 1243–1255.

Yamamoto, T., Juneja, L.R., Hatta, H. & Kim, M. (1997). *Hen Eggs. Their Basic and Applied Science*. Boca Raton, CRC Press.

RÉSUMÉ

Détection des virus endogènes et exogènes de la leucose aviaire à partir d'oûfs 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 oûfs de souches commerciales. Le programme d'éradication 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 limités aux dynamiques de transmission virale. Dans cet article, l'utilisation des nouvelles réactions de transcription 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'oûfs non embryonnés. Il a été observé que 95% des oûfs sur un total de 20, provenant de troupeaux commerciaux pouvant être infectés par des ALV et que 14,2% des ôufs 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 montrè que 4 ôufs 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 > ufs 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'éradication des ALV par l'industrie avicole.

ZUSAMMENFASSUNG

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

Aviäre Leukose-Retroviren (ALV) verursachen Lymphome und andere Tumoren bei Hühnern. Bei früheren Untersuchungen wurden ELISA- und indirekte Immunfluoreszenz (IFA)-Tests verwendet, um das gruppenspezifische ALV-Antigen (*gsa*) *p27* in kommerziellen Hühnereiern nachzuweisen. Im Tilgungsprogramm der Geflügelwirtschaft gegen exogenes ALV werden ELISA-Tests benutzt, um mit dem Virus infizierte Hühner zu erkennen. Das Unvermögen von ELISA- und IFA-Tests, zwischen ALV-*gsa* endogener oder exogener Herkunft und aktuellem Virus zu unterscheiden, hat exakten Einschätzungen der Virusübertragungsdynamik Grenzen gesetzt. Hier berichten wir über die Anwendung einer neu entwickelten Reverse-Transkriptase-Polymerase-Kettenreaktion (RT-PCR) mit direkter Sequenzierung des RT-PCR-Produktes, um endogenes und exogenes ALV im Eiklar von unbefruchteten Hühnereiern nachzuweisen. Wir fanden, daß 95% von 20 Eiern von ALV-exponierten kommerziellen Hühnern und 14,2% von 240 Eierproben aus 20 zufällig ausgewählten Einzelhandelsgeschäften in New Orleans in der RT-PCR ALV-positiv waren. Im Vergleich dazu waren nur 2,5% derselben Eierproben aus den Geschäften im ELISA positiv. Entsprechende direkte Sequenzierungen zufällig ausgewählter RT-PCR-Produkte zeigten, dass 4 von 6 Eierproben endogenes ALV enthielten, während 2 der 6 Proben in Bezug auf exogenes ALV der Untergruppe A positiv waren. Die Feststellung von endogenem ALV der Untergruppe E in unbefruchteten Hühnereirn unterstreicht, dass die Übertragung von endogenem ALV verbreitet ist und bei der Durchführung von ALV-Tilgungsprogrammen durch die Geflügelwirtschaft berücksichtigt werden sollte.

RESUMEN

Deteccion del virus de la leucosis aviar exogeno y endogeno en huevos comerciales utilizando técnicas de transcripcion inversa y reaccion de la polimerasa en cadena

Los retrovirus 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 avícola 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 avícola.