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Development of a Polymerase Chain Reaction to Differentiate Avian Leukosis Virus (ALV) Subgroups: Detection of an ALV Contaminant in Commercial Marek's Disease Vaccines

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SUMMARY. Avian leukosis viruses (ALVs) are common in many poultry flocks and can be detected using an enzyme-linked immunosorbent assay or any other test designed to identify p27, the group-specific antigen located in *gag*. However, endogenous retroviruses expressing p27 are often present and can be confused with exogenous ALVs. A more specific and informative assay involves targeting the variable envelope glycoprotein gene (gp85) that is the basis for dividing ALVs into their different subgroups. We designed polymerase chain reaction (PCR) primers that would specifically detect and amplify viruses from each of the six ALV subgroups: A, B, C, D, E, and J. Subgroup B and D envelopes are related, and our B-specific primers also amplified subgroup D viruses. We also designed a set of common primers to amplify any ALV subgroup virus. To demonstrate the usefulness of these primers, we obtained from the Center for Veterinary Biologics in Iowa culture supernatant from chicken embryo fibroblasts infected with an ALV that was found to be a contaminant in two commercial Marek's disease vaccines. Using our PCR primers, we demonstrate that the contaminant was a subgroup A ALV. We cloned and sequenced a portion of the envelope gene and confirmed that the ALV was a subgroup A virus. Unlike typical subgroup A viruses, the contaminant ALV grew very slowly in cell culture. We also cloned and sequenced a portion of the long terminal repeat (LTR) from the contaminant virus. The LTR was found to be similar to those LTRs found in endogenous ALVs (subgroup E) and very dissimilar to LTRs normally found in subgroup A viruses. The E-like LTR probably explains why the contaminant grew so poorly in cell culture.

RESUMEN. Desarrollo de una prueba de reacción en cadena por la polimerasa para diferenciar los subgrupos del virus de leucosis aviar: Detección de un virus de leucosis aviar contaminante en vacunas comerciales contra la enfermedad de Marek.

Los virus de leucosis aviar son comunes en muchas parvadas de aves domésticas y pueden detectarse mediante el uso de la prueba de inmunoensayo asociado a enzimas o cualquier otra prueba diseñada para identificar p27, que es el antígeno especifico de grupo localizado en el gen *gag*. Sin embargo, retrovirus endógenos expresando p27 están presentes comúnmente y pueden ser confundidos con virus exógenos de leucosis aviar. Una prueba más especifica e informativa implica la detección del gen para la glicoproteína de envoltura variable (gp85) en la que se basa la división de los virus de leucosis aviar en sus diferentes subgrupos. Se diseñaron iniciadores para la prueba de reacción en cadena por la polimerasa que específicamente detecten y amplifiquen virus de cada uno de los seis subgrupos del virus de leucosis aviar: A, B, C, D E y J. La envoltura de los subgrupos B y D están relacionadas, y nuestros iniciadores específicos diseñados para el subgrupo B también amplificaron virus del subgrupo D. A su vez, se diseñaron un par de iniciadores comunes para amplificar cualquier subgrupo de virus de leucosis aviar. Para demostrar la utilidad de estos iniciadores, se obtuvo del Centro de Biológicos Veterinarios en el estado de Iowa, sobrenadante de un cultivo de fibroblastos de embrión de pollo infectado con un virus de leucosis aviar contaminante de dos vacunas comerciales de la enfermedad de Marek. Utilizando nuestros iniciadores, se demostró que el virus contaminante era un virus de leucosis aviar perteneciente al subgrupo A. Se clonó y secuenció una porción del gen de envoltura y se confirmó que el virus de leucosis aviar era un virus del subgrupo A. A diferencia de los virus típicos del subgrupo A, el contaminante creció muy lentamente en cultivo celular. A su vez se clonó y secuenció una porción de las repeticiones terminales largas del virus contaminante. Las repeticiones terminales largas resultaron similares a las repeticiones terminales largas encontradas en los virus de leucosis aviar endógenos (subgrupo E) y muy diferentes a las repeticiones terminales largas normalmente encontradas en los virus del subgrupo A. Las repeticiones terminales largas tipo E probablemente explican la deficiencia en el crecimiento del contaminante en cultivos celulares.

Key words: PCR, ALV, diagnosis, vaccine, poultry, Marek's disease

Abbreviations: ADOL = Avian Disease and Oncology Laboratory; ALV = avian leukosis virus; CEF = chicken embryo fibroblast; CVB = Center for Veterinary Biologics; ELISA = enzyme-linked immunosorbent assay; LTR = long terminal repeat; MD = Marek's disease; PCR = polymerase chain reaction; RFS = rapid feathering susceptible

Avian leukosis viruses (ALVs) are members of the *Retroviridae* family (5). Based upon differences in their envelopes, ALVs are divided into six subgroups. Subgroups A, B, C, D, and J are classified as exogenous viruses and can induce B-cell lymphomas in susceptible chickens. Subgroup A viruses are the most common subgroup found in field flocks (3). However, in 1989, a subgroup J ALV (ALV-J) was isolated in the U.K. (6). Subsequently, ALV-J spread throughout the world, resulting in severe economic losses in the commercial broiler breeder industry. Subgroup E viruses are endogenous ALVs, present in nearly all chicken genomes, and they generally induce little to no oncogenicity. Traditionally, differentiation of subgroups has been accomplished with interference assays, host range assays using susceptible and resistant cells, or with subgroup-specific antisera (3). Although effective, these procedures are usually very time consuming, often requiring the use of multiple cell culture plates. In contrast, polymerase chain reaction (PCR) is very specific, and a PCR assay can be completed in a day. We describe a set of PCR primers and conditions that will selectively detect and amplify ALV subgroups.

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Table 1. Chicken lines.

Line	Subgroup resistance	Characteristics
SPAFAS	E or none	Some lines lack receptor for subgroup E
15B1	None	Contains endogenous subgroup E
Line 0	E	Lacks any endogenous viruses
RFS	None	Derived from line 0 and lacks any endogenous viruses

Rapid and efficient detection of ALV has recently been used to detect ALV contamination of a Marek's disease (MD) vaccine (4,13). Recently the USDA Center for Veterinary Biologics (CVB) isolated an ALV from commercial MD vaccines. We report utilizing our subgroup-specific PCR primers to identify the ALV isolate as a subgroup A ALV.

MATERIALS AND METHODS

Cell culture and viruses. Primary chicken embryo fibroblast (CEF) cultures were prepared as previously described (9) and were grown in Leibovitz L-15 medium plus McCoy 5A medium (1:1), supplemented with 2.5% bovine serum and antibiotics. Table 1 lists the CEFs used in this study. ALV subgroup J strain HPRS-103 was obtained from Dr. L. N. Payne (Institute of Animal Health, Compton, U.K.) (6). ALV subgroups A, B, C, D, and E were obtained by infecting susceptible CEFs with Avian Disease and Oncology Laboratory (ADOL) stocks of Rous-associated virus RAV-1, RAV-2, RAV-49, RAV-50, and RAV-0, respectively. After 7–9 days in culture, total DNA from CEF-infected cells was extracted using standard proteinase K, phenol–chloroform extraction procedures.

Growth of ALV isolates in cell culture. CVB isolated a subgroup A–like ALV from commercial MD vaccines from two different manufacturers (designated A and B in Table 2). At the ADOL, the supernatant fluids from the infected cultures were passed on line 0 (C/E) and rapid feathering susceptible (RFS) C/O line (developed at the ADOL) cells for four passages, using varying concentrations of chicken and calf serum. Following each passage, cell lysates were tested using an enzyme-linked immunosorbent assay (ELISA) for the presence of ALV group–specific antigen (p27), as previously described (11).

PCR. To generate PCR primers, we assembled and aligned available chicken ALV envelope sequences contained in GenBank. Based upon the alignment, we chose PCR primers (Oligo 4.0-s software; National Biosciences, Plymouth, MN) that would amplify a member of a specific ALV subgroup but that would not amplify viruses in different subgroups. Six pairs of PCR primers were chosen that were specific for the six different subgroups. One pair of PCR primers was designed to amplify all ALVs from all six ALV subgroups (Table 3). Each 50-µl PCR reaction contained 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 25 mM of each dNTP, 25 pmoles of each primer, 1.25 U Taq polymerase, and approximately 75 ng of template DNA. The following conditions were used with the two primer pairs designed to amplify all chicken ALVs and ALV-J; following an initial template melting step at 95 C for 3 min, the DNA was amplified during 30 cycles of 95 C for 1 min, 57 C for 1 min, and 72 C for 2 min, followed by a final elongation step at 72 C for 5 min. Different PCR conditions were used with the A–E subgroup–specific PCR primers, which comprised an initial template melting step at 95 C for 3 min, after which the DNA was amplified during 30 cycles of 95 C for 1 min, 60 C for 30 sec, and 72 C for 1.5 min, followed by a final elongation step at 72 C for 5 min.

DNA sequencing. Proviral DNA from infected CEFs was sequenced on an ABI Model 377 automatic DNA sequencer (Applied Biosystems, Foster City, CA), and contigs were constructed using Sequencer (Gene Codes Corp., Ann Arbor, MI). DNA was aligned using the Clustal W method, as implemented in MegAlign (DNASTAR Inc., Madison, WI). Phylogenetic relatedness of the isolates was also calculated using MegAlign. The following aligned sequences were obtained from GenBank: EV1 long terminal repeat (LTR), Accession number AY013303; EV3 LTR, Accession number AY013304; RAV1 LTR, Accession number M37980; RAV1 ENV; RAV-1 envelope, Accession number M37980; RAV0 ENV, RAV-0 envelope, Accession number M12172.

Table 2. MD vaccines contaminated with an ALV.

Manufacturer code	CVB No.	ADOL No.	Originally tested at ADOL
A	5060[A]	A46	Yes
A	5061[A]	A49	Yes
A	6508	None	No
B	5057	B39	Yes
B	5058	B53	Yes
B	5062	B50	Yes
B	7376[A]	None	No

[A]Selected for further studies at ADOL.

RESULTS

Replication of CVB isolates. CVB recently isolated a subgroup A ALV from MD vaccine vials from two companies (A and B in Table 2). We previously had identified and characterized ALV-A contaminants in some of the same vaccine batches that CVB later examined (indicated by a 'Yes' in the last column of Table 2). Three of the CVB isolates, as indicated in Table 2, were chosen for further characterization.

Initially the three isolates were passed on C/E CEFs and later in line 0 (C/E) and RFS (C/O) cells. The three isolates grew slowly in culture, and it was only at passage 4 that the isolates grew to a good titer, as measured by an ELISA assay (Table 4).

Table 3. PCR primers.

Subgroup	Forward primer[A]	Reverse primer[B]	PCR product (kilobase)
All[C]	CGAGAGTGGCTCGCGAGATGG	ACACTACATTTCCCCCTCCCTAT	2.4
A	CGAGAGTGGCTCGCGAGATGG	CCCATTTGCCTCCTCTCCTTGTA	1.3
B and D	CGAGAGTGGCTCGCGAGATGG	AGCCGGACTATCGTATGGGGTAA	1.1
C	CGAGAGTGGCTCGCGAGATGG	CCCATATACCTCCTTTTCCTCTG	1.4
E	CGAGAGTGGCTCGCGAGATGG	GGCCCCACCCGTAGACACCACTT	1.25
J[D]	CTTGCTGCCATCGAGAGGTTACT	AGTTGTCAGGGAATCGAC	2.3

[A]All forward primers, except for J, bind to the reverse transcriptase gene. The J forward primer binds immediately upstream of gp85.
[B]Reverse primer for "All" binds in gp37. Reverse primers for subgroups A, B, C, D, and E bind to unique regions in the gp85 gene. The reverse primer for J binds to the LTR.
[C]Subgroups A, B, C, D, E, and J.
[D]The subgroup J primers and PCR conditions were described in an earlier publication (8).

Table 4. Propagation of viruses isolated from MD vaccines by CVB.

CVB code (manufacturer)	Samples	Passage 0[A] VI Line 0[C]	Passage 0[A] PCR ALV-A	Passage 0[A] PCR ALV-E	Passage 1[B] VI Line 0	Passage 1[B] PCR ALV-A	Passage 1[B] PCR ALV-E
5060 (A)	S-1[D]	+2	+	−	+1	+	−
	S-2	+2	+	−	+4	+	−
5061 (A)	S-1	+1	+weak	−	+4	+	−
	S-2	+2	+	−	+4	+	−
7376 (B)	S-1	+1	+	−	+3	+	−
	S-2	+1	+	−	+3	+	−

[A]Passage 0 represents the initial inoculation of the sample in tissue culture.
[B]Passage 1 represents passing the supernatant from passage 0 onto fresh tissue culture cells.
[C]Numbers represent a subjective estimate of p27 ELISA intensities, from weakest, +1, to most intense, +4.
[D]CVP initially divided each isolate in duplicate and designated the isolates as either sample 1 or sample 2.

Subgroup-specific PCR. The PCR primers were designed to amplify a portion of the gp85 envelope gene of ALVs from different subgroups (Table 3). The subgroup J primers were described in an earlier publication (8) and are included here for the sake of completeness. With the exception of the subgroup B primers and the all-subgroup primer pair, the PCR primers were specific for only one subgroup (Fig. 1). The subgroup B primers also amplified subgroup D viruses (Fig. 1B). The subgroup E–specific PCR primers amplified the endogenous ALVs present in both SPAFAS and 15B1 cells (Fig. 1D).

Using our new PCR primer pairs, we were only able to PCR amplify a product from the three isolates using the ALV-A–specific primers and the all-subgroup primer pair. Cell culture passage 0 was positive with the ALV-A–specific PCR primers (Table 4), confirming the original findings by the CVB lab. To further confirm that the isolates contained an ALV-A, we cloned and sequenced the gp85 envelope gene from each isolate.

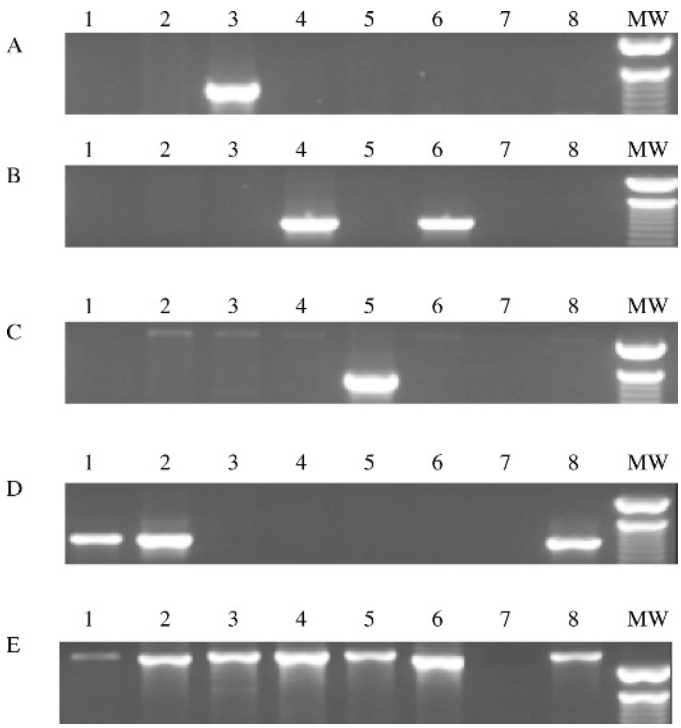

Fig. 1. PCR amplification of proviral DNA extracted from CEFs. Lane 1: noninfected 15B1 cells; Lane 2: RAV-0; Lane 3: RAV-1; Lane 4: RAV-2; Lane 5: RAV-49; Lane 6: RAV-50; Lane 7: noninfected line 0 cells; Lane 8: noninfected SPAFAS. (A) Subgroup A–specific primers; (B) subgroup B– and subgroup D–specific primers; (C) subgroup C–specific primers; (D) subgroup E–specific primers; (E) primers designed to amplify all chicken ALV subgroups.

Sequencing gp85 and the LTR. The gp85 genes from the three isolates were sequenced and aligned with the envelope sequences of RAV-1 (subgroup A) and RAV-0 (subgroup E) obtained from GenBank (data not shown). A phylogenetic tree generated from the alignments demonstrates that the envelope gene from the isolates is closely related to the envelope from a subgroup A virus and not a subgroup E virus (Fig. 2A).

Specific DNA motifs in the U3 region of the LTR function as transcriptional regulatory elements (1,2). Exogenous ALVs that replicate well usually have two CArG boxes [$CC(A/T)_6GG$], while the endogenous, poorly replicating, subgroup E viruses generally have only one or no CArG boxes (12). Similarly, endogenous ALV U3 elements generally contain one Y Box (ATTGG), while exogenous viruses often contain two Y Boxes. The slow cell-culture replication of our isolates indicated that their LTRs might lack one or more regulatory/enhancer elements. Therefore, we cloned and sequenced the LTRs from each isolate. We aligned the sequences with representative LTR sequences obtained from GenBank (Fig. 3). The U3 element in all three isolates contained only one CArG box and one Y Box. The DNA sequence, alignment, and resulting phylogenetic tree indicated that the contaminating isolates had

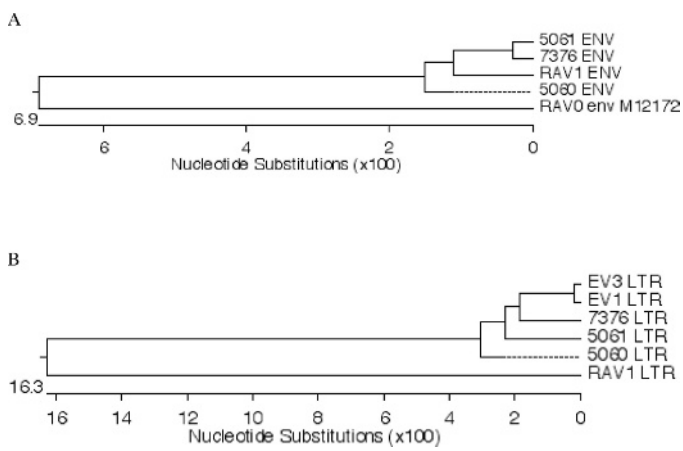

Fig. 2. Phylogenetic relationship of the viral envelopes and LTRs. Sequences in GenBank were aligned with the three official CVB isolates using Clustal W. A phylogenetic tree was prepared using Lasergene from DNASTAR. (A) Phylogenetic tree for the gp85 envelope. (B) Phylogenetic tree comparing the LTRs.

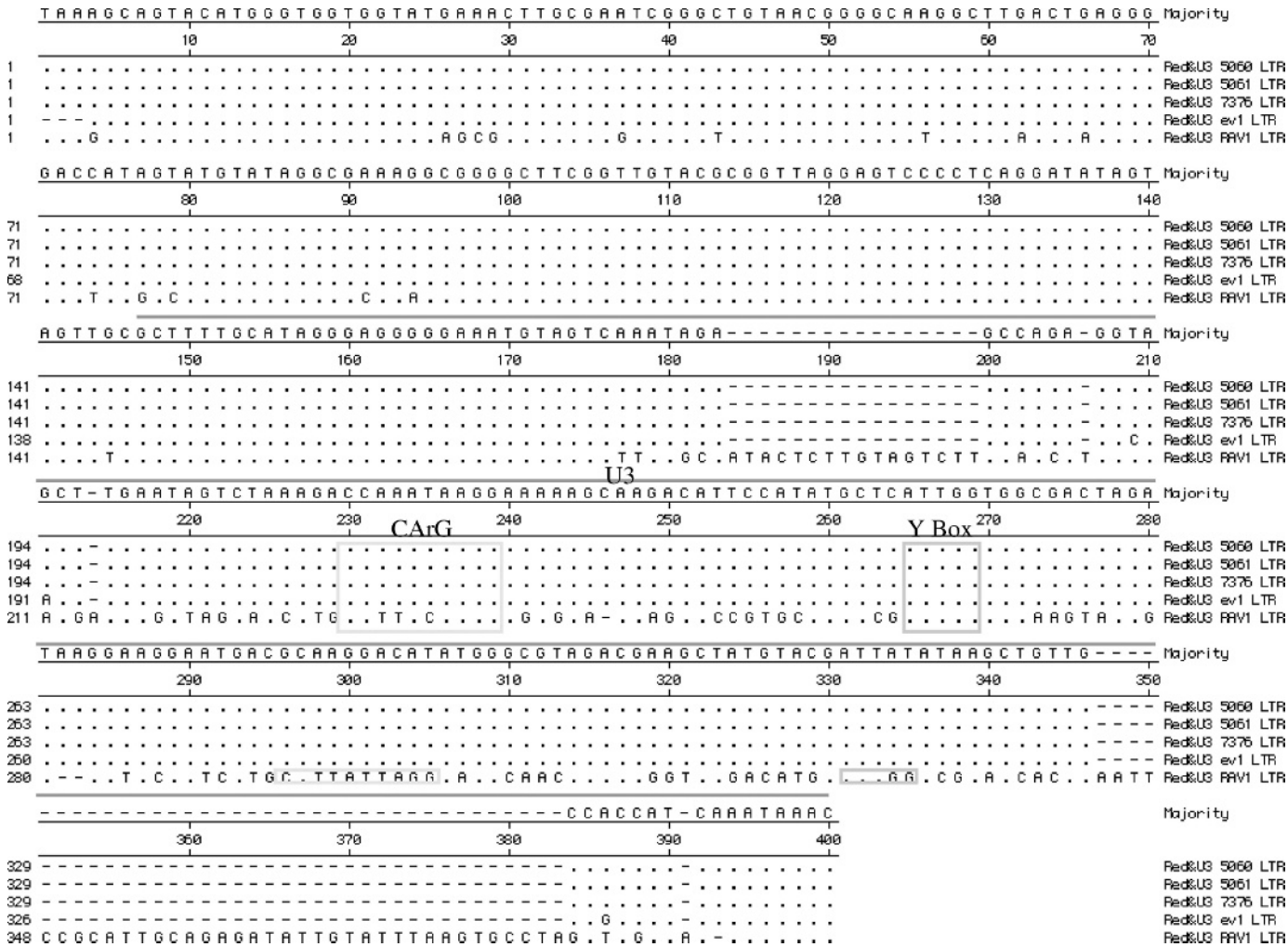

Fig. 3. LTR alignment. The noncoding redundant region, U3 (indicated by a line under sequences No. 76-330), R, and a portion of U5 were aligned using MegAlign. Sequences matching the majority sequence are shown as dots, and deleted sequences are shown as a dash. One CArG box (boxed sequences 230–239) and one Y box (boxed sequences 265–269) are located in all of the LTRs. RAV-1 contains a second CArG box (boxed sequences 295–305) and Y box (boxed sequences 331–335).

a LTR unrelated to exogenous LTRs and were more closely related to the LTRs associated with endogenous ALVs (Fig. 2B).

DISCUSSION

Detection of the p27 antigen is often used to diagnose ALV infections. While an ELISA assay for p27 is relatively fast and convenient, the test does not discriminate between the different ALV subgroups. Consequently, the frequent presence of endogenous subgroup E viruses in chickens can result in a positive p27 test that could erroneously be interpreted as being positive for an exogenous ALV. To differentiate between exogenous and endogenous ALVs, we designed PCR primers specific for the gp85 envelope gene, the subgroup-determining factor for ALVs. With one exception, the PCR amplifications were very subgroup specific. Our PCR designed to amplify subgroup B viruses also amplified subgroup D viruses. Since subgroup B and D envelopes are similar and share the same cellular receptor (10), the cross-reactivity was not unexpected. We believe the PCR primers described here will be useful for most if not all ALV isolates. Nevertheless, we have not determined the sensitivity of our PCRs and cannot be certain that there are some untested ALV isolates that will fail with these primers.

To evaluate the usefulness of the PCR primers, we used each PCR primer pair on cultures infected with an ALV-A contaminant provided by CVB. PCR amplification indicated that the contaminant was a subgroup A virus. Subsequent cloning and sequencing confirmed that the contaminant was an ALV-A. However, in the process of replicating the contaminant in cell culture, we found that the virus grew very slowly, unlike typical ALV-As. Since the ALV LTRs are critical elements that define their replicative ability (7,12), we cloned and sequenced the LTRs. Aligning the sequences from the contaminating viruses with sequences of ALV-A and ALV-E taken from GenBank clearly showed that the LTRs were not typical LTRs found in exogenous ALVs, such as ALV-A, but rather were closely related to LTRs found in subgroup E viruses. We believe the E-like LTR was responsible for the slow replication. The slow replication and low viral titers were probably the reasons the contaminant was missed during the initial screening.

The PCR primers reported here should be useful for anyone wishing to determine whether any unknown samples contain ALVs and, if so, what ALV subgroups are present. Our identification of an

ALV contaminant in commercial vaccines indicates that a slow-replicating subgroup A contaminant might escape detection if an insensitive assay, such as a COFAL assay, is used. Laboratories screening vaccines should consider adopting a more sensitive ELISA or PCR assay. Although we have not determined the sensitivity of our reported PCR, the PCR was sensitive enough to detect the slow-growing subgroup A contaminant in the commercial vaccines.

We also want to emphasize that all the PCR amplifications were performed on viruses replicating in tissue culture cells and have not been tried with tissues from infected poultry. Additional research will be needed before these PCRs can be used to diagnose ALV-induced tumors in chickens. Finally, the primers described here were designed to detect the ALVs commonly found in chickens. Nevertheless, a BLAST search of GenBank indicated that the subgroup A–specific primers might also be used to PCR amplify uncommon viruses such as myoblastosis-associated virus-1 (subgroup A).

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