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Avian oncogenesis induced by lymphoproliferative disease virus: a neglected or emerging retroviral pathogen?

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Abstract

Lymphoproliferative disease virus (LPDV) is an exogenous oncogenic retrovirus that induces lymphoid tumors in some galliform species of birds. Historically, outbreaks of LPDV have been reported from Europe and Israel. Although the virus has previously never been detected in North America, herein we describe the widespread distribution, genetic diversity, pathogenesis, and evolution of LPDV in the United States. Characterization of the provirus genome of the index LPDV case from North America demonstrated an 88% nucleotide identity to the Israeli prototype strain. Although phylogenetic analysis indicated that the majority of viruses fell into a single North American lineage, a small subset of viruses from South Carolina were most closely related to the Israeli prototype. These results suggest that LPDV was transferred between continents to initiate outbreaks of disease. However, the direction (New World to Old World or vice versa), mechanism, and time frame of the transcontinental spread currently remain unknown.

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Keywords

Lymphoproliferative disease virus (LPDV); alpharetrovirus; avian tumor viruses; exogenous retrovirus; oncogenesis; Order Galliformes

Introduction

Lymphoproliferative disease virus (LPDV) is an exogenous retrovirus that induces a neoplastic syndrome in domestic turkeys (*Meleagris gallopavo*), resulting in the formation of lymphoid tumors in multiple organs (Biggs et al., 1978). Although there is circumstantial evidence of LPDV-like outbreaks occurring in domestic turkeys in Europe prior to the 1970's, the virus was not identified as an etiological agent of disease until 1972 in the United Kingdom (Biggs et al., 1974). Within a few years of its recognition, LPDV outbreaks were documented in additional European countries as well as Israel (Biggs, 1997; Ianconescu et al., 1983). These outbreaks remained confined to the European-Middle Eastern region and the virus has never been detected outside of this area, including North America. Although LPDV is characterized as an oncogenic retrovirus, it does not contain a putative viral oncogene, nor does it appear to exert its oncogenic effects through insertional mutagenesis of a cellular proto-oncogene; thus, the mechanism by which lymphoproliferative disease is induced is unknown (Chajut et al., 1991; Sarid et al., 1994; Gazit and Yaniv, 1999).

In domestic turkeys, disease due to LPDV infection is generally first noted around eight to 10 weeks of age, with a variable flock mortality that can reach 25% (Gazit and Yaniv, 1999; Biggs, 1997). The characteristic lesion of LPDV infection is a pleomorphic infiltrate of lymphocytes, including lymphoblasts intermixed with plasma cells and monocytic reticular cells in multiple tissues. The most commonly affected organs are spleen, thymus, pancreas, and liver, although smaller focal lesions can be present in a variety of other tissues (Biggs, 1997). LPDV has been demonstrated experimentally to infect both turkeys and chickens (*Gallus gallus*), although not ducks or geese (species not listed; Ianconescu et al., 1983), suggesting that the natural host range of the virus may be restricted to birds of the order Galliformes, which also includes other game birds such as pheasants, quail, and grouse; however, reports of natural LPDV infections have been limited to domestic turkeys. Under experimental conditions, viremia occurs approximately two weeks after infection and may last for up to 10 months (Zimmer et al., 1983) and horizontal transmission can occur among birds in close contact (McDougall et al., 1978). How the virus is transmitted in nature (e.g., vector-borne, horizontal/vertical transmission) is uncertain.

Diagnostically, the identification of LPDV has been hampered by the inability to isolate and propagate the virus in an appropriate culture system, including embryonating eggs, primary cell cultures, and established cell lines (Gazit and Yaniv, 1999). However, inoculation of naive turkeys with lymphocytes transfected with an infectious clone resulted in the reproduction of disease and the detection of the virus in blood and tissues, indicating that LPDV is replication-competent (Gak et al., 1989; Gak et al., 1990). Thus, similar to 'slowly-transforming' exogenous strains of the related *avian leukosis virus (ALV)* (genus *Alpharetrovirus*), LPDV is a non-oncogene-containing, replication-competent, tumorigenic virus (Payne and Venugopal, 2000). Although a number of seminal studies conducted in Israel and the United Kingdom provided insights into the molecular genetics, pathogenicity, and cellular tropism of LPDV, the extent of its genetic diversity and source of emergence remains unknown (Chajut et al., 1992; Gak et al., 1989; Gak et al., 1991; Gazit et al., 1979; Gazit et al., 1982; Gazit et al., 1983; Gazit et al., 1986; McDougal et al., 1978; Yaniv et al., 1979; Zimmer et al., 1983).

In January 2009, an adult female wild turkey in Columbia County, Arkansas, was found recumbent and moribund. The turkey died shortly thereafter and was submitted to the Southeastern Cooperative Wildlife Disease Study (SCWDS) at the University of Georgia for diagnostic evaluation. Grossly, this turkey had marked splenomegaly, multiple demarcated tan foci scattered throughout the liver, and a diffuse thickening of the proximal small intestinal wall. Microscopically, infiltrating neoplastic lymphoid cells were identified in various organs, including the spleen, liver, intestines, kidney, pancreas, and lung. Attempts to isolate endemic oncogenic avian viruses, including *reticuloendotheliosis virus* (REV), *Gallid herpesvirus 2* (GaHV-2), and ALV, in a number of avian cell lines and specific-pathogen-free embryonating chicken eggs were unsuccessful. To further investigate a possible viral etiology for the lymphoid neoplasia, spleen, liver, and lung samples were screened for LPDV by PCR targeting a region spanning the p31 and capsid (CA) genes. Proviral LPDV DNA was detected in all three tissues, representing the first detection of LPDV outside of Europe and the Middle East, and the first natural LPDV infection in a wild avian host.

Following this initial identification, we attempted to determine whether LPDV may be a widespread, yet unrecognized, pathogen in the United States by screening select wild turkey diagnostic cases, as well as seasonally harvested wild turkeys, for the virus. In this report, we provide a description of natural LPDV infection in North American wild turkeys, including basic epidemiologic patterns, frequency and description of lesions associated with LPDV infection, the co-occurrence of LPDV with other common avian viral pathogens, and the prevalence of LPDV infection in apparently healthy birds. We conducted a comparative analysis of the proviral genome of North American LPDV and the Old World prototype strain from Israel and mapped the insertion site of LPDV into the host genome. We also performed a phylogenetic analysis of proviruses recovered from birds collected from 18 states between 2009-2012 with that of the Israeli prototype, and examined the evolutionary relationship of LPDV to other retroviruses.

Results

Detection of LPDV within the United States and lesions observed in diagnostic cases

Following the first recognition of LPDV in North America, we tested tissues from select clinically ill wild turkeys submitted to diagnostic laboratories throughout the eastern United States for LPDV proviral DNA. Including the prototype North American strain (12/AR/2009), LPDV was detected in 41 wild turkeys from 18 states, stretching from Maine to Louisiana and west to Colorado (Table 1), encompassing an area covering most of the natural geographic distribution of wild turkeys in the United States (Hatfield and Vance, 2009). The vast majority of LPDV-positive wild turkeys were adults (36/41; 87.8%), but five (12.2%) were hatch-year birds. There was a near even distribution of males (19/41; 46.3%) and females (18/41; 43.9%); sex was not determined for four wild turkeys. Tissues from two of the 41 LPDV-positive wild turkeys were not evaluated microscopically due to the poor post-mortem condition of the carcass. Of the remaining 39 LPDV-positive wild turkeys, only a small minority (6/39; 15.4%) had microscopic lesions consistent with LPDV infection in domestic turkeys (i.e., lymphoid neoplasia). However, as REV was also detected in two of these birds (196/NC/2012 and 453/NJ/2012), LPDV infection in the absence of any other detectable pathogens was diagnosed in only four birds with neoplasia-related mortality (12/AR/2009, 122/WV/2009, 152/GA/2011, 592/MS/2012; Table 1). The remaining 33 LPDV-positive wild turkeys were diagnosed with other causes of morbidity or mortality in the absence of lymphoid neoplasia, including *fowlpox virus* (FWPV) infection (16/39; 41.0%), bacterial infections, including systemic or skin infections (8/39; 20.5%), trauma (2/39; 5.1%), endoparasitism (1/39; 2.6%), toxicosis (1/39; 2.6%), or undetermined

(5/39; 12.8%). In addition to the two birds with lymphoid neoplasia that were positive for both LPDV and REV, another 17 of the LPDV-positive turkeys (19/41; 46.3%) also tested positive for REV proviral DNA and 12 of 41 turkeys (29.3%) were simultaneously positive for LPDV, REV, and FWPV (Table 1).

Of the six wild turkeys determined to have died from lymphoid neoplasia in which LPDV was detected, five were adults and one was a hatch-year bird. There was an even sex distribution among male and female birds. Each of the six cases were isolated disease events involving a single bird and all affected wild turkeys were found in a moribund condition exhibiting non-specific clinical signs including lethargy, disorientation, ataxia, and recumbency. Entire carcasses from five of the birds and tissues (skin, bone marrow, and liver) from one bird were submitted for diagnostic examination. All five of the examined wild turkey carcasses were emaciated. Other gross lesions included splenomegaly (3/5; 60%), tan nodules in the liver (2/5; 40%), proliferative growths in the unfeathered skin of the head and legs (2/5; 40%), and diffuse or nodular thickening of the intestinal wall (2/5; 40%) (Figure 1). Microscopically, the five wild turkey carcasses had infiltrates of neoplastic lymphoid cells in tissues from multiple organs, including liver (5/5; 100%), kidney (4/5; 80%), lung (4/5; 80%), spleen (3/5; 60%), gastrointestinal tract (2/5; 40%), skin (2/5; 40%), heart (2/5; 40%), brain (1/5; 20%), adrenal gland (1/5; 20%), pancreas (1/5; 20%), and skeletal muscle (1/5; 20%). The wild turkey for which only tissues were submitted had similar neoplastic infiltrates in the skin and bone marrow, but not in the liver. In all six cases, neoplastic round cells morphologically consistent with lymphocytes (often intermixed with variable numbers of plasma cells) formed densely cellular sheets that effaced and expanded the underlying parenchyma (Figure 2), concordant with lesions observed in LPDV-infected domestic turkeys (Biggs, 1997). In some cases, the neoplastic cells also formed coalescing nodules (most often in skin) and perivascular aggregates. Neoplastic cell populations were generally pleomorphic and cells had scant, eosinophilic cytoplasm and round, hyperchromatic nuclei with inapparent nucleoli and rare to occasional mitotic figures. Occasional necrotic foci were scattered among some of the more densely cellular areas in some tissues, most commonly in the liver and skin. The neoplastic lymphoid infiltrates were most reliably recognized in LPDV cases with little autolysis, as poor post-mortem condition limits detailed examination and interpretation of lesions and thus could hinder detection of cases. There were no obvious differences in the character, distribution, or frequency of lesions between the four LPDV-positive and two LPDV/REV-positive turkeys with lymphoid neoplasia.

Detection of LPDV in asymptomatic seasonally-harvested wild turkeys—To obtain preliminary insights into the prevalence of LPDV infection in apparently healthy wild turkeys, we tested livers from 74 hunter-collected turkeys from 10 different counties in South Carolina during 2010. Thirty-three (44.6%) of the birds tested were positive for LPDV proviral DNA by PCR. The remaining 41 (55.4%) were negative. Thus, nearly half of the birds tested in South Carolina were positive for LPDV infection, suggesting widespread prevalence within the state. None of the 74 wild turkeys were reportedly exhibiting overt signs of disease when harvested or had gross lesions identified during routine processing. However, microscopic examination of tissues for evidence of neoplasia was not performed on tissues from these birds.

Genomic analysis of the prototype North American LPDV—The complete proviral genome of the first LPDV case identified in North America (12/AR/2009) was PCR-amplified from liver tumor tissue. The proviral genome of 12/AR/2009 is 7,432 nucleotides in length (including both LTRs) and is flanked by two six-base pair repeats derived from the host (CCCAGC). The length of the LTRs, genes, and proteins are shown in Table 2. For 12/AR/2009, the tRNA^{Trp} primer binding site is ³²⁹-UGGUGACCCCGACGUGAU-³⁴⁶ and the

polypurine tract is ⁷⁰⁹⁶-GGGGAGGGGA⁻⁷¹⁰⁶, identical to the Israeli strain of LPDV and very similar to other alpharetroviruses (Gak et al., 1991; Whitcomb et al., 1995). Comparison of the 12/AR/2009 genome to the prototype Israeli strain (GenBank accession U09568) demonstrated a nucleotide identity of 87.9%, with both viruses containing the same genomic organization of 5'-LTR-*gag-pro-pol-env*-LTR-3'. Nucleotide conservation among the major ORFs in the two prototypes was similar, with *pol* exhibiting the highest degree of identity (90.1%), followed by *gag* (88.3%), *pro* (88.3%), and *env* (86.6%). Although most genes were of similar length, the predicted SU protein of strain 12/AR/2009 was 47 amino acids longer than the Israeli prototype due to an extended N-terminus. In this context, the start codon for SU in 12/AR/2009 is positioned one nucleotide downstream from the previously identified splice acceptor site for *env* (Sarid et al., 1994).

Similar to members of the *Alpharetrovirus* genus, the *pol* polyprotein of LPDV is produced by ribosomal frameshifting (Sarid et al., 1994). However, in contrast to alpharetroviruses, the viral protease (*pro*) of LPDV is also produced by a -1 ribosomal frameshift initiated by a conserved ²⁴⁴⁷-GGGAAAC⁻²⁴⁵³ signal sequence and predicted stem-loop structure, thus being more similar to betaretroviruses in this instance (Sarid et al., 1994; Gruber et al., 2008; Stoye et al., 2011). In conjunction with *pro* not being expressed as part of *gag* as it is in other alpharetroviruses, the LPDV protease contains an Asp-Thr-Gly active site rather than the thermodynamically less stable and less active Asp-Ser-Gly triplet (Ingr et al., 2003). Comparison of the *pol* polyprotein [reverse transcriptase (RT) and integrase (IN) proteins; 850 amino acids] of the North American prototype of LPDV to other retroviruses demonstrated that it shared the highest amino acid identity to the Israeli LPDV prototype (92.9%), followed by *Rous sarcoma virus* (RSV) (44.0%) and ALV (43.7%), in accordance with the phylogenetic analysis of *pol* (see *Evolutionary analysis of LPDV* below). Other LPDV proteins that are generally more conserved among the retroviruses, such as the CA and matrix (MA) proteins, also shared similar levels of amino acid identity as *pol* to other alpharetroviruses, although the nucleocapsid (NC; 98 amino acids) of LPDV exhibits a moderate amino acid identity (38.7%) to *simian immunodeficiency virus* (SIV) from NC residues 6-96, including similar zinc finger (Cys-X₂-Cys-X₄-His-X₄-Cys) motifs. Thus, although LPDV is most closely related to other avian oncogenic viruses of the genus *Alpharetrovirus*, it shares some identifiable characteristics with other closely related genera (i.e., *Betaretrovirus* frameshift strategies and *Lentivirus* NC homology).

A functional role for the p31 protein of LPDV (located between the MA and CA proteins) (Table 2) has not previously been ascribed (Sarid et al., 1994). However, two putative L domain motifs (PTAP and PPXY) separated by 11 amino acids were identified near the N-terminus, suggesting that p31 may recruit cellular factors that mediate budding of virions from infected cells (Freed, 2002; Pincetic and Leis, 2009). Additionally, the C-terminus of p31 in LPDV contains an LTDWGLVATEC motif similar to that of the RSV p10 protein that has been demonstrated to be critical for immature particle assembly, suggesting that p31 may also interact with CA (Phillips et al., 2008). The only large gap in the proviral genome sequence between the North American and Israeli LPDV prototypes was found in the U3 region of the LTR, where the approximate equivalent of nucleotide positions 21 to 49 (or 24 to 52) in the Israeli strain was missing in 12/AR/2009. Although this missing stretch of nucleotides is just upstream of direct repeat elements shown to be essential for efficient transcription, deletion of the distal 5' end of U3 in LPDV (which includes parts of the missing region) has been shown to increase transcriptional activity, suggesting that this region may act as a negative regulatory element (Sarid et al., 1995).

LPDV integration—Mapping of the insertion site of the North American prototype strain (12/AR/2009) demonstrated that it was integrated, in reverse orientation, at nucleotide position 45,329,568 in chromosome 6 of the turkey genome (GenBank accessions

NC_015016.1 and ADDD01042526), approximately 10.6 kB downstream of a C-C chemokine receptor type 4-like protein and 4.2 kB upstream of a beta-galactosidase-like protein. Although no known gene function has yet to be ascribed to the genomic region in which the provirus was integrated, BLAST analysis (using a Class Aves search set in the nucleotide collection database) of the integration site demonstrated the closest match was to a known oncogene, a serine/threonine kinase receptor-associated protein (GenBank accession XM_003202477), albeit only to the very 3'-terminus of the mRNA (Datta et al., 1998; Halder et al., 2006). Currently, it is unclear whether insertion at this site is related to the fatal oncogenesis observed in this case.

As both LPDV and FWPV were simultaneously detected in multiple birds (Table 1), we analyzed a subset of these cases to determine whether LPDV may be inserted into the genome of FWPV, similar to that observed in REV (Hertig et al., 1997). Although both LPDV and FWPV could be PCR amplified from original tissue samples, only FWPV could be detected after passage in avian cell culture, indicating that the isolated poxviruses did not contain LPDV sequences. This finding suggests that, in these cases, either the detection of both viruses is due to temporally distinct infections (e.g., LPDV integration into the host genome during a former infection followed by the diagnosis of an active FWPV infection) or that these were concomitant infections without recombination occurring between the two viruses.

Evolutionary analysis of LPDV—To determine the evolutionary relationships among the North American viruses, as well as to the prototype Israeli strain (GenBank accession U09568), we performed a phylogenetic analysis on the newly identified proviruses recovered from birds collected from 18 states between 2009-2012 (Table 1) using a portion of the *gag* polyprotein (partial p31/partial CA; 413 nt). A nucleotide and amino acid alignment of the sequences used to infer the p31/CA phylogeny is shown in Supplementary Figure 1. We also conducted a phylogenetic analysis of a subset of 15 proviruses using both a larger internal gene product [partial p31/full-length CA (977 nt)] and an external protein [partial surface (SU) glycoprotein; 530 nt] to determine if this provided greater resolution between the viruses or identified differences not observed in the partial p31/partial CA tree. Both the partial p31/full-length CA and SU trees had similar topologies to the 413 nt p31/CA phylogeny (data not shown; trees available from the authors on request). Sequences used in the inference of all three phylogenies have been deposited in GenBank under the accessions KC801949-KC802005 (p31/CA) and KC802006-KC802018 (SU).

The p31/CA phylogeny revealed the proviruses from North America could be grouped into two major clades, termed clades 1 and 2 (Figure 3). A small subset of four viruses from South Carolina (62-, 67-, 68-, 69/SC/2011) was shown to cluster with the Israeli prototype strain of LPDV in clade 2. Remarkably, while these four viruses exhibited between 83-87% nucleotide identity in their p31/CA sequences to other viruses from the United States, they shared a 97% identity to the Israeli virus. In contrast, the majority of the proviruses sampled in the United States were divergent from the Israeli strain and fell into clade 1. Although this clade showed some evidence of geographical clustering, these groupings were weak, as there was little phylogenetic resolution among strains (i.e., low bootstrap support values), indicative of relatively widespread viral gene flow. The overall lack of temporal structure in the data precluded a molecular clock dating analysis.

To better define the evolutionary relationship of LPDV to other retroviruses, we performed a phylogenetic analysis of LPDV with representative members from each of the six genera of *Retroviridae*, encompassing both *Orthoretrovirinae* and *Spumaretrovirinae* subfamilies, using conserved domains in RT within the *pol* polyprotein (Xiong and Eickbush, 1990). The amino acid RT phylogeny demonstrated that the North American and Israeli LPDV

prototypes grouped with ALV and RSV within the *Alpharetrovirus* clade with strong (85% bootstrap) support (Figure 4), thereby demonstrating that LPDV is most closely related to other oncogenic alpharetroviruses. Recently, a wide diversity of lineages of avian endogenous retroviruses (ERV) has been identified in galliform (chicken, turkey) genomes that have properties characteristic of alpha-, beta-, and gammaretroviruses, with LPDV being similar to some avian beta-like ERVs (Bolisetty et al., 2012). However, as alpharetroviruses are postulated to have evolved from beta-like precursors (Bolisetty et al., 2012), that LPDV retains some betaretrovirus properties (i.e., frameshift strategies) while also possessing alpharetrovirus motifs (e.g., tRNA^{T₁P} primer binding site) and clearly showing similar host associations (galliform birds) and a strong phylogenetic relationship with exogenous alpharetroviruses (ALV and RSV), suggests that its designation as an alpharetrovirus is the most appropriate given current data.

Discussion

This report is the first documentation of LPDV in the United States and provides the first description of the disease in naturally-infected wild birds, along with its genetic and phylogenetic relationship to viruses recovered in Israel in the 1990's. Although the broad geographical distribution of the virus (Maine to Louisiana and west to Colorado), coupled with the non-migratory nature of wild turkeys, tentatively suggests that LPDV may have been endemic in the United States for a considerable time, it is unclear if management practices such as the extensive intra- and interstate translocation of wild turkeys through restoration projects (Tapley et al., 2005) may have inadvertently facilitated the more recent spread of the virus. Additionally, because the full extent of the host range of LPDV (which may include additional wild galliform birds or even avian species within other orders) and the route of virus transmission in nature (e.g., direct contact, arthropod-borne, vertical/horizontal transmission) have not been clearly defined, whether this apparent endemicity in the United States predates the emergence of LPDV (or recognition of LPDV-like disease) in Europe is uncertain. Nevertheless, our analysis shows that LPDV is widespread in the wild turkey population in the United States, is responsible for sporadic fatal tumorigenic disease, and has likely been missed as the etiological agent in undiagnosed cases of lymphoid hyperplasia and neoplasia.

Although fatal tumorigenesis was attributable to LPDV infection in only four of 40 LPDV-positive birds (10.0%), this may be a conservative estimate. Other birds had lesions that were consistent with LPDV infection (e.g., multicentric neoplasia, multi-organ lymphoplasmacytic cellular infiltrates), but concurrent infection with other agents (e.g., REV, FWPV, bacteria) and the observation of additional lesions in these birds precluded a definitive diagnosis of LPDV infection as the cause of death. Presently, in the dual LPDV/REV-positive cases in which fatal tumorigenesis were observed, it is unclear which virus was responsible for the neoplasia. However, as one of our current objectives is the production of recombinant LPDV proteins for use as antigen in the development of specific antisera for immunohistochemistry, this (in conjunction with REV-specific antisera) will likely aid in providing a more definitive diagnosis in such cases. The high prevalence of viral co-infections in the diagnostic cases tested (i.e., 46.3% and 36.6% of LPDV-positive birds were also positive for REV or FWPV, respectively) further demonstrates that screening for multiple pathogens should be performed to definitively assess the cause(s) of mortality. Although the integration of complete or partial REV genomes into FWPV (including vaccine strains) has been extensively demonstrated (Hertig et al., 1997; Liu et al., 2009), we did not detect LPDV incorporation within FWPV genomes in dually-infected turkeys, possibly suggesting that the association between the two viruses may be due to the persistence of LPDV DNA in previously infected but recovered birds, such that the two infections may commonly occur independently. However, the potential spread of LPDV in

nature via integration into FWPV genomes, similar to that of REV, merits further investigation, as does the potential synergistic pathological effects of coinfection of LPDV with other pathogens.

The mechanism by which LPDV induces oncogenesis in its turkey host is unknown. Unlike RSV, LPDV does not carry an oncogene within its genome. However, LPDV is similar to the related ALV, as both viruses induce tumors in galliform birds independent of a transducing viral gene. In the case of ALV, although viral integration is a random process, insertion in the vicinity of the *c-myc* proto-oncogene has been demonstrated to lead to tumorigenesis (Neel et al., 1981; Hayward et al., 1981). Additionally, retroviral insertion into tumor-suppressor genes can be oncogenic, although this generally requires that both alleles be inactivated (e.g., Hicks and Mowat, 1988). Mapping of the insertion site of 12/AR/2009 demonstrated that it was integrated, in reverse orientation, into chromosome 6 of the turkey genome in a region of unknown function downstream of a C-C chemokine receptor type 4-like protein and upstream of a beta-galactosidase-like protein. Although LPDV integration into the turkey genome may be a random process, whether oncogenesis is triggered by insertion into a specific region, similar to ALV and *c-myc*, is currently unclear. As such, future research will be aimed at mapping the genomic integration site of a comprehensive set of proviruses in birds that were deemed to be unequivocal cases of LPDV-induced tumorigenesis, as well as other birds that died of apparently unrelated causes.

Other than the prototype Israeli strain, the lack of other sequences from historic LPDV outbreaks from Europe and Israel prohibits a detailed phylogenetic analysis of Old and New World viruses. However, it was striking that a subset of four proviruses sampled in South Carolina in 2011 (62-, 67-, 68-, 69/SC/2011) were more closely related genetically to the Israeli virus than to other North American viruses, forming a distinct monophyletic group in the p31/CA phylogeny (Clade 2 in Figure 3), as well as in the full-length CA or partial SU trees (data not shown). This grouping of South Carolina and Israeli isolates provides a direct link between viruses circulating in North America to those that first emerged in Europe, demonstrating that LPDV has been transferred between continents to result in outbreaks of disease. Despite their close evolutionary relationship, the route (North America to Europe or vice versa), approximate time frame, and mechanism by which the transcontinental spread of LPDV occurred is uncertain. However, if wild turkeys are the predominant natural host of the virus, and as their native range is exclusive to North America, it is possible that LPDV originated in North America and may have been inadvertently transferred to the Old World by the intercontinental movement of infected birds. Additionally, as no recent cases of LPDV have been detected in either Israel (personal communication, Irit Davidson, Kimron Veterinary Institute, Bet Dagan, Israel) or the United Kingdom (personal communication, Venugopal Nair, Pirbright Institute, Surrey, England), the sudden emergence of LPDV in the Old World followed by its apparent lack of endemic circulation or recurrent disease outbreaks is again compatible with the outbreak viruses originating from an exotic source. However, the evolutionary origins of LPDV and its precise transcontinental movements are currently unclear and will benefit from both retrospective and prospective analysis of additional viruses from the Middle East, Europe, and North America.

Whether the apparent widespread nature of LPDV in the United States is indicative of the recent identification of a long overlooked or misdiagnosed pathogen, or whether LPDV represents a newly emerging virus is uncertain. Tumorigenic diseases of unknown etiology have long been recognized in wild birds, including galliforms (Drew, 2007), suggesting that a long-standing failure to detect the virus is plausible. However, it is important to note that the population structure of wild turkeys within the United States over the last 50 years or so has undergone dramatic changes. Restoration projects, involving the movement of tens of

thousands of birds across the United States to boost dwindling population numbers, have substantially altered the population demographics of turkeys by displacing birds into new territories (Tapley et al., 2005). Although there is no evidence that these restoration projects have facilitated the rapid spread of the virus, the recognition of the emergence of viral diseases into new areas after the inadvertent translocation of infected wildlife is not novel, as epitomized by the rapid expansion and movement of raccoon rabies throughout the northeastern United States in the 1980'-1990's after the translocation of infected animals originating from the southeast during the 1970's (Rupprecht and Smith, 1994).

LPDV is currently not listed as a recognized viral species by the ICTV (Stoye et al., 2011). However, based on the genetic and evolutionary data presented here (Figure 4), in addition to previous phylogenetic analyses of Israeli viruses (Chajut *et al.*, 1992; Gazit and Yaniv, 1999), LPDV is clearly most closely related to avian leukosis-sarcoma viruses such as ALV and RSV. Accordingly, we suggest that it should be designated as a new species within the genus *Alpharetrovirus*, subfamily *Orthoretrovirinae*, family *Retroviridae*. Despite the apparent widespread geographic prevalence of the virus among wild turkeys, LPDV does not currently appear to be a serious threat to the North American poultry industry. However, this is conjectural until surveillance efforts have been made to screen commercial poultry for the presence of the virus and clinical trials are performed to definitively assess its pathogenic potential. As LPDV-associated lesions can superficially resemble other retroviral (e.g., reticuloendotheliosis) and non-retroviral (e.g. avian pox) diseases that are endemic to North America, in addition to our observation that dual infections with LPDV and these and other pathogens may be common, LPDV should be considered as a potential disease agent in both retrospective and prospective cases of leukocytic neoplastic or hyperplastic disease of unknown etiology.

Materials and Methods

Sample collection and virus testing

Following the first identification of LPDV in a North American wild turkey, we actively targeted the collection of tissues from clinically ill wild turkeys submitted to veterinary diagnostic laboratories throughout the eastern United States during 2009-2012. Samples were acquired from the diagnostic services at the SCWDS, the New York State Department of Environmental Conservation, the University of Maine Animal Health Laboratory, and the New Hampshire Veterinary Diagnostic Laboratory. These turkeys were submitted as entire carcasses and are designated as 'diagnostic cases'. Where possible, we targeted wild turkeys with gross or microscopic lesions consistent with the historical description of LPDV infection in domestic turkeys (see McDougall et al., 1978; Zimmer et al., 1983; Biggs, 1997) and/or proliferative skin lesions. Tissue samples acquired from diagnostic cases for LPDV testing varied, but when possible, efforts were made to test tissue collected directly from nodular lesions and/or lymphoid tissues. Unless stated otherwise, tissues from all diagnostic cases were examined by necropsy and fixed in 10% neutral-buffered formalin and processed for histopathological examination by standard hematoxylin and eosin staining. In certain cases, special histopathologic stains and ancillary diagnostic tests were performed as noted to assess for additional infectious agents such as bacteria, fungi, and other viruses. A diagnosis of LPDV infection as the sole cause of death was made when i) lymphoid infiltrates were detected in multiple tissues, ii) those tissues were PCR-positive for LPDV, and iii) other potential etiologies (e.g., bacteria, fungi, or other viruses) or alternative non-microbial causes of mortality (trauma, toxicosis) were not detected.

DNA was extracted from tissues in which lesions were observed using a QIAamp DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's guidelines. LPDV PCR was performed using primers based on the Israeli prototype strain of LPDV that covered a region

spanning the p31 and CA genes (5'-ATGAGGACTTGTTAGATTGGTTAC-3', 5'-TGATGGCGTCAGGGCTATTTG-3). PCR products were amplified using a GoTaq® Flexi DNA polymerase system (Promega, Madison, WI) and purified products were labeled for sequencing using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). All FWPV and REV cases were confirmed by PCR from tissue as previously described (Lüschow et al., 2004; Zavala et al., 2006) and also by histopathological examination of tissues. To determine if LPDV was incorporated into the genome of FWPV in birds where both viruses were detected, lesions that were PCR-positive for both viruses were mechanically homogenized in minimal essential media (Sigma-Aldrich, St. Louis, MO), clarified by centrifugation ($6700 \times g$ for 10 min), and an aliquot (100 μ l) was inoculated into DF-1 chicken fibroblast (CRL-12203), ConA-B1-VICK chicken T lymphocyte (CRL-12357), and/or Peking duck embryo fibroblast (CCL-141) cell cultures maintained at 37 °C in a 5% CO₂ atmosphere. Cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Each case was serially passaged at weekly intervals and then DNA was extracted from cell culture supernatant and screened for both LPDV and FWPV as stated above.

In addition to diagnostic cases, we acquired liver samples from 74 adult wild turkeys harvested in South Carolina during the 2011 spring hunting season. The 74 samples were acquired from 10 counties (Aiken, Anderson, Barnwell, Chesterfield, Darlington, Dorchester, Florence, Hampton, Marion, and Orangeburg). Liver samples were processed and tested for LPDV as in the diagnostic cases. These turkeys are designated as 'seasonally harvested' birds.

Genomic sequencing

The complete proviral genome of the index North American case of LPDV (strain 12/AR/2009) was PCR amplified from lung tumor tissue (deposited as GenBank accession number KC802224). Primers for genomic sequencing were developed based on the Israeli prototype strain (GenBank accession number U09568). Gaps in the genome due to areas of divergence were filled in using primers based on newly acquired sequences when necessary. Primers are available from the authors upon request. PCR products were cloned using a PCR Cloning Kit (Qiagen) and contigs were assembled using Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, MI), resulting in the full-length provirus. Mapping of the integration site of 12/AR/2009 into the turkey genome was performed by retroviral LTR arbitrarily primed PCR (RELAP-PCR) (Casper et al., 1998). RNA secondary structures were predicted using the RNAfold program (Gruber et al., 2008). Percent identities of genes and proteins among LPDV isolates and between different retroviruses were determined using the percent identity matrix in Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Theoretical molecular weights and isoelectric points of LPDV proteins were predicted using the Compute pI/MW tool in the ExPASy Bioinformatics Resource Portal (http://web.expasy.org/compute_pi/).

Phylogenetic analysis

We performed a phylogenetic analysis on the p31/capsid (CA) sequences (413 nt) of all LPDV strains recovered from wild turkeys in the United States during 2009-2012, combined with the prototype Israeli strain recovered in 1978. The 58 new proviruses used to infer the p31/CA tree were recovered from 58 individual birds (41 diagnostic cases and 17 seasonally harvested birds) and are designated as case number, followed by state and year of collection (as shown in Table 1). A maximum likelihood (ML) phylogeny was then estimated using PhyML 3.0 (Guindon et al., 2010) and employing the GTR+ Γ_4 model of nucleotide substitution (estimated Γ shape parameter of 0.549). The best tree was chosen after a combination of NNI and SPR branch-swapping. To infer support for individual nodes, a bootstrap analysis was conducted using 1000 replicate ML trees, again estimated using the

GTR+ Γ_4 substitution model (optimized for each run) and with NNI branch-swapping. An equivalent phylogenetic analysis was performed on a subset of 15 proviruses using (i) partial p31/full-length CA (sequence alignment of 977 nt) and (ii) partial surface (SU) glycoprotein (sequence alignment of 530 nt). All analyses were undertaken using the same ML methods as described above.

To determine the evolutionary relationship of LPDV to other extant retroviruses, we conducted an additional phylogenetic analysis using conserved regions of the *pol* polyprotein [reverse transcriptase (RT)]. Accordingly, conserved domains in RT (Xiong and Eickbush, 1990) were compiled for 21 retroviruses representing the genera Alpharetrovirus, Betaretrovirus, Deltaretrovirus, Epsilonretrovirus, Gammaretrovirus, Lentivirus and *Spumavirus*. These sequences were combined with two LPDV sequences representing the Israeli (U09568) and North American (12/AR/2009) prototype strains and aligned using MUSCLE (Edgar, 2004). Because of the large number of insertions/deletions and alignment uncertainties, we used Gblocks (Talavera and Castresana, 2007) to purge all such regions prior to phylogenetic analysis. This resulted in a final sequence alignment of 23 taxa, 191 amino acid residues in length. Phylogenetic analyses of these data were again undertaken using the ML method available in PhyML 3.0, this time employing the WAG+ Γ_4 model of amino acid substitution (estimated Γ shape parameter of 1.110), and selecting the best tree after a combination of NNI and SPR branch-swapping. A bootstrap resampling analysis was conducted using 1000 replicate ML trees, again inferred using the WAG+ Γ_4 substitution model and NNI branch-swapping.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- LPDV is responsible for periodic fatal tumorigenic disease in galliform birds
- First description of LPDV in North America
- First description of LPDV in wild birds
- Phylogenetic analysis suggests transcontinental spread between New and Old World
- LPDV has likely been the clandestine agent in many undiagnosed cases of neoplasia

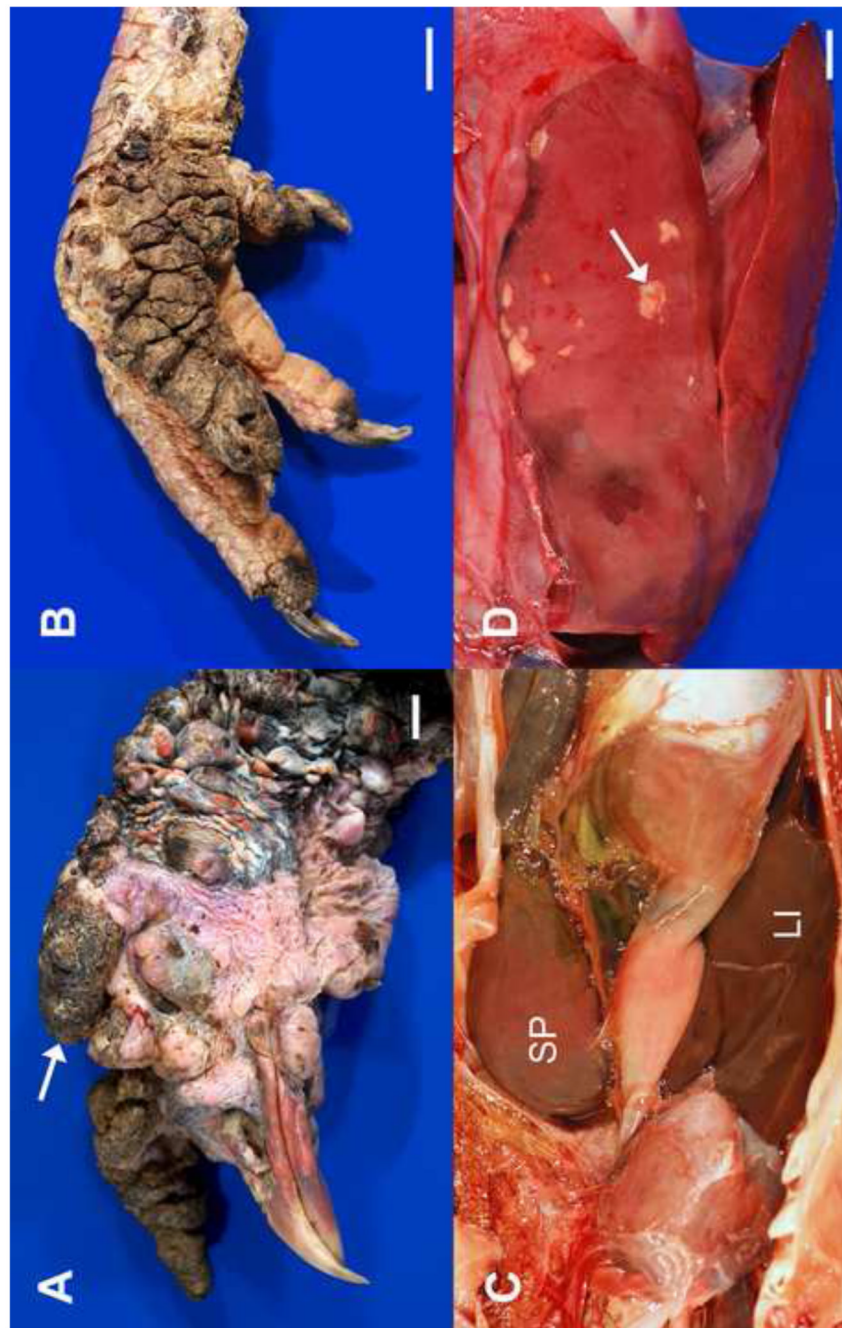


Figure 1.

Gross lesions in adult wild turkeys infected with LPDV. (A) The sparsely-feathered skin over much of the head and neck is covered by variably-sized, proliferative nodules, some of which contain superficial crusts (arrow); (B) The skin over two digits and the plantar aspect of the foot is severely thickened with multiple folds and is covered by dark, dry crusts; (C) The spleen (SP) is markedly enlarged ($8.0 \times 3.8 \times 3.3$ cm), a characteristic gross lesion also observed in domestic birds (see Gazit and Yaniv, 1999), (LI = liver); (D) The liver contains numerous, irregular, variably-sized pale foci, corresponding to aggregates of pleomorphic lymphoid cells (arrow). All scale bars = 1.0 cm.

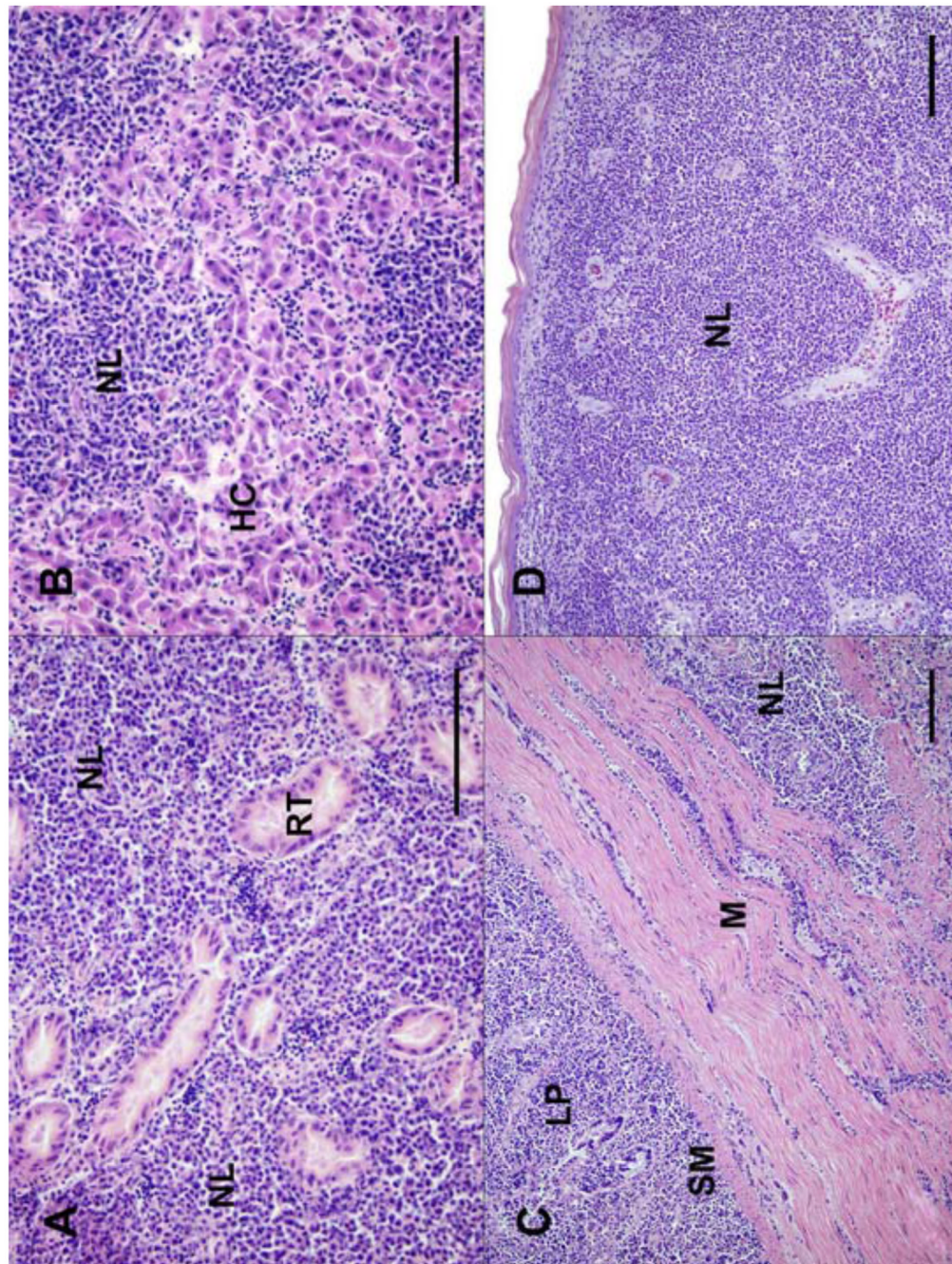


Figure 2.

Tissues from adult wild turkeys infected with LPDV. (A) Kidney: The interstitium is markedly expanded and effaced by sheets of neoplastic lymphocytes (NL). Remnant cross sections of renal tubules (RT) are completely surrounded by the neoplastic cells; (B) Liver: Most of the normal architecture is effaced by neoplastic lymphocytes. Remnant hepatic cords (HC) are compressed and distorted; (C) Intestine: There is transmural infiltration, marked expansion of the lamina propria (LP) and submucosa (SM), and multifocal perimysial expansion of underlying muscular tunics (M) by neoplastic lymphocytes; (D) Skin: The dermis is expanded and obscured by sheets of neoplastic lymphocytes that efface

adnexa and there is mild orthokeratotic hyperkeratosis of the overlying epidermis. All scale bars = 100 μ m. All images stained with hematoxylin and eosin.

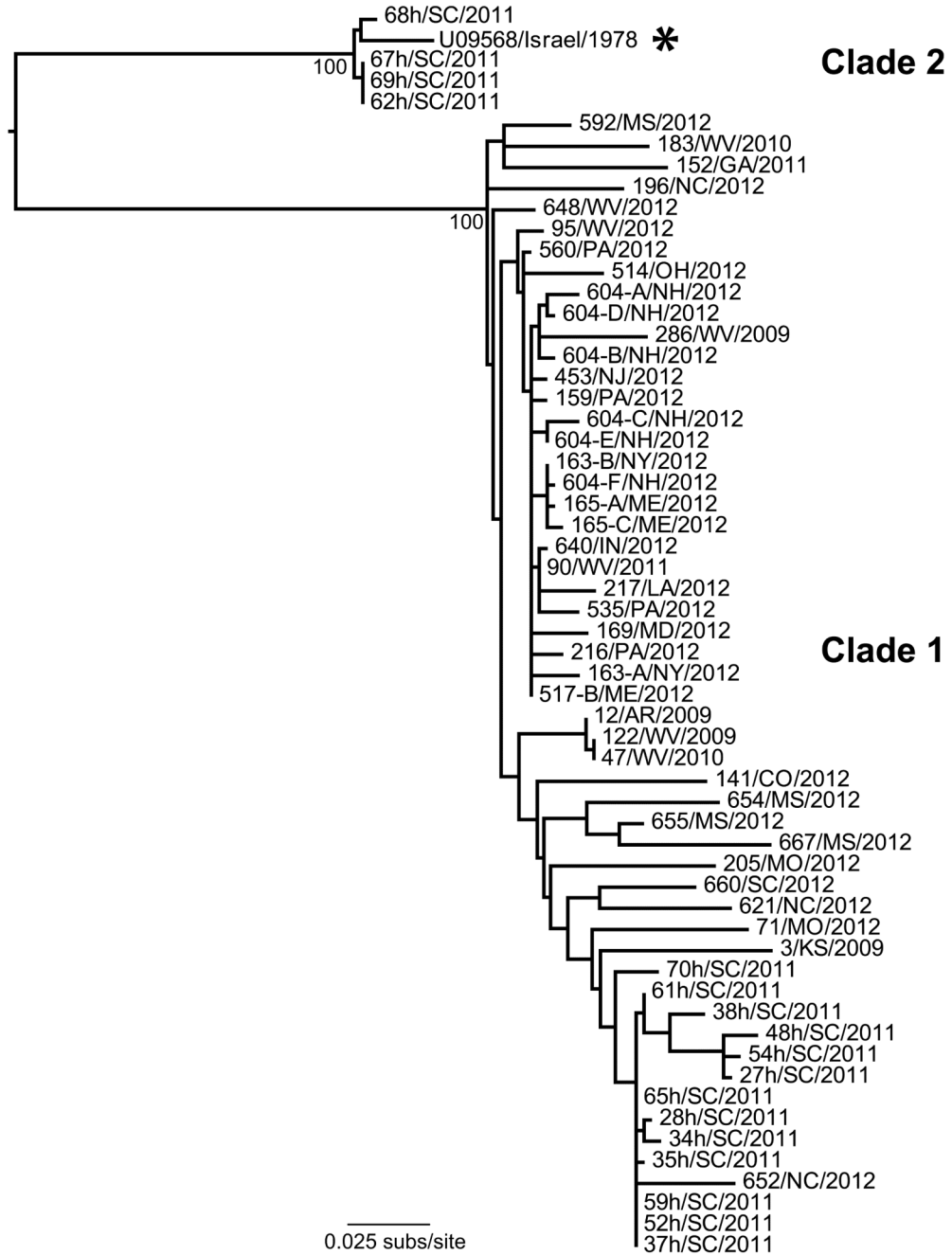


Figure 3.

Phylogenetic tree of p31/capsid sequences of LPDV strains recovered from wild turkeys in the United States during 2009-2012, along with the prototype Israeli strain from 1978 (highlighted with an asterisk). Bootstrap support values are shown for key nodes and all horizontal branch lengths are drawn to a scale of nucleotide substitutions per site. The tree is mid-point rooted for purposes of clarity only. The 58 viruses used to infer the tree are designated as case number, followed by state and year of collection (as shown in Table 2).

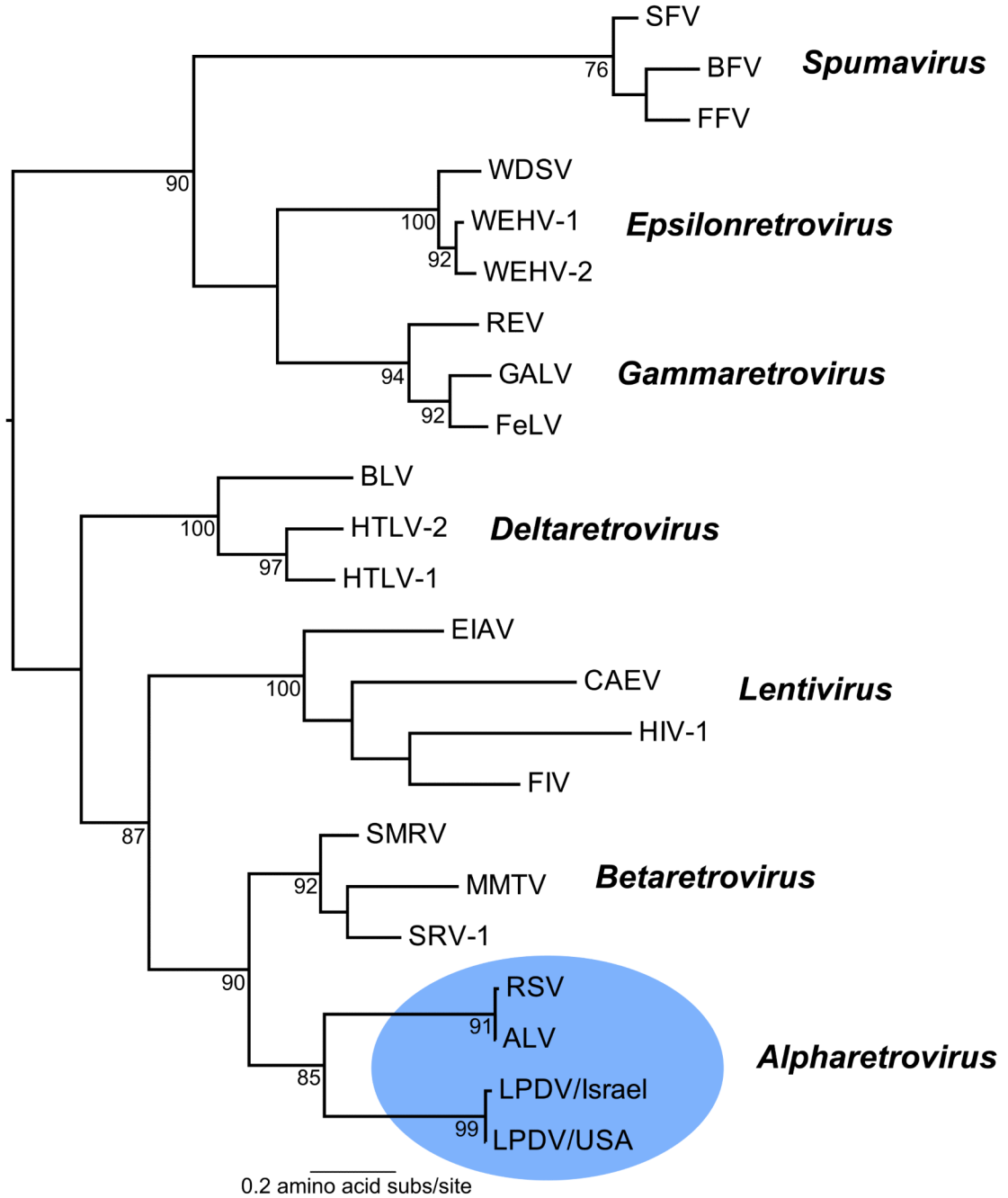


Figure 4.

Family-wide phylogeny of exogenous retroviruses based on conserved protein domains in reverse transcriptase. The clade containing LPDV and representing the *Alpharetrovirus* genus is shaded in blue. Bootstrap support values are shown for key nodes and all horizontal branch lengths are drawn to a scale of amino acid substitutions per site. The tree is mid-point rooted for purposes of clarity only. Viruses (and their GenBank accession numbers) used to infer the phylogeny were: ALV, *Avian leukosis virus* (ADO01000); BLV, *Bovine leukemia virus* (ACR15158); BFV, *Bovine foamy virus* (AAN08116); CAEV, *Caprine arthritis encephalitis virus* (AAG48629); EIAV, *Equine infectious anemia virus* (AAG02702); FeLV, *Feline leukemia virus* (BAM45657); FIV, *Feline immunodeficiency virus* (AAB49923);

FFV, *Feline foamy virus* (NP_056914); GALV, *Gibbon ape leukemia virus* (AAC80264); HIV-1, *Human immunodeficiency virus* (BAF32463); HTLV-2, *Human T-lymphotropic virus 2* (AAB59885); HTLV-1, *Human T-lymphotropic virus 1* (AAB20769); LPDV, *Lymphoproliferative disease virus* [KC802224 (USA) and U09568 (Israel)]; MMTV, *Mouse mammary tumor virus* (P03365); REV, *Reticuloendotheliosis virus* (ABD46829); RSV, *Rous sarcoma virus* (CAA48535); SFV, *Simian foamy virus* (AFX98084); SMRV, *Squirrel monkey retrovirus* (NP_041261); SRV-1, *Simian retrovirus 1* (ADI76909); WDSV, *Walleye dermal sarcoma virus* (NP_045937); WEHV-1, *Walleye epidermal hyperplasia virus 1* (AAC59310); WEHV-2, *Walleye epidermal hyperplasia virus 2* (AAD30054).

Table 1

LPDV strains recovered in the United States from 2009-2012 that were analyzed during the study. LPDV-positive birds that were also positive for *reticuloendotheliosis virus* (REV) and/or *fowlpox virus* (FWPV) are indicated.

Virus ^a	County	State	Date	Collection method ^b	GenBankID ^c	FWPV	REV
3/KS/2009	Logan	Kansas	2009	Diagnostic case	KC801949	×	×
12/AR/2009	Columbia	Arkansas	2009	Diagnostic case	KC802224	×	×
122/WV/2009	Randolph	West Virginia	2009	Diagnostic case	KC801950	×	×
286/WV/2009	Upshur	West Virginia	2009	Diagnostic case	KC801993	×	×
47/WV/2010	Upshur	West Virginia	2010	Diagnostic case	KC801994	×	×
183/WV/2010	Wayne	West Virginia	2010	Diagnostic case	KC801951	×	×
27h/SC/2011	Barnwell	South Carolina	2011	Seasonal harvest	KC801981	*	*
28h/SC/2011	Barnwell	South Carolina	2011	Seasonal harvest	KC802003	*	*
34h/SC/2011	Barnwell	South Carolina	2011	Seasonal harvest	KC801982	*	*
35h/SC/2011	Barnwell	South Carolina	2011	Seasonal harvest	KC801983	*	*
37h/SC/2011	Barnwell	South Carolina	2011	Seasonal harvest	KC801984	*	*
38h/SC/2011	Barnwell	South Carolina	2011	Seasonal harvest	KC801985	*	*
48h/SC/2011	Aiken	South Carolina	2011	Seasonal harvest	KC801986	*	*
52h/SC/2011	Aiken	South Carolina	2011	Seasonal harvest	KC801987	*	*
54h/SC/2011	Aiken	South Carolina	2011	Seasonal harvest	KC801988	*	*
59h/SC/2011	Aiken	South Carolina	2011	Seasonal harvest	KC801989	*	*
61h/SC/2011	Aiken	South Carolina	2011	Seasonal harvest	KC802004	*	*
62h/SC/2011	Aiken	South Carolina	2011	Seasonal harvest	KC801976	*	*
65h/SC/2011	Aiken	South Carolina	2011	Seasonal harvest	KC801990	*	*
67h/SC/2011	Dorchester	South Carolina	2011	Seasonal harvest	KC802005	*	*
68h/SC/2011	Dorchester	South Carolina	2011	Seasonal harvest	KC801975	*	*
69h/SC/2011	Dorchester	South Carolina	2011	Seasonal harvest	KC801991	*	*
70h/SC/2011	Dorchester	South Carolina	2011	Seasonal harvest	KC801992	*	*
90/WV/2011	Logan	West Virginia	2011	Diagnostic case	KC801995	*	×
152/GA/2011	Putnam	Georgia	2011	Diagnostic case	KC801952	×	×
141/CO/2012	Las Animas	Colorado	2012	Diagnostic case	KC801953	×	×
71/MO/2012	St. Clair	Missouri	2012	Diagnostic case	KC801954	×	×
95/WV/2012	Morgan	West Virginia	2012	Diagnostic case	KC801955	☑	☑
159/PA/2012	Clearfield	Pennsylvania	2012	Diagnostic case	KC801996	×	☑
163-A/NY/2012	Columbia	New York	2012	Diagnostic case	KC801997	×	☑
163-B/NY/2012	Columbia	New York	2012	Diagnostic case	KC801998	×	☑
165-A/ME/2012	Piscataquis	Maine	2012	Diagnostic case	KC801999	☑	☑
165-C/ME/2012	Piscataquis	Maine	2012	Diagnostic case	KC802000	×	×
169/MD/2012	Allegany	Maryland	2012	Diagnostic case	KC802001	*	☑
196/NC/2012	Bertie	North Carolina	2012	Diagnostic case	KC802002	×	☑
205/MO/2012	Howard	Missouri	2012	Diagnostic case	KC801956	*	×
216/PA/2012	Lackawanna	Pennsylvania	2012	Diagnostic case	KC801957	*	×

Virus ^a	County	State	Date	Collection method ^b	GenBankID ^c	FWPV	REV
217/LA/2012	East Feliciana	Louisiana	2012	Diagnostic case	KC801958	*	×
453/NJ/2012	Sussex	New Jersey	2012	Diagnostic case	KC801959	×	☑
514/OH/2012	Hancock	Ohio	2012	Diagnostic case	KC801977	×	×
517-B/ME/2012	Cumberland	Maine	2012	Diagnostic case	KC801960	×	☑
535/PA/2012	Blair	Pennsylvania	2012	Diagnostic case	KC801961	☑	×
560/PA/2012	Huntingdon	Pennsylvania	2012	Diagnostic case	KC801962	☑	☑
592/MS/2012	Monroe	Mississippi	2012	Diagnostic case	KC801963	×	×
604-A/NH/2012	Concord	New Hampshire	2012	Diagnostic case	KC801964	☑	☑
604-B/NH/2012	Sutton	New Hampshire	2012	Diagnostic case	KC801965	☑	☑
604-C/NH/2012	Lisbon	New Hampshire	2012	Diagnostic case	KC801966	☑	☑
604-D/NH/2012	Bradford	New Hampshire	2012	Diagnostic case	KC801967	☑	☑
604-E/NH/2012	Hinsdale	New Hampshire	2012	Diagnostic case	KC801968	☑	☑
604-F/NH/2012	Dublin	New Hampshire	2012	Diagnostic case	KC801969	☑	☑
621/NC/2012	Hertford	North Carolina	2012	Diagnostic case	KC801978	☑	×
640/IN/2012	Orange	Indiana	2012	Diagnostic case	KC801979	*	×
648/WV/2012	Braxton	West Virginia	2012	Diagnostic case	KC801970	*	×
652/NC/2012	Camden	North Carolina	2012	Diagnostic case	KC801971	☑	☑
654/MS/2012	Copiah	Mississippi	2012	Diagnostic case	KC801972	☑	×
655/MS/2012	Perry	Mississippi	2012	Diagnostic case	KC801973	×	×
660/SC/2012	Williamsburg	South Carolina	2012	Diagnostic case	KC801980	☑	☑
667/MS/2012	Stone	Mississippi	2012	Diagnostic case	KC801974	☑	☑

FWPV = *Fowlpox virus*; REV = *Reticuloendotheliosis virus*; * = not tested; × = negative; ☑ = positive

^aVirus identification: case number/state of collection/year of collection

^bCollection method: Diagnostic clinical case (entire bird) or seasonally harvested sample (liver only)

^cOther than the index case 12/AR/2009, GenBank accession numbers refer to p31/capsid sequences

Table 2

Genomic characteristics of the North American prototype strain of LPDV.

Gene/domain	Region	nt	aa	MW	pI	Position
U3	5' LTR	207	*	*	*	1-207
R	5' LTR	22	*	*	*	208-229
U5	5' LTR	96	*	*	*	230-326
Matrix (MA)	<i>gag</i>	459	153	16.67	5.12	384-842
p31	<i>gag</i>	708	236	25.78	5.14	843-1550
Capsid (CA)	<i>gag</i>	699	233	25.55	8.96	1551-2249
Nucleocapsid (NC)	<i>gag</i>	294	98	10.18	10.78	2250-2543
Protease (PR)	<i>pro</i>	444	148	15.98	8.87	2450-2893
Reverse transcriptase (RT)	<i>pol</i>	1743	581	65.34	6.75	2887-4629
RT domain	<i>pol</i>	834	278	*	*	2887-3720
Tether (t) domain	<i>pol</i>	519	173	*	*	3721-4239
RNase H domain	<i>pol</i>	390	130	*	*	4240-4629
Integrase (IN)	<i>pol</i>	810	270	30.10	9.92	4630-5439
Surface (SU)	<i>env</i>	972	324	35.99	9.20	5459-6430
Transmembrane (TM)	<i>env</i>	660	220	24.84	6.21	6431-7090
U3	3' LTR	207	*	*	*	7107-7313
R	3' LTR	22	*	*	*	7314-7335
U5	3' LTR	97	*	*	*	7336-7432

MW = molecular weight (kDa); pI = isoelectric point (pH); * = not applicable