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Phylogenetic Relationships among Virulent Newcastle Disease Virus Isolates from the 2002-2003 Outbreak in California and Other Recent Outbreaks in North America

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Isolates from the 2002-2003 virulent Newcastle disease virus (v-NDV) outbreak in southern California, Nevada, Arizona, and Texas in the United States were compared to each other along with recent v-NDV isolates from Mexico and Central America and reference avian paramyxovirus type 1 strains. Nucleotide sequencing and phylogenetic analyses were conducted on a 1,195-base genomic segment composing the 3′ region of the matrix (M) protein gene and a 5′ portion of the fusion (F) protein gene including the M-F intergenic region. This encompasses coding sequences for the nuclear localization signal of the M protein and the F protein cleavage activation site. A dibasic amino acid motif was present at the predicted F protein cleavage activation site in all v-NDVs, including the California 2002-2003, Arizona, Nevada, Texas, Mexico, and Central America isolates. Phylogenetic analyses demonstrated that the California 2002-2003, Arizona, Nevada, and Texas viruses were most closely related to isolates from Mexico and Central America. An isolate from Texas obtained during 2003 appeared to represent a separate introduction of v-NDV into the United States, as this virus was even more closely related to the Mexico 2000 isolates than the California, Arizona, and Nevada viruses. The close phylogenetic relationship between the recent 2002-2003 U.S. v-NDV isolates and those viruses from countries geographically close to the United States warrants continued surveillance of commercial and noncommercial poultry for early detection of highly virulent NDV.

Newcastle disease (ND) is one of the most serious infectious diseases of poultry, and virulent ND outbreaks require reporting to the Office of International Epizootes (OIE) by a member nation (3). The etiological agent, Newcastle disease virus (NDV), belongs to the *Avulavirus* genus within the family *Paramyxoviridae* in the order *Mononegavirales* and is designated avian paramyxovirus 1 (APMV-1), one of nine identified serotypes of APMVs (3, 9). The enveloped virus has a negative-sense, single-stranded RNA genome encoding six proteins, including the nucleocapsid, phosphoprotein, matrix (M), fusion (F), hemagglutinin-neuraminidase, and RNA-directed RNA polymerase proteins (3, 9). Currently, there are multiple NDV lineages circulating worldwide that are genetically highly diverse (1, 7, 8, 15, 27, 30, 36). Chickens are highly susceptible to virulent APMV-1, while ducks and geese may be infected but show few or no clinical signs (3). Also, members of the *Columbidae* family may be infected with APMV-1 adapted to those particular avian species, designated pigeon paramyxovirus 1 (PPMV-1), and are also susceptible to infection by NDV strains that produce clinical disease in chickens (4, 16, 36, 40). Erickson et al. (10, 11) reported that NDV can be shed from Amazon parrots for more than a year and from budgerigars, conures, mynahs, and black-headed nuns sporadically for up to 83 days.

Historically, NDV isolates were placed into one of three pathotypes, lentogenic, mesogenic, or velogenic, based on the results of in vivo pathogenicity tests (2, 3). The OIE now requires APMV-1 isolates to be characterized by virulence in day-old chicks and/or molecular determination of the F protein cleavage site rather than the severity of clinical disease. Thus, OIE defines ND as an infection caused by an APMV-1 virus with an intracerebral pathogenicity index (ICPI) of 0.7 or greater in day-old chicks or by multiple basic amino acids at the F protein cleavage site (21). An APMV-1 virus that does not meet the OIE definition for causing ND is referred to as a low-virulence APMV-1 or NDV. Highly virulent NDV (v-NDV) strains have two pairs of basic amino acids, either lysine (K) or arginine (R), at the F protein cleavage site at residues 112 to 113 and 115 to 116, as well as a phenylalanine at residue 117 (13, 18, 25). The presence of these basic amino acids in v-NDV permits the cleavage of the F_0 protein into two subunits (F_1 and F_2) by ubiquitous host proteases found in most tissues (14). The F_0 protein of low-virulence APMV-1 strains is cleaved only in cells containing unique trypsin-like enzymes, limiting infection to mucosal tissues of the respiratory or intestinal tracts of the host (23).

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TABLE 1. APMV-1 isolates utilized to compare U.S. and Mexico NDVs from outbreaks during 1998 through 2003

Isolate[a]	Pathotype[b]	Source[c]	GenBank accession no.	ICPI[d]	F_0 cleavage site sequence[e]
Chicken/Australia-Victoria/32	V	SEPRL	M16622, M21881	1.66	GGRRQKR FIG
Chicken/BeaudetteC/U.S./45	V	SEPRL	X04687, X04719	1.75	GGRRQKR FIG
Chicken/LaSota/U.S./46	L	SEPRL	AF077761	0.31	GGGRQGR LIG
Chicken/U.S./B1/48	L	SEPRL	NC_002617	0.13	GGGRQGR LIG
Chicken/U.S.(TX)/GB/48	V	SEPRL	U25835, M24698	1.74	GGRRQKR FIG
Chicken/Australia/QV4/66	L	SEPRL	U25834, AF217084	0.39	GGGKQGR LIG
Chicken/Ulster/Northern Ireland/67	L	SEPRL	U25837, Z12110	0.16	GGGKQGR LIG
Chicken/U.S.(CA)/1083(Fontana)/72	V	NVSL	AY288992, U25829	1.80	GGRRQKR FIG
Yellow nape parrot/U.S.(MA)/19120/87	V	NVSL	AY444496	nd	GGRRQRR FVG
Moluccan/Indonesia/904/87	V	NVSL	AY444497	nd	GGRRQKR FIG
Cockatoo/Indonesia/14698/90	V	NVSL	AF124446, AY288998	1.84	RGRRQKR FVG
Turkey/U.S.(ND)/43084/92	V	NVSL	AY289001, U25836	1.43	RGRRQKR FVG
Anhinga/U.S.(FL)/44083/93	M	SEPRL	AY288989, AF124450	1.54	RGRRQKR FVG
Chicken/Mexico/37821-550-1/96	V	Mexico	AF015518	1.75	GGRRQKR FVG
Chicken/Mexico/37821-550-2/96	V	Mexico	AF015520	1.75	GGRRQKR FVG
Parrot/U.S.(MO)/31378/96	V	NVSL	AY444498	1.84	GGRRQKR FVG
Chicken/Kenya/KRC-150/94	V	SEPRL	AY288997	1.89	GGRRQKR FIG
Yellow cheek/U.S.(TX)/27345/96	V	NVSL	AY444499	1.80	GGRRQKR FVG
Parrot/U.S.(OK)/32932/96	V	NVSL	AY444500	1.70	GGRRQKR FVG
Pigeon/U.S.(TX)/17498/98	PPMV-1	NVSL	AY444501	1.11	GERRQKR FIG
Game fowl/U.S.(CA)/24225/98	V	NVSL	AY444502	1.75	GGRRQKR FIG
Chicken/Mexico/6244/98	V	Mexico	AY246047	nd	GGRRQKR FVG
Chicken/Mexico/5166/98	V	Mexico	AY246042	nd	GGRRQKR FVG
Chicken/Mexico/6246/99	V	Mexico	AY246048	nd	GGRRQKR FVG
Chicken/Mexico/4100/99	V	Mexico	AY246044	nd	GGRRQKR FVG
Chicken/Mexico/3242/99	V	Mexico	AY246039	nd	GGRRQKR FVG
Chicken/Mexico/3310/00	V	Mexico	AY246035	nd	GGRRQKR FVG
Pigeon/Italy/1166/00	PPMV-1	SEPRL	AY288996	0.8	GGRRQKR FIG
Chicken/Mexico/3313/00	V	Mexico	AY246041	nd	GGRRQKR FVG
Dove/Italy/2736/00	PPMV-1	SEPRL	AY288995	1.20	GVRRKKR FIG
Chicken/Honduras/44814/00	V	NVSL	AY288993	1.61	GGRRQKR FVG
Broiler chicken/Italy/3286/00	V	SEPRL	AY288994	1.86	GGRRQRR FIG
Chicken/Mexico/290/00	V	Mexico	AY246036	1.675	GGRRQKR FVG
Chicken/Mexico/1/00	V	Mexico	AY246040	1.66	GGRRQKR FVG
Chicken/Mexico/2/00	V	Mexico	AY246046	nd	GGRRQKR FVG
Game fowl/U.S.(CA)/211472/02	V	NVSL	AY246050	1.75	GGRRQKR FVG
Pet bird/U.S.(CA)/169302/02	V	NVSL	AY246053	1.75	GGRRQKR FVG
Dove/U.S.(CA)/9547-3/03	V	CDFA	AY438667	1.68	GGRRQRR FVG
Chicken/U.S.(CA)/5634/03	V	CDFA	AY43628	nd	GGRRQRR FVG
Game fowl/U.S.(NV)232947/03	V	NVSL	AY246052	1.75	GGRRQKR FVG
Game fowl/U.S.(AZ)236498/03	V	NVSL	AY246049	1.75	GGRRQKR FVG
Game fowl/U.S.(TX)248306/03	V	NVSL	AY438627	1.81	GGRRQKR FVG
Chicken/U.S.(CA)/229808/03	V	CDFA	AY246051	1.13	GGRRQKR FVG

[a] Isolate names are given as bird type/country of origin/accession number/year of isolation, as designated by regulatory agencies or by historical reference. CA, California; TX, Texas; AZ, Arizona; NV, Nevada; OK, Oklahoma; MO, Missouri; FL, Florida; ND, North Dakota; MA, Massachusetts.
[b] L, lentogen; M, mesogen; V, velogen.
[c] SEPRL, Southeast Poultry Research Lab; CDFA, California Department of Food and Agriculture.
[d] See reference 2. nd, not determined.
[e] Deduced amino acid residues 110 to 119 of the fusion protein, as numbered from the NH_2-terminal end. Spaces indicate where cleavage occurs.

Exotic Newcastle disease (END), a term used in the United States to define the illness caused in birds by a v-NDV infection from an area where the disease is not considered endemic, was confirmed on 1 October 2002 in the state of California. During the course of the next 6 months, v-NDV was also detected in Nevada, Arizona, and Texas. Infection was confined to backyard fowl in Nevada, Arizona, and Texas (19). However, in California, v-NDV was eventually transmitted to commercial poultry, and approximately 3.5 million birds were depopulated in an effort to eradicate the disease in affected states (20). Previously, v-NDV has been introduced into the United States through the importation of exotic avian species (6, 12, 24, 29, 30, 32, 37) and by water birds (5, 30, 31, 39). During the last several years starting from 1998, v-NDV has infected commercial poultry in Mexico, Honduras, and the United States (22). Phylogenetic analyses of the recent U.S., Mexico, and Honduras v-NDV strains presented here resulted in a close epidemiological relationship among these North and Central American isolates.

Virus isolates were examined genetically and their pathotypes are presented in Table 1. v-NDV isolates were obtained from chickens in Mexico (37821-550-1/96 and 37821-550-2/96) isolated during 1996 (provided by Moises Fraire Cachon, Mexico-U.S. Commission for the Prevention of Foot and Mouth and Other Diseases, Palato, Mexico). Viruses that were obtained during an outbreak of v-NDV among commercial poultry in Mexico were the 6244/98, 5166/98, 4100/99, and 3242/99 isolates, along with 290/00, 3310/00, 313/00, 6246/00, 2/00, and

1/00. v-NDV isolates derived from imported pet birds, free-living birds, and poultry and isolated by the Diagnostic Virology Laboratory, National Veterinary Services Laboratories (NVSL), Animal and Plant Health and Inspections Services, and U.S. Department of Agriculture, Ames, Iowa, were 904/87, 24225/98, 19120/87, 31378/96, 32932/96, 27345/96, 17498/98, 169302/02, 248306/03, 232947/03, 236498/03, 229808/03, and the index virus from California, 211472/02. Also included for analysis was a velogenic virus from the 1972-1974 U.S. outbreak, 1083(Fontana)/72 (37), and a neurotropic velogenic NDV isolate from the 1992 outbreak involving cormorants and turkeys in the north-central United States (43084/92) initially submitted to NVSL by the National Wildlife Health Research Center, Madison, Wis. (31), along with a mesogenic anhinga isolate, 44083/93 (30). The well-characterized standard U.S. vaccine challenge virus GB/48 and BeaudetteC/45 (28) were included in the analysis. For comprehensive comparison, lentogenic live-NDV vaccine strains including LaSota/46, B1/48, Ulster/67, and QV4/66 were also examined (30, 31). Two other NDV isolates, dove/U.S.(CA)/9547-3/03 and chicken/U.S.(CA)/5634/03, from the outbreak of v-NDV in backyard flocks in California were provided by the San Bernardino Branch of the California Animal Health and Food Safety Laboratory System in California. The PPMV-1 viruses, including 2736/00, 11660/00 (35), and v-NDV 3286/00 (7), were provided by the National Reference Laboratory for Newcastle Disease and Avian Influenza, Istituto Zooprofilattico Sperimentale delle Venzie, Legnaro, Italy. The chicken/Honduras/44814/00 isolate, a v-NDV, was provided to the NVSL and isolated from imported tissue specimens (21, 22).

Initial biological characterization, including intracerebral inoculation of day-old chicks and the hemagglutination-inhibition test, was completed for the Mexico, Honduras, and U.S. isolates as described previously (2, 21). All Mexico 1996-2000, Honduras 2000, and California, Nevada, Arizona, and Texas 2002-2003 viruses and U.S. v-NDV psittacine viruses were inhibited by APMV-1 polyclonal antisera. The ICPI values of all virulent isolates examined ranged from 1.13 to 1.81 (Table 1) and were above the OIE value of equal to or greater than the 0.7 standard for v-NDV (21). Genomic RNA was extracted directly from infectious allantoic fluid (31) for each isolate and reverse transcription (17) with PCR (26) amplification methods (RT-PCR) was conducted as previously described (29, 30, 31). Amplification was completed utilizing primers M629F (5′-TCGAGICTGTACAATCTTGC-3′) at positions 3884 to 3903 and F581R (5′-CTGCCACTGCTAGTTGIGATAATCC-3′) at positions 5054 to 5078 on the full-length NDV genome. This yielded a 1,195-bp product including the 3′ region of the M gene coding for the nuclear localization signal, a 5′ F gene portion coding for the cleavage activation site, and the M-F intergenic sequence (30, 31). Direct double-stranded nucleotide sequencing was completed using fluorescently labeled dideoxynucleotide terminators (33) with the oligonucleotide primers used for RT-PCR. Nucleotide sequence editing, analysis, prediction of amino acid sequences, and alignments were conducted with the LaserGene sequence analysis package version 5 (DNASTAR, Inc., Madison, Wis.) and IntelliGenetics GeneWorks version 2.5.1 (IntelliGenetics, Mountain View, Calif.) software. Phylogenetic analyses were completed with phylogenetic analysis using parsimony (PAUP) version 4.0b (34) software with a neighbor-joining algorithm using absolute distances following 1,000 bootstrap replicates. GenBank, EMBL, and DDBJ accession numbers for sequences used in the analysis are provided in Table 1.

The deduced amino acid sequence at the fusion protein cleavage site for all viruses is presented in Table 1. The predominant amino acid sequence among the virulent viruses analyzed was ^{110}GGRRQKR/FVG^{119}, including all Mexico 1996-2000 isolates, chicken/Honduras/44814/00, the 1996 U.S. psittacine isolates, most of the isolates from the outbreak in the southwestern United States during 2002 to 2003, along with the turkey/U.S.(ND)/43084/92 to isolate contracted from cormorants and the anhinga isolate from 1993. Interestingly, two isolates from the 2002-2003 outbreak in the southwestern U.S., dove/U.S.(CA)/9547/03 and chicken/U.S.(CA)/5634/03, had a single nonsynonymous nucleotide substitution at the F gene cleavage site resulting in the amino acid sequence GGRRQ*R*R (with the substitution indicated in italics). The OIE molecular definition for v-NDV is any APMV-1 virus that has three basic amino acids (R or K) at the fusion protein cleavage site between residues 113 and 116 at the C terminus of the F_2, as well as phenylalanine at residue 117 of F_1. Failure to demonstrate the characteristic pattern of amino acids requires characterization of the isolates by the ICPI assay (21). Therefore, all the isolates were considered virulent by those standards.

Recent v-NDV isolates obtained from outbreaks in the United States, Mexico, and Central America were phylogenetically compared to other worldwide strains (Fig. 1). Reference lentogenic chicken/U.S./LaSota/46 and chicken/U.S./B1/48 vaccine viruses clustered together and were most closely related to chicken/U.S.(TX)/GB/48 and chicken/U.S./BeaudetteC/45, two U.S. neurotropic velogenic viruses isolated prior to 1970. This group of viruses clearly separated from the other lentogenic isolates chicken/Australia/QV4/66 and chicken/N.I./Ulster/67, two viruses used as live-virus vaccines outside the United States. Virulent chicken/Australia/Victoria/32 separated as a potential progenitor for the remainder of the phylogram, which included the v-NDV isolates currently circulating worldwide and isolates from pigeons or doves considered PPMV-1. All the PPMV-1 isolates (dove/Italy/2736/00, pigeon/Italy/1166/00, and pigeon/U.S./TX/17498/98) clustered together and were most closely related to the virulent chicken/Kenya/KRC150/94 and chicken/U.S.(CA)/1083(Fontana)/72 isolates. Despite the amino acid sequence identity and phylogenetic similarity between the PPMV-1 clade and these strains, there are clear virulence differences in poultry (4, 16, 36, 40). The turkey/U.S.(ND)/43084/92, a neurotropic velogenic virus, and the mesogenic anhinga/U.S.(FL)/44083/93 virus formed a separate clade representing viruses from water birds other than ducks or geese. The chicken/Italy/3286/00 isolate was most closely related to two viruses, yellow nape parrot/Indonesia/904/87 and cockatoo/Indonesia/14698/90, isolated from psittacine birds in U.S. quarantine stations. The chicken/Mexico/37821-550-1/96, chicken/Mexico/37821-550-2/96, chicken/Mexico/3242/99, and chicken/Mexico/5166/98, along with three psittacine strains isolated in the United States during 1996 [parrot/U.S.(OK)/32932/96, parrot/U.S.(MO)/31378/96, and yellow cheek/U.S.(TX)/27345/96], form a unique phylogenetic clade closely related to yellow nape parrot/U.S.(MA)/19120/87 and chicken/Honduras/44814/00.

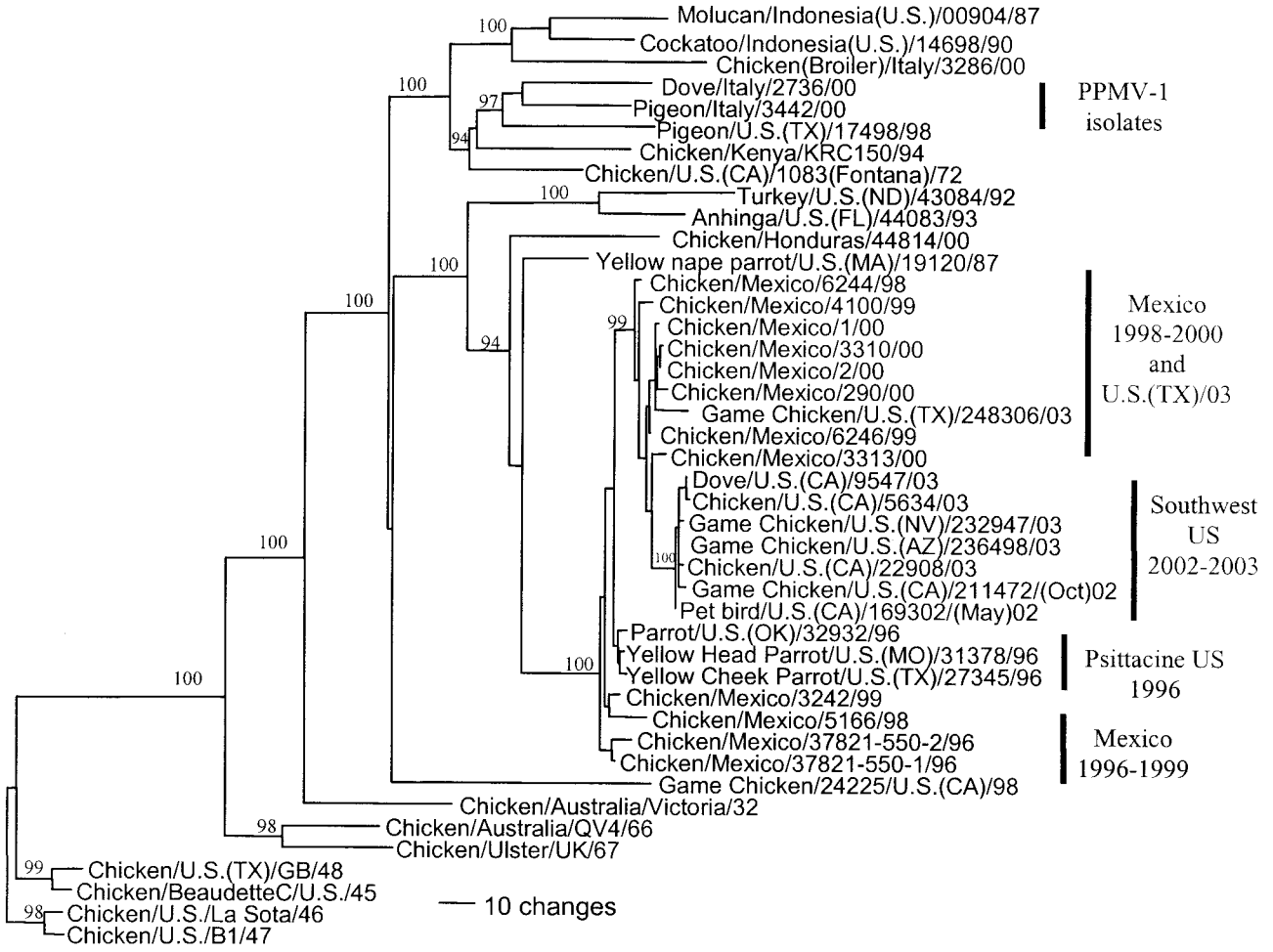

FIG. 1. Phylogenetic relationships among APMV-1 isolates obtained from the southwestern United States during the 2002-2003 outbreak and from Mexico during the 1998-2000 outbreak of virulent ND. Analysis was completed following alignment of nucleotide sequences from the 3′ portion of the matrix protein gene to the 5′ region of the fusion protein gene. The phylogram was generated by a neighbor-joining algorithm utilizing an absolute distance matrix. Numbers at the phylogram nodes indicate bootstrap confidence limits after 1,000 samplings and values of >90 are noted, indicating the strongly supported branches.

Nucleotide sequence comparisons resulted in chicken/Mexico/37821-550-1/96 and -2/96 having 98% identity with the three U.S. 1996 psittacine isolates mentioned in the previous paragraph and a 91% nucleotide sequence identity with the v-NDV chicken/Honduras/44814/00. A second distinct phylogenetic group was composed of two 1998-1999 Mexico chicken isolates, along with the Mexico 2000 viruses and game fowl/U.S.(TX)/248306/03. The nucleotide sequence similarity for the game fowl/U.S.(TX)/248306/03 virus isolated in El Paso, Tex., during the 2002-2003 v-NDV outbreak was 98% when compared to the other 2002-2003 NDV isolates from the United States. Despite this high degree of similarity, game fowl/U.S.(TX)/248306/03 was phylogenetically more closely related to the Mexico 2000 viruses than the California outbreak isolates. A third unique phylogenetic cluster was composed of the California 2002-2003 END isolates as well as the Arizona and Nevada viruses. The Arizona, Nevada, chicken/U.S.(CA)/22908/03, and game fowl/U.S.(CA)/211472/02 isolates had nearly 100% nucleotide sequence identity for the amplification product analyzed and formed a unique clade. Interestingly, the pet bird/U.S.(CA)/169302/02 virus was isolated from a sick bird in a pet shop during May of 2002, 5 months prior to the California outbreak. The dove/U.S.(CA)/9547-3/03 and chicken/U.S.(CA)5634/03 isolates, two viruses which were part of this cluster, had 99% nucleotide sequence identity to other members of the clade. Despite this high degree of nucleotide sequence similarity, there was a single nonsynonymous nucleotide substitution at the F gene cleavage site coding sequence of these two viruses. This resulted in an arginine (R)-for-lysine (K) substitution at amino acid position 115, in the predicted amino acid sequence GGRRQRR/FVG at the F cleavage site. An amino acid sequence change from GGRRQKR/FVG to GGRRQRR/FVG did not alter the virulence of the viral isolates, but it does support the possibility that mutations occur among isolates during an outbreak. This was clearly demonstrated during an ND outbreak in Australia during 1998 through 2000, wherein low-virulence viruses evolved to a more virulent form during a several-month time period (15).

The 2002-2003 v-NDV outbreak in the southwestern United States was initially detected in game fowl and later spread to

commercial poultry (19, 20). Phylogenetic analyses of the 2002-2003 U.S. NDV isolates suggest that the California, Nevada, and Arizona viruses represented a single introduction of virus. Despite the close nucleotide sequence identity (98%) among these viruses and the END virus isolated in El Paso, Tex., the game fowl/U.S.(TX)/248306/03 virus was most closely related to the v-NDV isolated in Mexico during 2000. Thus, this strain appears to be a separate introduction and not the result of movement of virus from the affected areas in California, Nevada, or Arizona. The California, Nevada, and Arizona viruses formed their own phylogenetic cluster that was most closely related to the Mexico/00-Texas/03 clade, as well as the v-NDV isolated during 1996 from pet birds in the United States. These viruses were also closely related to isolates from poultry in Honduras during 2000 and Mexico during 1996 to 1999. However, 5 months prior to the initial detection of v-NDV in California, a phylogenetically similar virus [pet bird/U.S.(CA)/169302/02] was isolated from a psittacine bird in a northern California pet shop. Several species of psittacine birds can intermittently shed virus in excess of 83 days postexposure (10, 11). For this reason, nonclinically infected psittacine birds can potentially transmit NDV, especially since psittacine birds and game fowl are often located on the same premises.

Multiple outbreaks of v-NDV were reported by Mexico to the OIE during 1996 to 2001 (22). Due to geographical proximity, the relationship of the 2002-2003 U.S. viruses to the Mexico v-NDV lineage is not surprising. However, it further emphasizes the necessity for an ongoing NDV surveillance program throughout the United States, Mexico, and Central America. Development and validation of a real-time RT-PCR assay (38) for the detection of APMV-1 may make this surveillance more feasible.

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