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Molecular Characterization of Avian Paramyxovirus 1 Isolates Collected from Cormorants in Canada from 1995 to 2000

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Sequences encompassing cleavage sites of fusion protein genes were obtained for avian paramyxovirus 1 isolates from cormorants in Canada. All isolates have the virulent cleavage site SRGRRQKR*FVG. They form a distinct cluster within isolates obtained around the world and may represent a novel genotype closely related to genotype V.

Avian paramyxovirus 1 (APMV1) has been isolated from a variety of species of free-living and domestic birds worldwide. The enveloped virus, belonging to the family *Paramyxoviridae*, has an over 15,000-nucleotide-long, negative-sense single-stranded RNA genome (7). Virus infections range from inapparent to severe (e.g., Newcastle disease in poultry), depending on the number of viral and host factors (2). The amino acid composition of the fusion protein cleavage site is the major determinant in the virulence of the virus (22). Wild bird species, especially aquatic birds, are considered to be more resistant to the disease and could serve as a virus reservoir (9, 14, 16, 17). However, high mortalities from APMV1 occurred in double-crested cormorants in Canada and the United States in 1990 and 1992 (8, 10, 21, 28). Based on fusion and matrix protein sequence data, the viruses from cormorants were related to viruses of psittacine origin and to viruses isolated during the 1970s California outbreak (24, 25, 26).

Canada has experienced several smaller outbreaks in cormorants since 1992. Relatively large outbreaks occurred in 1995 in Saskatchewan (18, 19), in 1996 in Ontario, and in 1999 in Alberta (6). Isolated submissions were also received from different parts of Canada throughout the years 1995 to 2000.

The purpose of this work was to characterize on the molecular level cormorant isolates submitted from 1995 to 2000, confirm their in vivo pathotype by using predicted amino acid composition of the fusion protein cleavage site, and determine their genotype.

Archived cormorant APMV1 isolates (allantoic fluid [AF] from embryonated chicken eggs inoculated with 0.1 ml of 10% homogenate from pooled cormorant tissues), stored at the National Centre for Foreign Animal Disease, Winnipeg, Manitoba, Canada, were used in the molecular analysis. AF was tested for the presence of APMV1 by the hemagglutination-inhibition test (5). AFs from dead embryonated chicken eggs yielding negative or weak hemagglutinating activity were retested on Vero cells by the indirect immunoperoxidase assay

TABLE 1. Elucidated fusion protein amino acid cleavage site sequences of the APMV1 cormorant isolates, with the GenBank accession numbers[a]

Isolate identification (reference)	ICPI (8 days)	IVPI (10 days)	Fusion protein cleavage site (molecular pathotyping)	GenBank accession no.
APMV1/CT/Sask/1479/90 (8)	1.55	2.08	SGGRRQKR*FVG	AF448223
APMV1/CT/Sask/2585/92 (8)	1.6	1	SRGRRQKR*FVG	AF448844
APMV1/CT/Man/3298/92	1.3	0.8	SRGRRQKR*FVG	AF448843
APMV1/CT/Ont/2150/95	1.6	1.2	SRGRRQKR*FVG	AF448486
APMV1/CT/Ont/2344/95	1.71	0.89	SRGRRQKR*FVG	AF448486
APMV1/CT/Ont/2575/95	1.88	0.12	SRGRRQKR*FVG	AY063493
APMV1/CT/Sask/2035/95 (12)	1.61	1.23	SRGRRQKR*FVG, nonhemagglutinating	AF448845
APMV1/CT/Ont/39/96	1.15	0.86	SRGRRQKR*FVG	AF448842
APMV1/CT/Ont/48/96	1.66	1.41	SRGRRQKR*FVG	AY063492
APMV1/CT/Sask/3-1125/98	1.56	1.09	SRGRRQKR*FVG, nonhemagglutinating	AY063494
APMV1/CT/Alb/35/99 (4)	1.07	0.41	SRGRRQKR*FVG, nonhemagglutinating	AF448841
APMV1/CT/Sask/45/99			SRGRRQKR*FVG	AF263615
APMV1/CT/Ont/378/00	1.3	1.64	SRGRRQKR*FVG, nonhemagglutinating	AY063123
APMV1/CT/Ont/429/00	ND	ND	SRGRRQKR*FVG	AY063123
APMV1/CT/Ont/410/00	ND	ND	SRGRRQKR*FVG, nonhemagglutinating	AY063123

[a] CT, cormorant. In ICPI the most virulent APMV1 isolates give indices close to 2, in IVPI the most virulent APMV1 isolates give indices close to 3, and in both ICPI and IVPI the avirulent viruses give values close to 0. ND, not done.

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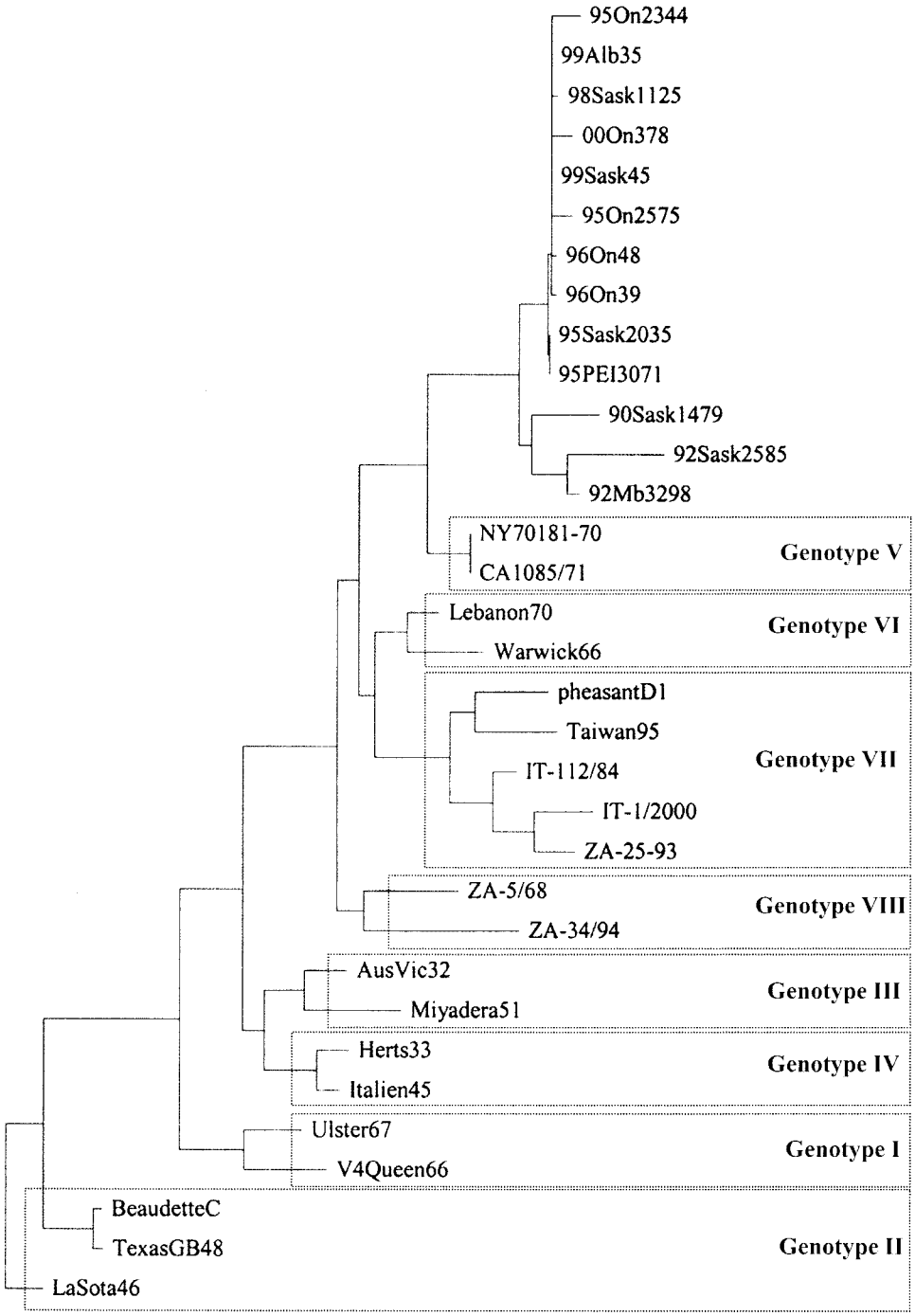

FIG. 1. Phylogenetic relationships among APMV1 isolates representing individual genotypes and the cormorant isolates from Canada based on a 374-nucleotide sequence including the fusion protein cleavage site. Sequences previously published in GenBank are as follows: NY70181/70, parrot, United States, AF001105; CA1085/71, fowl, United States, AF001106; Lebanon70, AF001110; Warwick66, Z12111; pheasantD1, D-16/93, pheasant, Germany, AF001113; Taiwan95, U62620; IT-112/84, turkey, Bergamo, Italy, AF218127; IT-1/2000, chicken, Padua, Italy, AF293350; ZA-25-93, South AFrica, AF13676; ZA-5/68, ZA 5/(South Africa)/68, AF136762; ZA-34/94, ZA-34/(South Africa)/94, AF136773; AusVic32, Australia, Victoria/32, M21881; Miyadera51, M18456; Herts33, M24702; Italien45, Italien/45, M17710; Ulster67, Ulster 2C/67, D00243; V4Queen66, V4 Queensland/66, M24693; BeaudetteC, X04719; TexasGB48, Texas GB/48, M24692; LaSota46, LaSota/46, U22292.

adapted from the work of Afshar et al. (1). The intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI) were determined as described in the Office International des Epizooties manual of standards (29). Viral RNA was extracted from AF by using TriPure isolation reagent (Boehringer Mannheim). One-step reverse transcription-PCR (RT-PCR) was performed with the Qiagen OneStep RT-PCR kit and the following RT-PCR program: 50°C for 30

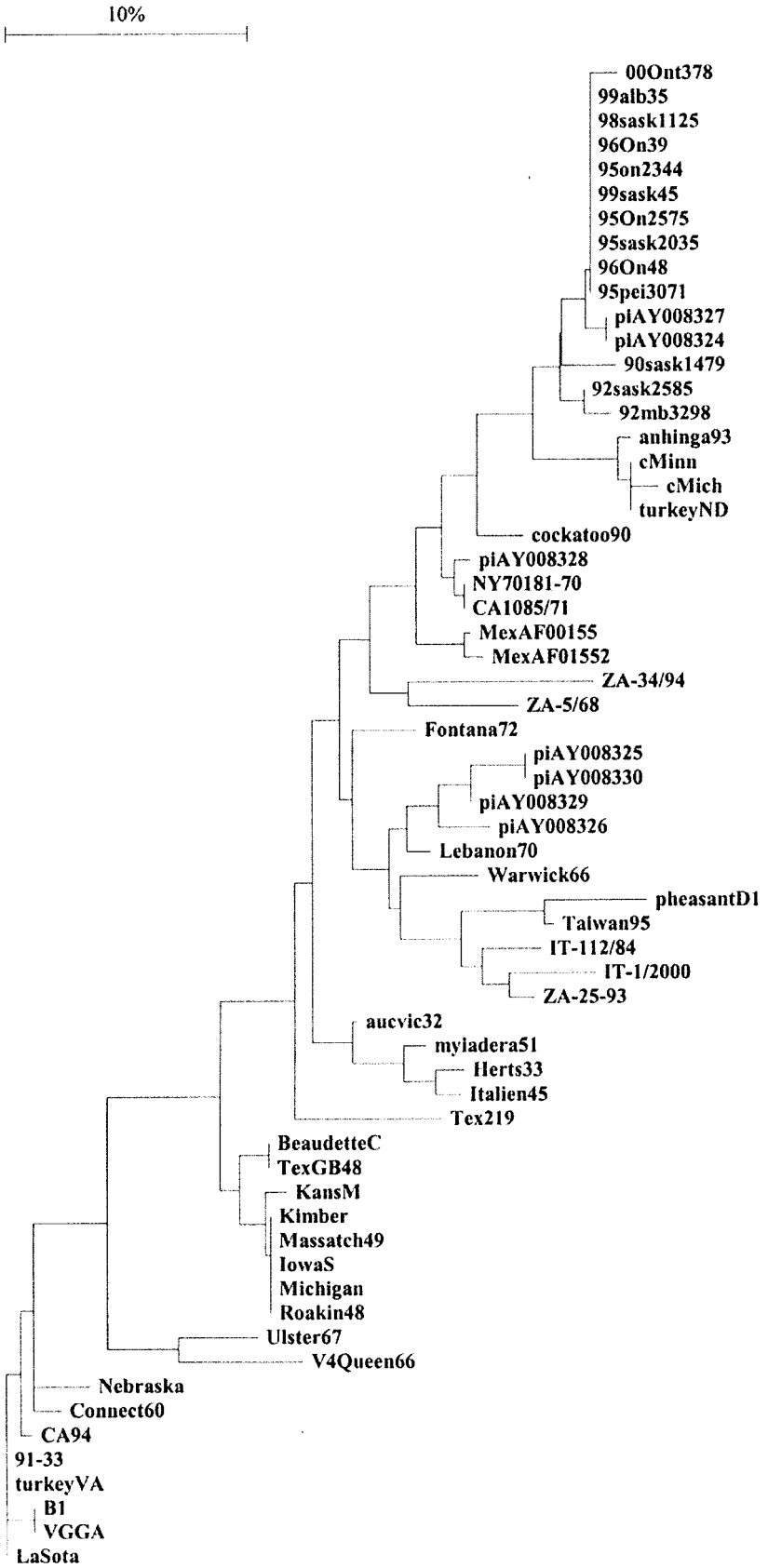
10%
00Ont378
99alb35
98sask1125
96On39
95on2344
99sask45
95On2575
95sask2035
96On48
95pei3071
piAY008327
piAY008324
90sask1479
92sask2585
92mb3298
anhinga93
cMinn
cMich
turkeyND
cockatoo90
piAY008328
NY70181-70
CA1085/71
MexAF00155
MexAF01552
ZA-34/94
ZA-5/68
Fontana72
piAY008325
piAY008330
piAY008329
piAY008326
Lebanon70
Warwick66
pheasantD1
Taiwan95
IT-112/84
IT-1/2000
ZA-25-93
aucvic32
myiadera51
Herts33
Italien45
Tex219
BeaudetteC
TexGB48
KansM
Kimber
Massatch49
IowaS
Michigan
Roakin48
Ulster67
V4Queen66
Nebraska
Connect60
CA94
91-33
turkeyVA
B1
VGGA
LaSota

FIG. 2. Phylogenetic relationships among the North American isolates of APMV1 and the representative isolates for each genotype based on a 93-nucleotide sequence including the fusion protein cleavage site. Sequences previously published in GenBank are as follows: cMich 92, cormorant, Michigan, 1992, U22270; cMinn 92, cormorant, Minnesota, 1992, U22269; turkeyND 92, turkey, North Dakota, 1992, U22289; anhinga93, anhinga (exotic—South America)/USA/93, U22265; cockatoo90, cockatoo (Indonesia)/USA/90, AF015508; piAY008326, pigeon/USA/1984, AY008326; c99Sask45, cormorant/Saskatchewan (Canada)/45/1999, AF263615; piAY008329, pigeon/USA/1984, AY008329; piAY008324, pigeon/USA/1975, AY008324; piAY008330, pigeon/USA/1998, AY008330; piAY008327, pigeon/USA/1984, AY008327; piAY008327, pigeon/USA/1984, AY008327; piAY008328, pigeon/USA/1975, AY008328; Fontana, Fontana 72 (CA1083), U22274; Tex219, Texas 219, U22286; Connect60, Connecticut 60, AF206617; CA94, California/1994, U22267; VGGA, VGGA/turkey/USA/1989, U22273; 91-33, isolate 9133, U22288; turkeyVA, turkey, Virginia, U22285; Nebraska, Nebraska, U22282; IowaS, Iowa/Salsbury/1949, U22276; Kimber, Kimber/1947, U22278; Michigan, Michigan, U22281; Massatch49, Massachusetts/MK/1949, U22280; Roakin, Roakin 48 (United States), U22284; KansasM, Kansas/Manhattan/1948, U22277; MexAF015520, Mexico/1996, AF015520; MexAF015518, Mexico/1996, AF015518; B1, B1, U22266.

min; holding at 95°C for 5 min; and 30 cycles of 95°C for 60 s, 56°C for 30 s, and 72°C for 30 s. The forward (5′-TGTCGCA GTGACTGCTGACC) and reverse (5′-GTCAGTGACCTCG TGCACAG) primers yielded an amplicon of approximately 750 nucleotides, which was sequenced with the RT-PCR primers. The ClustalW multiple alignment algorithm (European Bioinformatics Institute) was used to align the sequences, and the obtained PHYLIP output file (J. Felsenstein, Department of Genetics, University of Washington) was applied in TREECON, version 1.3b (27) to construct a dendrogram, with the neighbor-joining algorithm (23), based on a distance estimation model of Jukes and Cantor (15).

The 1995 to 2000 AMPV1 isolates from cormorants appear to form a separate cluster from the viruses isolated from cormorants in 1990 and 1992, based on the analysis of the 643-nucleotide sequence spanning from the 5′-terminal nucleotides of the matrix protein gene past the fusion protein cleavage site coding sequence and supported by a decrease or lack of hemagglutinating activity not observed for the earlier isolates (Table 1) (11). The phylogenetic analysis is graphically presented in Fig. 1. The molecular pathotyping (amino acid sequence of the fusion protein cleavage sites) corresponded with the in vivo pathotyping (Table 1). Compared to over 400 fusion protein cleavage site sequences published in the GenBank database, this type of cleavage site was identified only in isolates from cormorants, two isolates from exotic birds, and two isolates from pigeons in the United States.

Genetic characterization of the cormorant isolates was performed by evaluation of 374-nucleotide partial sequences of the variable region of the F gene (Fig. 1), by using corresponding sequences previously published in GenBank. The cormorant isolates appear to form a group related to genotype V in the system proposed by Ballagi- Pordany et al. (3), Lomniczi et al. (20), and Herczeg et al. (12). The phylogenetic distance from the "classical" genotype V ranges from 6.15 to 10.2% (distance matrix not shown).

Despite the short sequence used in the analysis, the grouping of the viruses illustrated in Fig. 2 remained close to the grouping in Fig. 1. (The longest published common nucleotide sequence for the representative viruses of the individual genotypes and the North American isolates of AMPV1 is only 93 nucleotides.) Again all cormorant isolates fell into one subgroup, distinct from the remaining viruses in genotype V. Only the anhinga and two pigeon isolates clustered together with cormorant sequences.

The finding is supported by previous work of other authors. Seal et al. (24, 25, 26) grouped the APMV1 isolated from parrots imported from South America and viruses isolated in the 1971 California epizootic in a way which in essence agrees with genotype V within the system used by European groups (3, 12, 13, 20). Considering the 6 to 10% phylogenetic distance from the "classical" genotype V, the cormorant isolates may represent a new genotype or a subtype within genotype V (Fig. 1). Clinical signs in cormorants reported for all the outbreaks indicated involvement of the central nervous system, and the isolates were considered neurotropic, velogenic, or mesogenic (4, 6, 10, 11, 18, 19, 21). Regretfully, Canada does not employ an in vivo pathogenicity test for chickens which allows distinction between viscerotropic and neurotropic isolates.

This work suggests that the 1995 to 2000 APMV1 isolates from cormorants may represent an indigenous APMV1 genotype present in the free-living wild bird population in North America, possibly waterfowl (14, 17), which intermittently causes high mortality in species such as cormorants or pigeons. Despite the rigorous biosecurity measures within the poultry industry, there is always the possibility of further spillover into domestic poultry as suggested during the 1992 cormorant outbreak, when a virus simultaneously isolated from a Newcastle disease virus outbreak in turkeys was closely related to the cormorant isolate from Minnesota (24).

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