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Characterization of Newcastle Disease Virus Isolates by Reverse Transcription PCR Coupled to Direct Nucleotide Sequencing and Development of Sequence Database for Pathotype Prediction and Molecular Epidemiological Analysis

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Degenerate oligonucleotide primers were synthesized to amplify nucleotide sequences from portions of the fusion protein and matrix protein genes of Newcastle disease virus (NDV) genomic RNA that could be used diagnostically. These primers were used in a single-tube reverse transcription PCR of NDV genomic RNA coupled to direct nucleotide sequencing of the amplified product to characterize more than 30 NDV isolates. In agreement with previous reports, differences in the fusion protein cleavage sequence that correlated genotypically with virulence among various NDV pathotypes were detected. By using sequences generated from the matrix protein gene coding for the nuclear localization signal, lentogenic viruses were again grouped phylogenetically separate from other pathotypes. These techniques were applied to compare neurotropic velogenic viruses isolated from an outbreak of Newcastle disease in cormorants and turkeys. Cormorant NDV isolates and an NDV isolate from an infected turkey flock in North Dakota had the fusion protein cleavage sequence ^{109}SRGRRQKRFVG^{119}. The R-for-G substitution at position 110 may be unique for the cormorant-type isolates. Although the amino acid sequences from the fusion protein cleavage site were identical, nucleotide sequence data correlate the outbreak in turkeys to a cormorant virus isolate from Minnesota and not to a cormorant virus isolate from Michigan. On the basis of sequence information, the cormorant isolates are virulent viruses related to isolates of psittacine origin, possibly genotypically distinct from other velogenic NDV isolates. These techniques can be used reliably for Newcastle disease epidemiology and for prediction of pathotypes of NDV isolates without traditional live-bird inoculations.

Newcastle disease virus (NDV), a member of the *Paramyxoviridae* family, is designated avian paramyxovirus 1. The enveloped virus has a negative-sense single-stranded genome of approximately 15 kb which codes for six proteins, including an RNA-directed RNA polymerase, hemagglutinin-neuraminidase protein, fusion protein, matrix protein, phosphoprotein, and nucleocapsid protein (3). Outbreaks of Newcastle disease were first reported for poultry from Java, Indonesia, and Newcastle-upon-Tyne, England, in 1926. The disease currently has a worldwide distribution with a wide host range in which all orders of birds have been reported to be infected by NDV. Infectious virus may be ingested or inhaled, which results in its transmission and is the basis for mass-application vaccination procedures for poultry (reviewed in reference 3). Isolates of NDV are categorized into three main pathotypes depending on the severity of disease produced by the isolate in chickens (2, 3). Lentogenic isolates do not usually cause disease in adult birds and are considered avirulent. Viruses of intermediate virulence that cause respiratory disease are termed mesogenic, while virulent viruses that cause high mortality are termed velogenic. Neurotropic and viscerotropic forms of velogenic viruses have been reported worldwide (3). Viscerotropic velogenic viruses have entered the United States via importation of psittacines (13, 47, 57) and were the causal agent of the major outbreak in southern California during the early 1970s (55, 63). Recent outbreaks of Newcastle disease in cormorants in the north central United States and southern Canada have been attributed to neurotropic velogenic viruses (9, 65).

Differential diagnosis of NDV involves hemagglutination inhibition with polyclonal NDV-specific antisera (2, 3) or use of the enzyme-linked immunosorbent assay (28, 41, 64). Oligonucleotide probes (26, 27) and viral genomic RNA fingerprint analysis (40, 46) have been used to identify and differentiate NDV strains but with limited success. Monoclonal antibodies are now used to identify antigenic groups (6, 18, 25, 34), but pathotyping NDV isolates still involves labor-intensive procedures. Pathotype prediction (2, 3) initially involves NDV inoculation of embryonated eggs to determine the mean time of death of the embryo. Further testing entails inoculation of chickens to determine the intracerebral pathogenicity index and the intravenous pathogenicity index. In the United States, the intracloacal inoculation pathogenicity test is used to distinguish viscerotropic velogenic NDV from neurotropic velogenic viruses (2). Additionally, virulent NDV can be differentiated by its ability to replicate in most avian and mammalian cell types without the addition of trypsin (30, 31, 43). Although all NDV isolates can replicate in chicken embryo kidney cells, lentogens require trypsin for replication in avian fibroblasts or mammalian cell types (31).

The molecular basis for NDV pathogenicity is dependent on the fusion protein cleavage site amino acid sequence (21, 35, 43) and the ability of specific cellular proteases to cleave the fusion proteins of different pathotypes (23, 45). Fewer basic amino acids are present in the fusion protein cleavage site of

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TABLE 1. NDV isolates examined phylogenetically

Isolate	Country	Yr of isolation	Pathotype[a]
D26	Japan	1978	L
Queensland/V4	Australia	1966	L
Ulster	Northern Ireland	1964	L
England/F	United Kingdom	1949	L
LaSota	United States	1946	L
B1	United States	1948	L
VGGA	United States	1989	L
Nebraska	United States	1954	L
Turkey/VA	United States	1985	L
CA4963	United States	1994	L
91/33	United States	1991	L
England/PR	Israel	1945	M
93/44083 (anhinga)	United States	1994	M
Massachusetts/MK	United States	1949	M
Michigan	United States	1946	M
Roakin	United States	1946	M
Kimber	United States	1947	M
Texas/DK	United States	1958	M
Australia/Victoria	Australia	1932	NV
Cormorant/MN	United States	1992	NV
Turkey/ND	United States	1992	NV
Cormorant/MI	United States	1992	NV
Iowa/Salsbury	United States	1949	NV
Texas/GB	United States	1948	NV
Beaudette/C	United States	1959	NV
California/RO	United States	1944	NV
Kansas/Manhattan	United States	1948	NV
Herts/33	United Kingdom	1933	VV
Italy/Milano	Italy	1945	VV
Texas/219	United States	1970	VV
Fontana	United States	1971	VV
FL80	United States	1980	VV
Largo	United States	1971	VV

[a] L, lentogen; M, mesogen; NV, neurotropic velogen; VV, viscerotropic velogen.

lentogenic NDV isolates than in the proteins of either mesogenic or velogenic strains, which have similar cleavage site sequences (21). Reverse transcription coupled to PCR (RT-PCR) was used to amplify fusion protein gene sequences of various NDV strains (29). However, when the amplification products were analyzed by agarose gel electrophoresis following restriction enzyme digestion, the results were inconsistent. Collins et al. (19) amplified a portion of the NDV fusion protein gene, and the cleavage activation site was deduced from nucleotide sequences of the amplification product. However, several sets of oligonucleotide primers were needed to amplify sequences from different pathotypes. In this study, we used a set of degenerate oligonucleotide primers for consistent single-tube RT-PCR coupled to direct automated nucleotide sequencing of the amplified product. Subsequent phylogenetic analysis allowed for pathotype prediction of NDV isolates and use in molecular epidemiology of Newcastle disease.

MATERIALS AND METHODS

History of virus isolates. A listing of virus strains with their pathotypes, countries of origin, and years of isolation is presented in Table 1. More-extensive histories follow in the text. Pathogenic (neurotropic velogenic) NDV isolates were obtained from cormorants in Michigan (cormorant/MI from the Animal Health Diagnostic Laboratory, Lansing, Mich.) and Minnesota (cormorant/MN from the National Wildlife Health Research Center, Madison, Wis.) during outbreaks in 1992 (9, 65). Subsequently, neurotropic velogenic NDV was isolated from turkeys (turkey/ND) in a commercial flock in North Dakota (provided by the Diagnostic Virology Laboratory, [DVL], National Veterinary Services Laboratory [NVSL], Animal and Plant Health Inspection Service [APHIS], U.S. Department of Agriculture [USDA], Ames, Iowa). Several nonpathogenic (lentogenic) field isolates (turkey/VA from the Poultry Diagnostic Research Center, College of Veterinary Medicine, University of Georgia, Athens; 91/33, a rhea isolate from the Georgia Poultry Diagnostic Laboratory, Oakwood; CA/4963, from turkeys provided by the Fresno Branch, California Veterinary Diagnostic Laboratory System; and Nebraska virus from the Southeast Poultry Research Laboratory, USDA, Athens, Ga.), viscerotropic velogenic isolates from psittacines (FL80 and Largo [12]), and an NDV isolate from the outbreak of Newcastle disease in California from 1972 to 1974 (Fontana, or CA/1083 [55]) as well as a viscerotropic velogenic virus isolate made in the early 1970s, Texas/219, with biological properties similar to those of Largo (12), were also used for analysis. Strains B1 (24), LaSota (22), VGGA (vaccine strain provided by P. Villegas, Poultry Diagnostic Research Center, College of Veterinary Medicine, University of Georgia), England/F (8), Ulster (37, 42), and Queensland/V4 (20, 58) were analyzed as lentogenic vaccine strains used in the poultry industry worldwide. The lentogenic waterfowl virus D26 was isolated and characterized in Japan (53). Several isolates of intermediate (mesogenic) virulence used for sequence analysis included the Massachusetts/MK, Michigan, Kimber, England/PR, and Texas/DK viruses (56). The mesogenic vaccine strain Roakin (10) and a mesogenic anhinga isolate (93/44083) from a captive population (DVL, NVSL, APHIS, USDA) were included for analysis. Velogenic viruses examined included well-characterized isolates, such as the viscerotropic velogenic Herts/33 (4) and Italy/Milano (4, 56) isolates as well as the neurotropic velogenic Australia/Victoria (1, 8, 39), Beaudette/C (11, 14), and Texas/GB (54, 56) isolates. Less well characterized neurotropic velogenic viruses included for analysis were the California/RO, Kansas/Manhattan (55), and Iowa/Salsbury (Southeast Poultry Research Laboratory, USDA) isolates. Pigeon NDV isolates (7, 48) and avian paramyxoviruses 2 and 3 (3) were also included for analysis.

Initial characterization of all viral isolates was accomplished by hemagglutination inhibition with NDV-specific polyclonal antisera (2). Virulence of NDV isolates not previously evaluated was assessed by standard procedures (2).

Replication of isolates and RNA extraction. Isolates of NDV were replicated in embryonated eggs (2). Viruses were purified by sucrose gradient centrifugation (43), and genomic RNA was acid-guanidinium-phenol extracted (16). Also, RNA was extracted (16) directly from allantoic fluid of infected embryonated eggs following inoculation with different NDV isolates. For use as negative controls during RT-PCR, RNA was extracted (16) from allantoic fluid of embryonated eggs infected with infectious bronchitis virus (33) or avian influenza virus (49).

Oligonucleotide primers and RT-PCR. Degenerate oligonucleotide primer pairs (fusion protein: 5′-CCTTGGTGAITCTATCCGIAG-3′ [sense] and 5′-CTGCCACTGCTAGTTGIGATAATCC-3′ [antisense]; matrix protein: 5′-TCGAGICTGTACAATCTTGC-3′ [sense] and 5′-GTCCGAGCACATCACTGAGC-3′ [antisense]; I stands for inosine) were generated by alignment of published NDV nucleotide sequences followed by analysis of conserved nucleotide sequences using the PRIMER2 (Scientific & Educational Software, Stateline, Pa.) computer program. Oligonucleotide RT-PCR primers were designed to amplify regions of the fusion protein gene, including the fusion protein cleavage site (61, 62), and the matrix protein gene (15, 39, 53) region encoding the nuclear localization signal of the matrix protein (17). A single-tube RT-PCR for genomic NDV RNA was completed essentially as described previously (36), except that initial RT was conducted by use of Superscript (Life Technologies, Gaithersburg, Md.) for 1 h at 45°C (32) followed by denaturation at 95°C for 5 min. Twenty-five cycles of denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s, and primer extension at 72°C for 10 min were completed with 3 U of Amplitaq (Perkin-Elmer) polymerase (50). Amplification products were separated by gel electrophoresis in 1.0% agarose using Tris-borate buffer (50 mM Tris, 50 mM boric acid, 10 mM EDTA; pH 8.4), stained with ethidium bromide, and photographed during UV transillumination (36).

Direct nucleotide sequencing of RT-PCR products and phylogenetic analysis. Resultant RT-PCR products were purified with Microcon spin filters and quantitated spectrophotometrically. Direct double-stranded nucleotide sequencing (52) was completed by using *Taq* polymerase (Applied Biosystems) with the oligonucleotide primers used for RT-PCR, fluorescently labelled dideoxynucleotides, and an automated nucleic acid sequencer (59).

Nucleotide sequence editing, analysis, prediction of amino acid sequences, and alignments were conducted by using IntelliGenetics GeneWorks 2.4 software (IntelliGenetics/Betagen, Mountain View, Calif.). Phylogenetic analysis included in the GeneWorks package was completed by using the unweighted-pair group method with arithmetic means (UPGMA) (44), which is based on pairwise comparisons of most-similar sequences. Additionally, phylogenetic trees were constructed by use of PAUP (60) software with a heuristic search and 1,000 bootstrap replicates.

Nucleotide sequence accession numbers. The nucleotide sequences of the fusion protein and matrix protein genes from the RT-PCRs were submitted to GenBank as a single sense-strand contiguous sequence for each NDV isolate. The accession numbers assigned to the isolates are U22265 through U22294.

RESULTS

Evaluation of RT-PCR for NDV isolates. Products from genomic RNA RT-PCR amplification representing the three

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CormorantMN-NV        .........G .A..R..... ...V.....S I......... .......... N.........
TurkeyND-NV           .........G .A..R..... ...V.....S I......... .......... N.........
Anhinga-M             .........G .A..R..... ...V.....S I......... V......... N.........
FL80-VV               ..D....T.G .A........ ...V.....S .......... .......... N.........
Michigan-M            .......... .......... .......... .......... .......... K.........
Roakin-M              .......... .......... .......... .......... .......... K.........
TexasGB-NV            ..D....... .......... .......... .......... .......... K.........
Fontana-VV            .........G ..S....... .........S .......... .......... N.........
Herts33-VV            ..D...K... .......... R........S .......... ....S..... N.........
EnglandF-L            .......... .......G.. G.L....... .......... .......... K.........
LaSota-L              .......... .......G.. G.L....... .......... .......... K.........
91/33-L               .......... .......G.. G.L....... .......... .......... K.........
B1/47-L               ..D....... .......G.. G.L....... .......... .......... K.........
QueenslandV4-L        ..D....... .......GK. G.L....... .......... ....S..... N.........

Consensus          95 LGESIRRIQE SVTTSGGRRQ KRFIGAIIGG VALGVATAAQ ITAAAALIQA .QNAANILRL 154
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FIG. 1. Predicted amino acid sequence alignment of NDV fusion protein cleavage sites. Partial sequences for 14 representative isolates are shown. The sequences were derived by translating primary nucleotide sequences obtained by direct sequencing of the RT-PCR products. The fusion protein cleavage site sequence from positions 109 to 119 is underlined. Abbreviations: L, lentogen; M, mesogen; NV, neurotropic velogen; and VV, viscerotropic velogen.

pathotypes of NDV were produced by using degenerate oligonucleotide primers for the fusion protein gene and matrix protein gene. Amplification from coding sequences surrounding the fusion protein cleavage site resulted in a 254-bp product, while amplification products from the region surrounding nucleotide sequences encoding the matrix protein nuclear localization signal were 232 bp long (data not shown). No products were obtained when RNA from infectious bronchitis virus or avian influenza virus was used for RT-PCR with the NDV-specific primers (data not shown).

Amplification products were produced by using the fusion protein gene RT-PCR primers with pigeon NDV or avian paramyxovirus 2 and 3 RNAs with this protocol. However, no matrix protein gene amplification products were obtained when RNAs from these virus types were used in the respective RT-PCRs (data not shown).

Predicted amino acid sequences of RT-PCR products. Predicted amino acid sequences of the fusion protein cleavage site for several representative NDV isolates examined were computer generated from the nucleotide sequences of the amplified products and are presented in Fig. 1. The amino acid sequence begins with the nucleotide at position 2 of the sense-strand oligonucleotide primer used for RT-PCR and sequencing of the fusion protein gene. The lentogenic vaccine isolates LaSota, England/F, B1, and Queensland/V4 and the lentogenic field isolate 91/33 all have the sequence ^{109}SGGGRQGRLIG^{119} at the fusion cleavage site, while the mesogenic and velogenic viruses have the sequence ^{109}SGGRRQR(K)RFIG^{119} containing the two diagnostic pairs of dibasic amino acids associated with virulence. More-virulent isolates have the RR sequence instead of the GR sequence at positions 112 and 113 as well as the RR or RK pair rather than the GR sequence at positions 115 and 116. The turkey/ND isolate shared 100% similarity at the nucleotide sequence level and corresponding amino acid sequence with the cormorant/MN virus. Both isolates had a conserved V-for-I substitution at position 118 that was shared with the psittacine isolates FL80 and Largo. The cormorant, turkey/ND, and anhinga isolates also shared an R-for-G substitution at position 110 and an I-for-V substitution at position 125 that were unique to these isolates, resulting in the ^{109}SRGRRQKRFVG^{119} fusion protein cleavage site sequence.

For the matrix protein gene, translation of the RT-PCR product nucleotide sequences begins at frame 1 of the sense-strand oligonucleotide primer and encompasses the nuclear

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QueenslandV4-L        ........K. .......... .......... ....S...K. .......... R...R....E
Herts33-VV            ........K. .......... .......... ....S...K. .......... ....R.....
FL80-VV               .......SK. .......... .......... ....S.I.KR .......... R...R.N...
Roakin-M              ........R. .......... .......... ....T...R. .......... ....S.....
B1/47-L               ..N.....R. .......... .......... ....T...R. .......... ....S.....
EnglandF-L            ..N.....R. .......... .......... ....T...R. .......... ....S.....
TexasGB-NV            ..N.....R. .......... .......... ....T...RR .......... ....S.....
Michigan-M            ..N.....R. .......... .......... ....T...RR .......... ....S.....
LaSota-L              ..N.....R. .......... .......... ....T...R. .......... ....S.....
91/33-L               ..N.....R. .......... .......... ....T...R. .......... ....S.....
TurkeyND-NV           ........K. .......... G......... ....S...K. .......... E...R.N...
CormorantMN-NV        ........K. .......... G......... ....S...K. .......... E...R.N...
Anhinga-M             ........K. ......T... G......... ....S...S. .......... E...R.....
Fontana-VV            .F......K. .......... .......... ....S...K. ........I. G...R.N...

Consensus         210 TIDVEVDP.S PLVKSLSKSD SGYYANLFLH IGLM.TVD.K GKKVTFDKLE KKIR.LDLSV 268
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FIG. 2. Predicted amino acid sequence alignment of NDV matrix proteins. Partial sequences for 14 representative isolates are shown. The sequences were derived by translating primary nucleotide sequences obtained by direct sequencing of the RT-PCR products. The nuclear localization signal from positions 247 to 263 is underlined. Abbreviations: L, lentogen; M, mesogen; NV, neurotropic velogen; and VV, viscerotropic velogen.

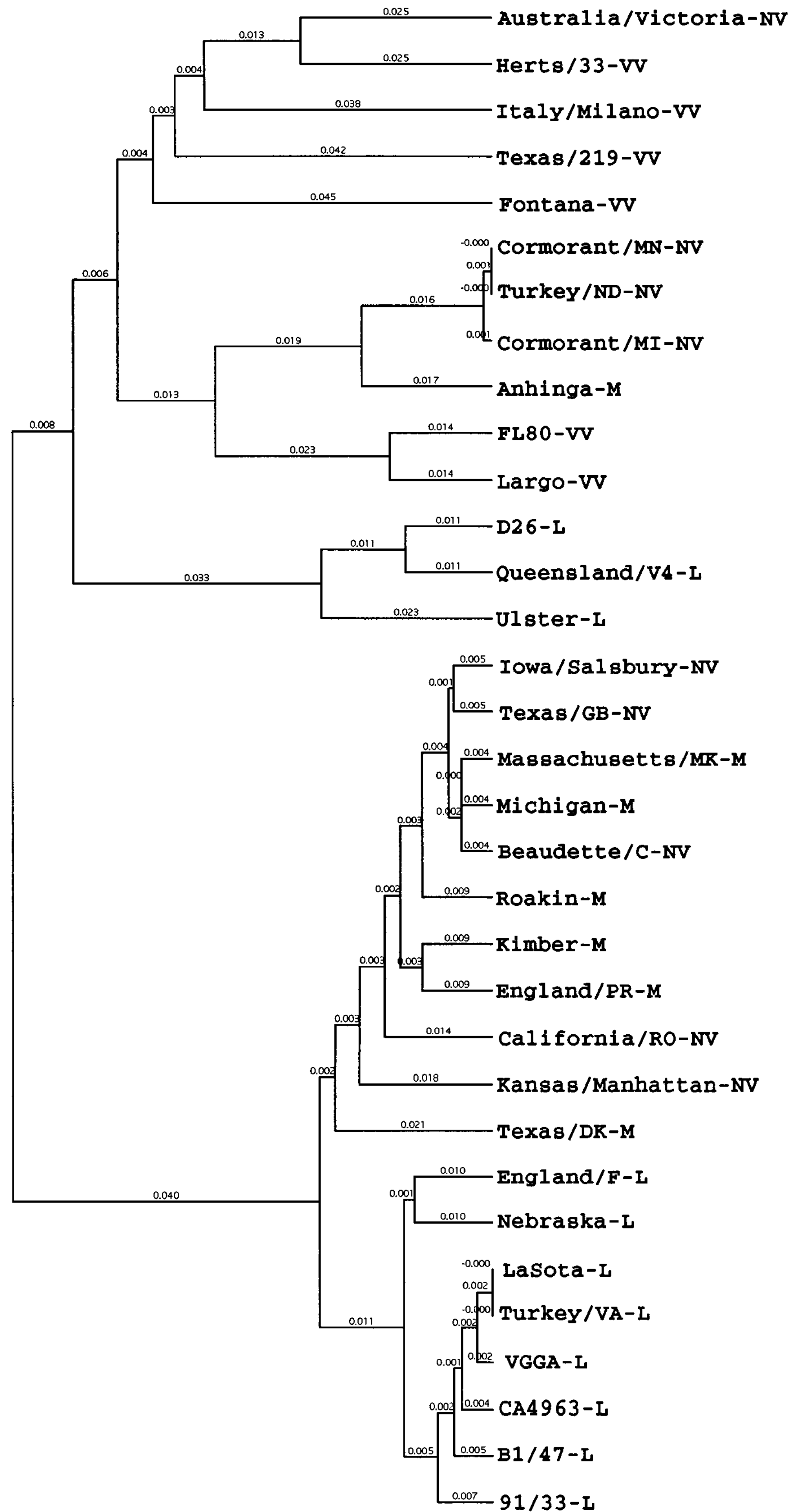

FIG. 3. Phylogenetic analysis of contiguous nucleotide sequences from the amplified products of NDV fusion protein and matrix protein genes. A phylogenetic tree was generated by UPGMA following alignment of sequences. Numbers represent the percent chance of a nucleotide substitution at one site. Abbreviations: L, lentogen; M, mesogen; NV, neurotropic velogen; and VV, viscerotropic velogen.

localization signal of the matrix protein (Fig. 2). Both cormorant NDV isolates share 100% amino acid sequence similarity with the turkey/ND virus and share a D-for-N substitution at position 212 with the anhinga isolate and psittacine isolates Largo and FL80. At position 259 an E-for-K substitution occurs in the cormorant, turkey/ND, and anhinga isolates, while the psittacine viruses, Largo and FL80, have an R-for-K substitution at this position. An R-for-D substitution at residue 263, also present in the anhinga sequence, and the N-for-D substitution at position 265 are shared by the cormorant and psittacine viruses. No particularly distinguishing sequence variations were identified among the lentogenic viruses in comparison with the mesogenic and velogenic pathotypes other than the sequence differences observed among the psittacine- and cormorant-derived isolates.

Phylogenetic relationships among NDV isolates based on nucleotide sequences of RT-PCR products. Contiguous nucleotide sequences from the fusion protein and matrix protein genes were subjected to phylogenetic analysis following multiple alignment (Fig. 3). Similar results were obtained when either nucleotide or predicted amino acid sequences from either the fusion protein gene or matrix protein gene were analyzed separately by UPGMA or by maximum parsimony (data not shown). The NDV isolates can be separated into two major groups by phylogenetic analysis. One branch contained isolates such as neurotropic velogenic Texas/GB, mesogenic Kimber, and the lentogenic vaccine strains B1 and LaSota. The second branch includes the viscerotropic velogenic Herts/33 and neurotropic velogenic Australia/Victoria isolates. Only one known mesogenic isolate was found in this group. This virus was isolated from an anhinga in a captive bird collection in the southeastern United States. Isolates recently made from Newcastle disease outbreaks in cormorants and turkeys from the north central United States fall into this second branch. The cormorant and turkey/ND viruses are most closely related genotypically to the psittacine isolates Largo and FL80. The Fontana and Texas/219 isolates also belong to the group including Herts/33 and Australia/Victoria.

Lentogenic isolates characterized by vaccine strains B1 and LaSota can be subdivided separately from mesogenic or velogenic viruses, such as Roakin and Texas/GB. However, the recent field isolates turkey/VA, 91/33, and CA4963 do not share 100% sequence identity to the vaccine strains commonly used. Vaccine strains not utilized in the United States, Queensland/V4 and Ulster, also phylogenetically separate independently from mesogenic and velogenic viruses in the group containing Herts/33, Australia/Victoria, and the psittacine isolates. The lentogenic duck isolate, D26 from Japan, is genotypically similar to the lentogenic Queensland/V4 and Ulster viruses.

DISCUSSION

The use of degenerate oligonucleotide primers for amplification of nucleotide sequences from the genomes of divergent NDV isolates is reported here. Following RT-PCR, the same primers were subsequently used to directly sequence the amplified products with an automated nucleotide sequencer. Using phylogenetic analysis following alignment of the nucleotide or predicted amino acid sequence, avirulent lentogenic NDV isolates could be routinely separated from the more virulent mesogenic and velogenic isolates. Similar results were obtained for the matrix protein gene RT-PCR product sequence and the fusion protein gene sequence. Consequently, these methods may eventually result in partial reduction in traditional live-animal testing for pathotyping of NDV isolates. It is important that the phylogenetic relationships based on the sequences of the RT-PCR products conform to what was reported previously for the entire fusion protein gene (61) and the hemagglutinin-neuraminidase gene (51) of divergent NDV isolates.

On the basis of nucleotide sequence information obtained during these studies, the fusion protein cleavage site amino acid sequence differences for all lentogenic versus more-pathogenic isolates correspond to what has been demonstrated previously (21). However, the recent cormorant isolates (9, 65), as well as a virus from a turkey flock infected with the cormorant-like virus, had fusion protein cleavage site amino acid sequences different from those of neurotropic velogenic isolates Texas/GB and Beaudette/C. The sequence of the fusion protein cleavage site from the cormorant-type isolates more closely resembles that of highly virulent viruses isolated from chickens during the 1990 outbreak of Newcastle disease in the Republic of Ireland (5, 19). The V-for-I substitution at position 118 of the fusion protein is unique to the psittacine-type and cormorant NDV isolates and is also similar to the protein sequences of antigenically distinct waterfowl isolates reported in Europe (19). The R-for-G substitution at residue 110 of the fusion protein is unique to the cormorant-derived isolates from North America.

Phylogenetic analyses not only can be applied to pathotype prediction of NDV isolates, but also may be used to determine viral origins. The Fontana NDV isolate obtained from chickens during the major 1970s Newcastle disease outbreak in southern California (55) was also genotypically related to the Largo and FL80 (12) psittacine-type isolates. This further supports the epidemiologic information implicating pet birds as the cause of that outbreak (63). On the basis of the nucleotide sequence analysis presented here, a virus like the cormorant isolate from Minnesota and probably not the virus from Michigan was the cause of the outbreak in a North Dakota turkey flock. These viruses are also most closely related genotypically to NDV isolates of psittacine origin and not to the traditional neurotropic velogenic viruses, i.e., Texas/GB, previously isolated from poultry in North America. The Texas/219 and anhinga isolates also are genotypically related to psittacine-type viruses and were isolated at the same time as the Fontana and cormorant viruses, respectively. Since these viruses were isolated at different times and from different locations in the United States, these virus types may have been introduced from psittacine sources or are circulating in bird populations of North America. Finally, the data indicate that there are two major phylogenetic groups of NDV isolates. One group includes viruses common worldwide, including to North America, while the second group contains viruses from psittacines and isolates considered exotic to the United States (12, 13, 47, 57). The psittacine-type viruses fall into their own subset of the second group, possibly unique in comparison with the original subtypes proposed for NDV isolates (51, 61).

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