PATHOTYPING OF NEWCASTLE DISEASE VIRUS ISOLATES FROM PET BIRDS

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Summary. – Four Newcastle disease virus (NDV) isolates obtained from a pigeon, lory, parrot, and love bird were subjected to biological and molecular characterization. All the isolates were identified as velogenic with intracerebral pathogenicity indices (ICPI) of 1.9–2.0. All the isolates had a ^{112}RRQKRF^{117} motif in the fusion protein cleavage site (FPCS), typical for pathogenic NDV. Phylogenetic analysis placed the isolates along with a velogenic Indian isolate of C1 group recovered during 1987.

Key words: Newcastle disease virus; pet birds; pathotyping; fusion protein cleavage site; phylogenetic analysis; RT-PCR; sequencing

Introduction

Newcastle disease (ND), defined as a List A disease by Office International des Epizooties (OIE), is a highly contagious and devastating avian disease caused by NDV (the *Newcastle disease virus* species, *Rubula virus* genus, family *Paramyxoviridae* (van Regenmortel *et al.*, 2000; Mayo, 2002). It is characterized by respiratory distress, diarrhea, depression and impairment of central nervous system. NDV has a negative-sense single-stranded RNA genome of approximately 15 kb, encoding six major proteins – fusion (F) protein, hemagglutinin-neuraminidase (HN), matrix (M) protein, phosphoprotein (P), RNA polymerase (L), and nucleoprotein (NP). The F protein, which is responsible for initiation of infection by the fusion of virion membrane with host cell membrane and penetration of the virus into the cell (Marin *et al.,* 1996), is synthesized as a F_0 precursor, which is activated only after cleavage into F_1 and F_2 fragments by host cell proteases. Based on pathogencity, NDV has been categorized into lentogenic, mesogenic and velogenic pathotypes, which also differ in the number of basic amino acids (Seal *et al.,* 1995). NDV has a wide host range, which embraces domestic and free-living birds, water fowls, commercial and pet birds (Seal *et al*., 1998; Aldous and Alexander, 2001; Alexander, 2003; Kumanan *et al*., 2003; Wehmann *et al*., 2003; Mathivanan *et al*., 2004).

In India, pigeons and psittacine birds (lory, parrot, and love bird) are reared as pets for a number of reasons, including a wide range of sizes, colors, and temperaments. Isolation of a virulent NDV from caged birds (Senne *et al*., 1983) and spread of NDV by this source (Utterback and Schwartz, 1973) have been reported earlier. Virulent NDV has been isolated regularly from pet birds imported illegally or held in quarantine in the USA (Panigrahy *et al*., 1993). There was a particular concern in 1991 when outbreaks occurred in illegally imported pet birds in six States of USA. (Bruning-Fann *et al*., 1992; Panigrahy *et al*., 1993), but no spread to poultry occurred. Besides, clinically healthy captive birds belonging to the *Columbiformes, Psittaci formes, Phasianiformes* and *Passeriformes* in India were found to be shedding velogenic NDV in their feces without showing clinical signs (Roy *et al*., 1998). Thus, ND in pet birds assumes considerable significance on economic,

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Abbreviations: AAF = amnioallantoic fluid; FPCS = fusion protein cleavage site; HA = hemagglutinin, hemagglutination; HAI = hemagglutination-inhibition; ICPI = intracerebral pathogenicity index; MAb = monoclonal antibody; ND = Newcastle disease; NDV = ND virus; OIE = Office International des Epizooties

ecological and emotional grounds. Further, these birds are not vaccinated against ND. There are only few reports on pathotyping of NDV isolates from these birds. Hence, a study on the prevalence of NDV in these birds deemed necessary to evaluate the ecological distribution and their possible role in the spread of the disease. In this context, this study describes isolation of virulent NDV from a pigeon, lory, parrot and love bird, submitted for necropsy, followed by pathotyping based on biological and molecular characterization.

Materials and Methods

Virus isolation and identification. Spleen, brain, proventriculus and tracheal samples were collected from a pigeon, love bird (*Agapornis* spp.), lory (*Domicella* spp.), and parrot (*Psittacus* spp.) during necropsy at the Department of Veterinary Pathology, Madras Veterinary College, Madras, India. Virus isolation was done by inoculating pooled suspensions of the samples into the allantoic cavity of 9-day-old embryonated SPF chicken eggs as described by Alexander (1988). Five blind passages were done before regarding a sample as negative in virus isolation. The amnioallantoic fluid (AAF), harvested from eggs with dead embryos that died 24 hrs after inoculation, was tested for hemagglutination (HA) with 1% chicken erythrocytes. A hemagglutination inhibition (HAI) test was done by using a NDV-specific antiserum for the samples with HA activity (Alexander, 1988, 1989).

Biological characterization. ICPI in one-day-old chicks (OIE, 2000), HA of mammalian (cattle, goat, horse, and human 'O' group) erythrocytes (Winslow *et al*., 1950), adsorption of HA by chicken brain cells (Hanson, 1980), and thermostability of HA at 56°C (Hanson *et al*., 1949) were assessed using bacteria-free-AAF as described earlier.

RT-PCR. Total RNA was extracted from both tissue samples and clarified AAF as described by Chomczynski and Sacchi (1987). cDNA was synthesized from total RNA using the Revert Aid First Strand cDNA Synthesis Kit from MBI Fermentas (USA) according to the manufacturer's instructions. In PCR, the FPCS sequence of 254 bp was amplified in a thermal cycler (Perkin Elmer, USA). The reaction mixture (50 µl) contained 2 µl of cDNA, 1 µl (10 picomoles) each of forward (5'-CCTTGGTGAITCTATCCGIAG -3') and reverse primer (5'-CTGCCACTGCTA GTTGIGATAAT CC-3'), 5 µl of the 10x reaction buffer, 2 µl of 10 mmol/l dNTPs (Finnzymes), 0.5 µl of DyNAzyme™ II DNA polymerase (Finnzymes) (Seal *et al*., 1995). Initial denaturation at 94°C for 5mins was followed by 25 cycles of denaturation at 95°C for 30 secs, annealing at 52°C for 40 secs, and extension at 70°C for 1 min, and final extension at 70°C for 7 mins. The PCR products were electrophoresed in 2% agarose gels, purified using the QlAquick Gel Extraction Kit from Qiagen (Germany).

Nucleotide sequencing and analysis. Purified RT-PCR products were sequenced in an automated DNA sequencer (ABI prisms, Version 3, Applied Biosystems, USA). The sequences were deposited at the GenBank and compared with those of reference isolates/strains, retrieved from the GenBank. The Gene Tool Lite Software Package Version 1.0 was used for editing nucleotide sequences and deducing amino acid sequences. All the nucleotide and deduced amino acid sequences were aligned using the Clustal X Version 1.8 (Jeannmougin *et al*., 1998).

Phylogenetic analysis and amino acid sequence variation estimation was carried out using the Molecular Evolutionary Genetics Analysis (MEGA) software Version 1.02 (Sudhir *et al*., 1993).

Results

Virus isolation and identification

NDV isolation attempts resulted in the recovery of four isolates from a pigeon, lory, parrot, and love bird. Whereas the pigeon and love bird isolates took three passages to kill the embryo, the lory and parrot isolates took five passages. All the isolates agglutinated chicken erythrocytes, with the HA titers ranging from 256 to 4096. HA agents present in the allantoic fluid were identified as NDV by the HAI test using a NDV-specific antiserum.

Biological characterization

The results of biological characterization are presented in Table 1. The intracerebral pathogenicity indices were found to range between 1.9 and 2.0. None of the isolates agglutinated equine erythrocytes. The HA of the isolates

Table 1. Biological characterization of the NDV isolates from pet birds

Host	ICPI	HA adsorbed by chicken brain cells (%)	Stability of HA at 56°C	Agglutination of mammalian erythrocytes (HA titers)			
				Cattle	Goat	Horse	Human[a]
Pigeon (*Columba livia*)	1.9	96.8	6 hrs	128	8	<2	8
Lory (*Domicella* spp.)	2.0	95.0	6 hrs	2	2	<2	4096
Parrot (*Psittacus* spp.)	1.9	87.5	85 mins	4	8	<2	2048
Love bird (*Agapornis* spp.)	2.0	93.7	90 mins	4	4	<2	2048

[a]'O' group.

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Pigeon(AY378322)                          SVSTSGGRRQ KRFIGAVIGS VALGVATAAQ ITAAAALIQA NQNAANILRL KESIAATNEA VHE
Lory (AY378321)                           .......... .......... .......... .......... .......... .......... ...
Parrot(AY378320)                          .......... .......... .......... .......... .......... .......... ...
Love bird(AY378326)                       .......... .......... .......... .......... .......... .......... ...
African Green Timneh/Zimbabwe(AF015513)   ..T....... R.....I... .......... .......... .......... .......... ...
Amazon/U.S.(AF015516)                     .AT....... ...V..I... .......... .......... K......... .......... .L.
Anhinga (AY562986)                        .AT..R.... ...V..I... I......... V......... .......... .......... ...
Cormorant Minnesota(U22269)               .AT..R.... ...V..I... I......... .......... .......... .......... ...
Essex/U.K. (AF355275)                     .AT....... ...V..I... .......... .......... .......... .......... ...
Finch/Tanzania(AF015519)                  .AT....... ...V..I... A......... .......... .K....V... .......... ...
Herts33(U22275)                           ..T....... R.....I... .......... ....S..... .......... .......... ...
LaSota (U22292)                           ..T....G.. G.L...I..G .......... .......... K......... .......... ...
Nebraska(U22282)                          ..T....G.. G.L...I..G .......... .......... K......... .......... ...
Parrot/U.S. (AF015512)                    .AT....... ...V..I... .......... .......... .......... .......... ...
Queensland V4(U22283)                     ..T....GK. G.L...I..G .......... ....S..... .......... .......... ...
Roakin(U22284)                            ..T....... ......I..G .......... .......... K......... .......... ...
Mukteshwar(R2B)Vaccine Strain(AF204755)   .LT....... ......I... .......... .......... L........I .......... ...
Texas GB (U22293)                         ..T....... ......I..G .......... .......... K......... .......... ...
Ulster (U22290)                           ..T....GK. G.L...I..G A......... ....S..... .......... .......... ...
Yellow Headed Parrot/U.S.(AF015511)       .PT....... ...V..S... .......... .......... .......... .......TD. ...
F strain                                  ..T....G.. G.L...I..G .......... .......... K......... .......... ...
K strain                                  ..T....... ......I..G .......... .......... K......... .......... ...
Layer Chicken(NDV-2) India1(AF204741)     ...A.RR... R..V...... .T......G. ...V...... S........I .....S...H QL.
Layer Chicken(NDV-3) India2(AF204742)     ..T....G.. G.....L..G .......S.. .......... .......... .........P ...
UP-2(Pigeon) India3(AJ245812)             ..T....G.. G.L...I..G .......... .......... K.......L. .......... ...
TN1- India4(AJ249528)                     ..T....G.. A.L...I..G .......... .......... ........L. .......... ...
WB1-94  India5(AJ249530)                  .......... R......... .......... .......... ........L. .......... ...

                    CONSENSUS       105  SVSTSGGRRQ KRFIGAVIGS VALGVATAAQ ITAAAALIQA NQNAANILRL KESIAATNEA VHE 167
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Fig. 1

Alignment of deduced amino acid sequences surrounding FPCS of the four NDV isolates with those of other isolates retrieved from GenBank

The FPCS sequence is in bold. Only the changed nucleotides are indicated, while the unchanged ones are dotted.

were found to be stable at 56°C from 85 mins to 6 hrs. The HA adsorption percentage was found to be between 87.5 and 96.8.

Molecular characterization

An RT-PCR product of approximately 254 bp was detected by agarose gel electrophoresis for all the isolates. The deduced amino acid sequences of these isolates were compared with a few Indian and exotic NDV isolates (Fig. 1). The predicted amino acid sequence (aa 105–167) surrounding the FPCS region was identical for all the isolates with ^{112}RRQKRF^{117} motif indicating the velogenic nature of the isolates. A phylogenetic tree was generated using nucleotide sequences (Fig. 2) which placed the isolates with a velogenic Indian isolate recovered from a layer chicken from 1987. That isolate has been reported as belonging to C1 group by monoclonal antibody (MAb) typing.

Discussion

It has been reported that more than 250 species of birds are susceptible to NDV as a result of natural or experimental infection; it is probable that many more susceptible species do exist but have not yet been identified (Aldous and Alexander, 2001). In an attempt to isolate NDV from pet birds such as pigeon and psittacine birds (lory, parrot, and love bird) which were submitted for diagnostic investigation, four isolates were obtained. All of them were confirmed as NDV based on HA and HAI test results (Alexander, 1988, 1989). The ICPI values of the isolates were 1.9 or close to the maximum score of 2.0, placing the isolates in velogenic group of NDV (OIE, 2000). Although the isolates agglutinated the erythrocytes of cattle, goat and human, they failed to do so with equine erythrocytes, indicating that they are not lentogenic (Winslow *et al*., 1950; Kumanan *et al*., 1992). Some of the earlier reports have indicated that the HA of virulent isolates were more stable than those of less virulent ones at 56°C. In this study, the HA of the isolates were relatively stable at 56°C and their ability to get adsorbed to the chicken brain cells was also high as observed with velogenic isolates (Hanson *et al.*, 1949; Kumanan *et al*., 1992).

The amplification of the 254 bp product by RT-PCR using NDV-specific primers further confirmed that all the four isolates were indeed NDV (Seal *et al*., 1995; Mathivanan *et al*., 2004). The deduced amino acid FPCS sequences of the isolates were typical for more pathogenic NDV and were conserved for all the four isolates irrespective of the species of the bird involved. The ^{112}RRQKRF^{117} motif indicated that the isolates were velogenic (Meulemans *et al*., 2002). The isolates had at the C-terminus of the F2 protein a multiple basic amino acid (arginine) at the positions 112, 113 and 116, lysine at the position 115, and phenylalanine at the

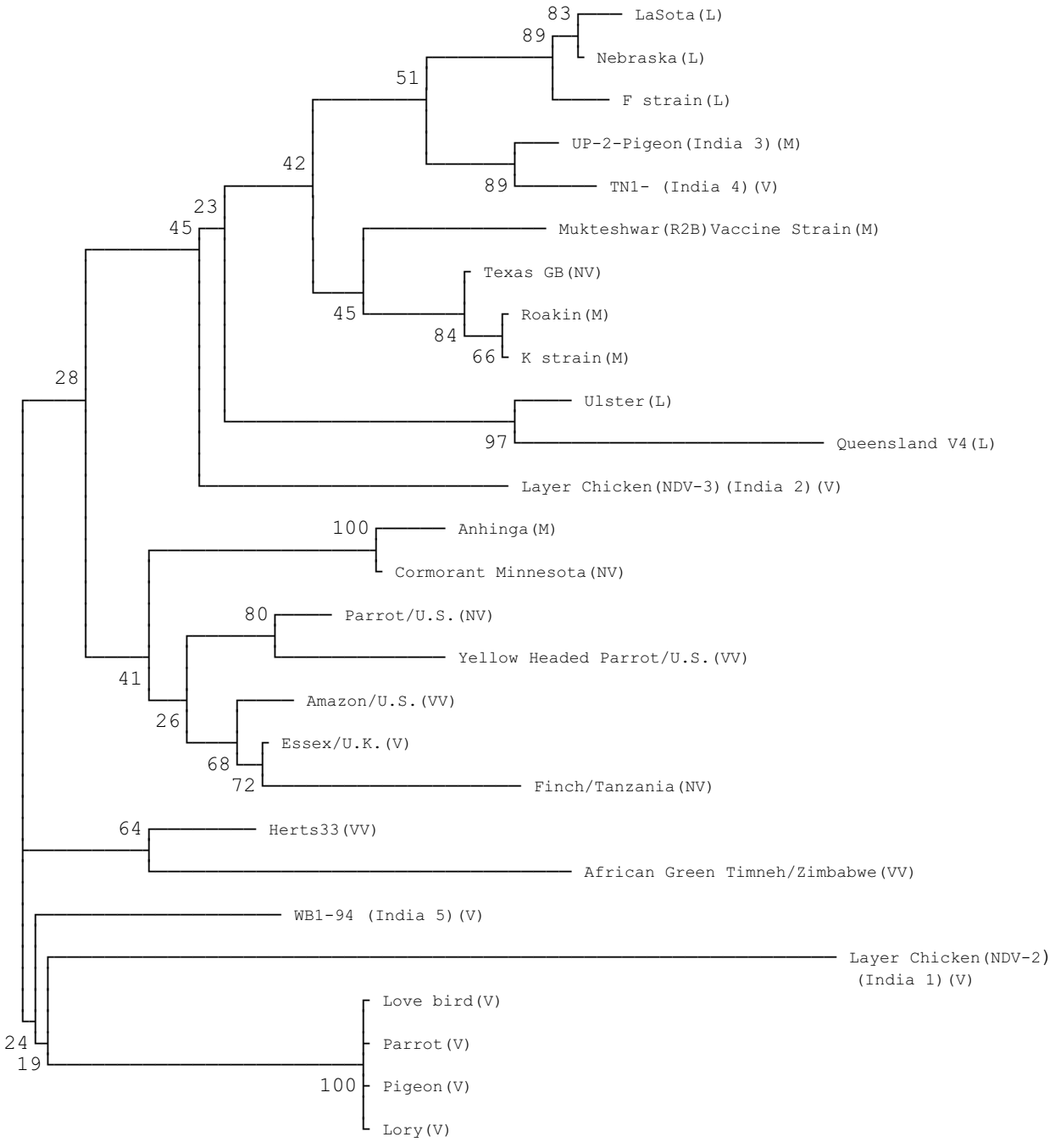

Fig. 2

Phylogenetic tree of NDV isolates based on nucleotide sequences from F protein gene

Numbers represent bootstrap confidence levels following 1000 replications. Lentogenic (L), mesogenic (M), neurotropic velogenic (NV), velogenic (V), and viscerotropic velogenic (VV).

position 117 (the latter being the N-terminus of the F1 protein (OIE, 2000), which is characteristic of velogenic NDV.

Phylogenetic analysis has been exploited by various authors for pathotype prediction and assessing the evolutionary background (Seal *et al*., 1995, 1998; Mathivanan *et al*., 2004). Having confirmed the velogenicity of the isolates, we analyzed their relatedness with other Indian and global NDV isolates based on their FPCS sequences. The phylogenetic tree placed the four isolates along with an Indian NDV isolate of layer chicken origin from 1987. This velogenic Indian isolate has been reported to belong to C1 group by MAb assay (Kumanan *et al*., 1992). NDV isolates of C1 group were found to be velogenic and of psittacine origin (Alexander *et al*., 1987). The findings of this study which link the four isolates with that of C1 group from poultry may represent introduction of such isolates from feral birds to poultry and other unvaccinated pet birds. Moreover, the isolates were found to be phylogenetically related to another Indian velogenic isolate (WB1-94) from early nineties from a different geographical location. From these observations it is clear that NDV belonging to the C1 group are circulating among different avian species throughout the country over a long period of time. The presence of viruses of this type clearly indicates a possible threat to the large commercial poultry population of the country. Nevertheless, widescale spread could be avoided with routine prophylactic vaccinations of all susceptible avian species and following hygienic measures. Thus, the results of this study indicate that highly virulent forms of NDV are circulating among pet birds in India which are not routinely vaccinated against ND.

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