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## Isolation of Newcastle Disease Viruses of High Virulence in Unvaccinated Healthy Village Chickens in South India

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**Abstract:** Three isolates of Newcastle disease virus obtained from normal village chickens, which were never vaccinated against Newcastle disease were characterized. These isolates namely D8, D53 and D58 were found to have an ICPI value of 1.59, 0.50 and 0.14 respectively. The deduced amino acid sequence at the FPCS of these three isolates between positions 112 and 119 were G-R-R-Q-K-R-F-I-G- -G-R-R-Q-K-R-F-I-G- and – G-G-R-Q-G-R-L-I-G- respectively. The two isolates namely D8 and D53 had phenylalanine (F) residue at position 117, confirming their high virulence to chicken, whereas the isolate D58 had leucine (L) at 117 confirming their low virulence to chicken. The existence of NDV with high ICPI confirms the circulation of virulent NDV in village chicken raised in the backyard, which could be a threat to commercial chicken.

**Key words:** Newcastle disease, avian paramyxovirus 1, fusion protein cleavage site, virulence, ICPI value, village chicken

### Introduction

Newcastle disease (ND) - a List A infection is considered as one of the two most important diseases of chickens along with highly pathogenic avian influenza (Aldous and Alexander, 2001). It is an economically important disease causing heavy production loss to the farmers besides high mortality. The disease affects 27 of the 50 orders of birds (Schelling *et al.*, 1999; Alexander, 2000). Depending upon the pathotype and susceptibility of birds the mortality varies from zero to 100% (Nanthakumar *et al.*, 2000a). The disease is present in endemic form with frequent outbreaks in commercial poultry. Besides commercial poultry the disease also affects the village and it remains as a constant threat to the backyard poultry (Spadbrow, 1993; Westbury, 2001). While the commercial poultry are routinely vaccinated, the village chickens are not normally vaccinated due to social and financial reasons (Spadbrow, 1992; Alexander, 2001). The village chickens with their apparent disease resistance capacity survive the disease but they are also believed to act as source/carrier to commercial poultry (Echeonwu *et al.*, 1993; Kamaraj *et al.*, 1998; Bell and Mouloudi, 1998). Hence, it is clear that to eradicate ND in poultry, it is imperative to control the spread of infection from village chicken and other birds to commercial poultry flocks.

The infection is caused by Avian Paramyxo virus type 1 (APMV 1) of the genus *Avulavirus* under the family *Paramyxoviridae*. The virus has a negative sense, single stranded - RNA genome that consist of approximately 15 kbp (15,186 bp), which is located in the central hollow of the herringbone like nucleocapsid. The genome contains six major genes that encode the structural proteins in the order 3'-NP-P-M-F-HN-L-5'

(Chambers *et al.*, 1986) as well as two non-structural proteins W and V (McGinnes *et al.*, 1988). Among these, the fusion protein (F) mediates the fusion of virus with the cellular membranes and it originates from its precursor F<sub>0</sub>. The cleavability of F<sub>0</sub> protein is the major determinant for virulence (Peeters *et al.*, 1999). The variation in the virulence NDV is mediated by amino acid variation at the cleavage site of F<sub>0</sub> and availability of protease enzymes to cleave F<sub>0</sub> in different tissues (Nagai *et al.*, 1976; Chambers *et al.*, 1986; Glickman *et al.*, 1988; Alexander, 2001). Technologies based on genome are now being increasingly used to detect and differentiate pathogens of avian species worldwide (Cavanagh, 2001). The detection, characterization and epidemiology of the virus can be studied reliably by genome-based technology (Aldous and Alexander, 2001). The molecular techniques like restriction enzyme analysis (Nanthakumar, 2000a) and nucleotide sequencing (Nanthakumar *et al.*, 2000b) has also been used for characterizing NDV isolates. The method of studying the amino acid sequence at 112 to 117 of F<sub>0</sub> cleavage site also offers valuable information for molecular epidemiology of spread of ND (Nagai *et al.*, 1976; Chambers *et al.*, 1986; Glickman *et al.*, 1988; Seal and Benmnett, 1995). Based on these facts, in the present study we have used a set of published oligonucleotide primers for F<sub>0</sub> cleavage site (Seal and Benmnett, 1995) for a two-step RT-PCR coupled to automated sequencing of the amplified PCR product. Subsequent analysis for pathotype determination of NDV helped us to confirm the presence of virulent NDV in healthy village chickens, which have never been given any vaccine against ND.

## Materials and Methods

**Virus isolates:** A total number of three live NDV isolates named D8, D53 and D58 obtained from the healthy village chickens with no history of vaccination against ND were used in this study. These isolates were obtained from cloacal swabs obtained from village chicken by following standard methods of virus isolation as specified in the Terrestrial Manual of OIE. These birds had no history of vaccination against any type of ND vaccine. These isolates were propagated in the allantoic cavity of nine-day old embryonated chicken eggs. The amnioallantoic fluid obtained after the death of embryo 24 hours post infection or 72 hours after infection was used for the estimation of intra cerebral pathogenicity index (ICPI) and for RNA extraction.

**Estimation of ICPI:** The ICPI was estimated to find out the pathogenicity of isolates obtained from normal village chickens. The test was performed on day old chicken as per the standard procedure mentioned in Terrestrial Manual of OIE. Fresh infective allantoic fluid obtained after passaging the NDV isolates in embryonated eggs with a HA titre  $>2^4$  ( $>1/16$ ) is diluted 1/10 in sterile isotonic saline with no additives, such as antibiotics. 0.05 ml of the diluted virus was injected intracerebrally into each of ten chicks between 24 to 40 hours after hatch. The birds were examined every 24 hours for a observation period of eight days. At each observation, the birds were scored: 0 if normal, 1 if sick, and 2 if dead. The intracerebral pathogenicity index (ICPI) was calculated as the mean score per bird per observation over the 8-day period.

**Viral RNA extraction and RT-PCR:** Viral RNA was extracted from total one ml of infected amnioallantoic fluid (AAF) using acid-guanidium-thiocyanate method Chomczynski and Sacchi (1987). The resultant RNA was dissolved in a final volume of 20 $\mu$ l of DNase-RNase free water. The two-step RT-PCR reaction was carried out using enhanced Avian HS RT-PCR kit (M/s.Sigma-Aldrich, USA, Cat #1001) as described by the manufacturer with minor modifications. For cDNA synthesis, 5  $\mu$ l of RNA was mixed with 1  $\mu$ l of random nanomer and 2  $\mu$ l of dNTP mix. After incubation at 70°C for 10 minutes snap chilled on ice for 1 minute. Following this, cDNA synthesis was carried out in a 20 $\mu$ l reaction volume containing 2 $\mu$ l 10X AMV-RT buffer, 1 $\mu$ l RNase inhibitor and 8 $\mu$ l DNase-RNase free distilled water. After this, reaction mixture was incubated at 25°C for 10 minutes followed by addition of 1 $\mu$ l of reverse transcriptase (RT). The tube was then incubated at 42°C for 60 minutes for reverse transcription followed by heating at 65°C for 10 minutes to inactivate unused reverse transcriptase. Consensus primers namely sense, 5'-CCT TGG TGA' ITC TAT CCG IAG-3 and antisense, 5'-CTG CCA CTG CTA GTT GIG ATA ATC C-

3'), for a 254 bp nucleotide sequence of the fusion protein cleavage site (FPCS) of F gene of NDV (Seal and Bennet, 1995) were used for amplification of cDNA. For PCR, 3  $\mu$ l of cDNA was amplified using the PCR-master mix for a reaction volume of 50  $\mu$ l containing 2  $\mu$ l each primer (20 p mol), 5  $\mu$ l 10 X PCR buffer (with MgCl<sub>2</sub>), 2  $\mu$ l MgCl<sub>2</sub> (50mM), 2  $\mu$ l dNTP mix (10 mM), 1  $\mu$ l Taq polymerase (2.5 units) and 33  $\mu$ l DNase RNase free distilled water. Amplification was carried out in a thermal cycler (Perkin-Elmer, USA, Model #2400) with an initial denaturation at 94°C for 5 minutes followed by a sequence of 25 cycles (denaturation - 94°C for 30 seconds; annealing-52°C for 30 seconds; extension-72°C for 60 seconds) and final extension hold at 72°C for 7 min, then stored at -20°C. The PCR product was detected in 2% agarose gel electrophoresis after staining with ethidium-bromide.

**Nucleotide sequencing / DNA sequencing:** The FPCS amplicons were purified using AuPrep PCR purification kit of Life Technologies, India (Cat# PP28-104 LT) according to the manufacturers instruction. The purified PCR products with a concentration equal to or more than 50ng/ $\mu$ l were used for sequencing. The sequencing was done by Big dye termination chemistry method in an automated sequencer (ABI Prism, Version 3, Applied Biosystems, USA). Multiple sequence alignment and analysis of the nucleotide sequence and deduced amino acid sequence of F gene was performed with Bioedit software supplied by North Carolina State University, USA. The phylogenetic tree was developed using Neighbour joining (NJ) algorithm with bootstrap values and distance using Mega 3.1 software. The F gene sequences of different NDV strains available in GenBank were used for comparison.

## Results and Discussion

**ICPI:** The ICPI values obtained for D8, D53 and D58 isolates were 1.59, 0.50 and 0.14 respectively. The ICPI value of D8 isolate was close to virulent strains, ICPI value of D53 was above that lentogenic strains and that of D58 was close to 0.0 indicating its less virulence to chickens. As per the Terrestrial Manual of OIE, the most virulent viruses will give indices that approach the maximum score of 2.0, whereas lentogenic strains will give values close to 0.0.

**RT-PCR and DNA sequencing:** Three isolates obtained from normal village chicken were subjected to RT-PCR. All the three isolates were amplified in RT-PCR. The PCR products from three NDV were sequenced. The sequence was analyzed by Bio-edit software. The nucleotide and amino acid sequences of FPCS region of the three NDV isolates and other common vaccine strains, their GenBank accession number and ICPI values are provided in Table 1. The percentage of

Table 1: Comparison of sequences of FPCS region of NDV isolates obtained from village chicken with different strains of NDV

Strains	GenBank Accession Number	Nucleotide sequence (from 4868-4900)	Amino acid (109-119 of F gene)	ICPI Value	Pathotype	Virulence to chicken
Consensus		<b>TCTGGAGGGG GGAGACAGGG ACGCCTTATA GGC</b>	<b>SGRRRQGRFI G</b>			
LaSota	EF440344	.....G.....G.....	..G...L..	0.40	Lentogenic	Low
Clone 30	Y18898	.....G.....G.....	..G...L..	0.40	Lentogenic	Low
F	EF440343	.....G.....G.....	..G...L..	0.25	Lentogenic	Low
B1	AF309418	.....G.....G.....	..G...L..	0.20	Lentogenic	Low
HB92	AY225110	.....G.....G.....	..G...L..	0.00	Apathogenic	Low
V4	AF217084	.C...A..A.....T.....	..GK...L..	0.00	Apathogenic	Low
Ulster	M24694	.....A..A.....	..GK...L..	0.00	Apathogenic	Low
K	EF440345	.....A.....AA.....T.....	.....K....	1.45	Mesogenic	High
Beaudette C	X04719	.....A.....AA.....T.....	.....K....	1.56	Mesogenic	High
R2B	AJ249527	.....A.....AA.....T.....	.....K....	1.20	Mesogenic	High
Essex 70	AJ629062	.....AA.....AA.....T..G.. ..T	.....K...V.	1.95	Velogenic	High
Texas GB	M33855	.C.....AA.....A.....T.....T	.....R....	1.74	Velogenic	High
Herts 33	U22275	.C.....AA.....A.....T.....T	.....R....	1.90	Velogenic	High
<b>D8</b>	<b>AY365028</b>	..... <b>A</b> ..... <b>AA</b> ..... <b>T</b> .....	..... <b>K</b> ....	<b>1.59</b>	<b>Velogenic</b>	<b>High</b>
<b>D53</b>	<b>AY365029</b>	..... <b>A</b> ..... <b>AA</b> ..... <b>T</b> .....	..... <b>K</b> ....	<b>0.50</b>	<b>Mesogenic</b>	<b>High</b>
<b>D58</b>	<b>AY365028</b>	..... <b>A</b> ..... <b>G</b> .....	... <b>G</b> ... <b>L</b> ..	<b>0.14</b>	<b>Lentogenic</b>	<b>Low</b>

\* Sequences of NDV isolates obtained are provided in bold letters

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Table 2: Identity similarity (%) between amino acid nucleotide composition of FPCS region of Newcastle disease virus (NDV) isolates obtained from village chicken with different strains of NDV

Seq->	LaSota	Clone		B1	HB92	V4	Ulster	K	Beaudette C	R2B	Essex	Texas	Herts	D8	D53	D58
		30	F								70	GB	33			
LaSota		<b>100.00</b>	<b>100.00</b>	<b>100.00</b>	<b>100.00</b>	<b>84.80</b>	<b>90.90</b>	<b>84.80</b>	<b>84.80</b>	<b>84.80</b>	<b>75.70</b>	<b>78.70</b>	<b>78.70</b>	<b>84.80</b>	<b>84.80</b>	<b>100.00</b>
Clone 30	100.00		<b>100.00</b>	<b>100.00</b>	<b>100.00</b>	<b>84.80</b>	<b>90.90</b>	<b>84.80</b>	<b>84.80</b>	<b>84.80</b>	<b>75.70</b>	<b>78.70</b>	<b>78.70</b>	<b>84.80</b>	<b>84.80</b>	<b>100.00</b>
F	100.00	100.00		<b>100.00</b>	<b>100.00</b>	<b>84.80</b>	<b>90.90</b>	<b>84.80</b>	<b>84.80</b>	<b>84.80</b>	<b>75.70</b>	<b>78.70</b>	<b>78.70</b>	<b>84.80</b>	<b>84.80</b>	<b>100.00</b>
B1	100.00	100.00	100.00		<b>100.00</b>	<b>84.80</b>	<b>90.90</b>	<b>84.80</b>	<b>84.80</b>	<b>84.80</b>	<b>75.70</b>	<b>78.70</b>	<b>78.70</b>	<b>84.80</b>	<b>84.80</b>	<b>100.00</b>
HB92	100.00	100.00	100.00	100.00		<b>84.80</b>	<b>90.90</b>	<b>84.80</b>	<b>84.80</b>	<b>84.80</b>	<b>75.70</b>	<b>78.70</b>	<b>78.70</b>	<b>84.80</b>	<b>84.80</b>	<b>100.00</b>
V4	90.90	90.90	90.90	90.90	90.90		<b>93.90</b>	<b>75.70</b>	<b>75.70</b>	<b>75.70</b>	<b>72.70</b>	<b>81.80</b>	<b>81.80</b>	<b>75.70</b>	<b>75.70</b>	<b>84.80</b>
Ulster	90.90	90.90	90.90	90.90	90.90	100.00		<b>81.80</b>	<b>81.80</b>	<b>81.80</b>	<b>78.70</b>	<b>81.80</b>	<b>81.80</b>	<b>81.80</b>	<b>81.80</b>	<b>90.90</b>
K	72.70	72.70	72.70	72.70	72.70	63.60	63.60		<b>100.00</b>	<b>100.00</b>	<b>90.90</b>	<b>87.80</b>	<b>87.80</b>	<b>100.00</b>	<b>100.00</b>	<b>84.80</b>
Beaudette C	72.70	72.70	72.70	72.70	72.70	63.60	63.60	100.00		<b>100.00</b>	<b>90.90</b>	<b>87.80</b>	<b>87.80</b>	<b>100.00</b>	<b>100.00</b>	<b>84.80</b>
R2B	72.70	72.70	72.70	72.70	72.70	63.60	63.60	100.00	100.00		<b>90.90</b>	<b>87.80</b>	<b>87.80</b>	<b>100.00</b>	<b>100.00</b>	<b>84.80</b>
Essex 70	63.60	63.60	63.60	63.60	63.60	54.50	54.50	90.90	90.90	90.90		<b>90.90</b>	<b>90.90</b>	<b>90.90</b>	<b>90.90</b>	<b>75.70</b>
Texas GB	72.70	72.70	72.70	72.70	72.70	63.60	63.60	90.90	90.90	90.90	81.80		<b>100.00</b>	<b>87.80</b>	<b>87.80</b>	<b>78.70</b>
Herts 33	72.70	72.70	72.70	72.70	72.70	63.60	63.60	90.90	90.90	90.90	81.80	100.00		<b>87.80</b>	<b>87.80</b>	<b>78.70</b>
D8	72.70	72.70	72.70	72.70	72.70	63.60	63.60	100.00	100.00	100.00	90.90	90.90	90.90		<b>100.00</b>	<b>84.80</b>
D53	72.70	72.70	72.70	72.70	72.70	63.60	63.60	100.00	100.00	100.00	90.90	90.90	90.90	100.00		<b>84.80</b>
D58	100.00	100.00	100.00	100.00	100.00	90.90	90.90	72.70	72.70	72.70	63.60	72.70	72.70	72.70		

Amino acid variations are indicated with normal font and nucleotide variations are indicated in bold letter

nucleotide and amino acid sequence variations between the three isolates with standard vaccine strains of NDV are provided as Table 2.

**Phylogenetic analysis of the NDV isolates:** The three isolates namely D8, D53 and D58 had ICPI value of 1.59, 0.50 and 0.14 respectively. The amino acid sequence at the FPCS of these three isolates namely D8, D53 and D8 were -G-R-R-Q-K-R-F-I-G-, -G-R-R-Q-K-R-F-I-G- and -G-G-R-Q-G-R-L-I-G- respectively. Taking cognizance of the findings that the F gene of NDV determines the virulence (Nagai *et al.*, 1976; Glickmann *et al.*, 1988; Gotoh *et al.*, 1992; Peeters, 1999) and the amino acid sequences between positions 112-117 of FPCS gene serve as a tool for pathotyping for NDV (Collins *et al.*, 1993; Seal and Benmnett, 1995;) further analysis of the sequences was carried out. The amino acid sequences of D 58 matched with other less virulent strains like LaSota (Colins *et al.*, 1993) F (Nanthakumar *et al.*, 2000b), D26 (Toyoda *et al.*, 1989), MC 110 (Collins *et al.*, 1993) and 1154/98 (Alexander, 2001). It has been already reported that all the less virulent strains have the amino acid leucine (L) at position 117 and glycine (G) or glutamic acid (E) at position 115 (Collins *et al.*, 1993). In the present study also, the D58 isolate with an ICPI value of 0.14 has leucine (L) at 117 and glycine (G) at 115 confirming its status as an isolate of low virulence to chickens. These observations are in accordance with ICPI value as already explained by Toyoda *et al.* (1989) and Collins *et al.* (1993).

The amino acid sequence of NDV isolates D8 and D53 obtained from normal village chicken matched with sequences of other mesogenic strains like K, R2B and Beaudette C. The amino acid sequence was also found to match with velogenic strains of NDV like Essex 70 (Collins *et al.*, 1993) and Herts 33 (Toyoda *et al.*, 1989) with minor variation at position 118, whereas for Essex

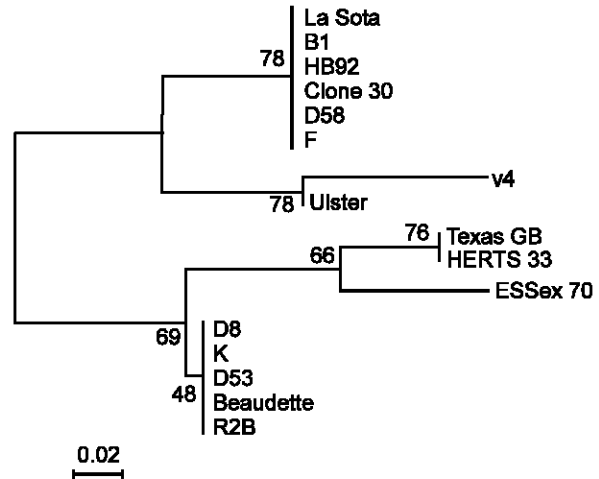


Fig. 1: Phylogenetic tree (NJ) with distance and bootstrap value for FPCS region of Newcastle disease virus NDV isolates obtained from village chicken with different strains of NDV.

70 the amino acid isoleucine (I) found in all strains has been replaced by the amino acid valine (V). Further, the two isolates namely D53 and D8 had phenylalanine (F) residue at position 117, which was also found in other mesogenic and velogenic strains, which have high virulence to chicken.

It had been already established that a pair of basic amino acids lysine (K) and/or arginine (R) at positions 116 and 115 with a phenylalanine (F) at position 117 and arginine at position 113 determine the virulence for chickens. (Aldous and Alexander, 2001). In the present study, also the isolates D8 and D53 had basic amino acids lysine (K) and arginine (R) and phenylalanine (F) at positions 115-117 respectively and basic amino acid arginine (R) at position 113 confirming their status as

ND virus of high virulence to chickens. These findings were also in agreement with ICPI values.

Based on the phylogenetic tree (Fig. 1) constructed by neighbour joining (NJ) algorithm with bootstrap values along with distance the isolate D58 with an ICPI value of 0.14 was positioned in a group comprising of other lentogenic NDV strains like LaSota, Clone 30, B1, HB92 and F. This group has a common node with another group consisting of apathogenic strain Ulster and V4. The isolates D8 and D53 with ICPI values 1.59 and 0.50 were positioned in a group comprising of mesogenic strains like K, R2B (Mukteswar) and Beaudette C. This group also has a common node with highly virulent velogenic strains of NDV like Essex 70, Texas GB and Herts 33.

Based on the sequence identity matrix, it is clear at both nucleotide and amino acid levels that the lentogenic strains like LaSota, Clone 30, F, B1 and HB92 do not differ among them. The D58 isolate obtained in this study, which has an ICPI value of 0.14, which is grouped with these lentogenic strains in phylogenetic tree was found to be 100% similar at FPCS region with these lentogenic strains. In the same way, at both nucleotide and amino acid level, the mesogenic strains like K, R2B and Beaudette C do not differ among themselves. Two isolates of NDV obtained in this study namely D8 and D53 that have an ICPI value of 1.59 and 0.50 respectively and are grouped with these mesogenic strains in phylogenetic tree was found to be 100% similar at FPCS region with these mesogenic strains.

Our findings besides clearly establishing the utility of amino acid sequence of FPCS region in pathotyping of NDV and its correlation with conventional ICPI technique, also confirmed the presence/circulation of ND viruses of different virulence in village chicken. This observation strengthens the possibility of village chicken acting as host/carrier of NDV. The existence of NDV with high ICPI value like 1.59 and 0.50, which were later confirmed by FPCS sequencing confirms the circulation of virulent NDV in village chicken. It could be concluded that village chicken by their natural resistance withstand the infection and do not show any clinical symptoms. But these birds could act as potential source of infection for commercial chicken. In India or in countries where village chicken are also raised in the backyard of farm workers, threat of ND outbreaks could be high.

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