Analysis of the Fusion Protein Cleavage Site of Newcastle disease virus Isolates from India Reveals Preliminary Evidence for the Existence of II, VI and VII Genotypes

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Abstract *Newcastle disease virus* (NDV) has been a threat to poultry industry in most of the developing countries with a wide variety of avian species being susceptible, coupled with the presence of mobile wild bird reservoirs contributing not only to the vast genomic diversity of this virus but also to the diagnostic failures. NDV of multiple genotypes (I–XI) is known to be prevalent and reported worldwide. However, there is a paucity of information on the circulating genotypes of NDV in India. This study utilized the fusion protein cleavage site (FPCS) sequence to determine the different genotypes of NDV circulating in India. Our results indicate that majority of NDV isolates from southern states of India namely, Tamil Nadu, Kerala and Karnataka were found to belong to genotype II. However, some of the strains from Tamil Nadu and most from Uttar Pradesh belong to genotype groups VI and VII. Interestingly, three isolates recovered from Tamil Nadu grouped with genotype IV viruses (namely Herts/33) which had not been hitherto reported to GenBank since 1989. This preliminary information points to the existence of multiple genotypes and also the need for efficacy studies with vaccines incorporating multiple genotypes in controlling virulent NDV (vNDV) outbreaks in India.

Keywords APMV-1 · FPCS · Pathotyping · Genotype · Class II-APMV

Newcastle disease virus (NDV) is a member of the family Paramyxoviridae and grouped under the genus *Avulavirus*. Till date ten different serotypes of avian paramyxovirus (APMV) has been identified and designated APMV-1 to APMV-10 with ND being referred to as APMV-1. Newcastle disease (ND) has been isolated so far from at least 241 different species of birds [16]. ND occurs in at least six of the seven continents with the disease being enzootic in many countries. The genome of NDV is a negative sense single stranded RNA of approximately 15 kilobases (15186 in early genotypes and 15192 in recent genotypes). Six different proteins in the order namely nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), Haemagglutinin neuraminidase (HN) and large polymerase protein (L) are encoded by the genome [4]. The key contributor to APMV-1 pathogenicity is the formation of an active fusion protein (F) upon cleavage of the F protein precursor (F₀) [33, 35] as well as the number of basic amino acid residues in the fusion protein cleavage site (FPCS) [12, 39]. The FPCS sequence has not only been utilized to pathotype the isolates into lentogenic, mesogenic or velogenic [1, 10, 36] but also to group the APMV-1 that are circulating worldwide into class I (with nine genotypes) and class II (with eleven genotypes) [11]. The class I viruses are distributed worldwide in wild birds and generally avirulent to chickens [19] have been isolated from live bird market samples [19, 20]. The class II viruses consists of eleven (I–XI) genotypes with some viruses (genotype I—chicken/Australia/QV4/1966 and Ireland/Ulster/1967; genotype II—Lasota) used as live vaccines in different parts of the world.

With respect to NDV in India there have been several studies to show the outbreak of this disease in commercial chicken and their impact on production [22, 30]. Most of the reports had not only looked into the biological

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characteristics of the virus but also sequenced the FPCS to determine the virulence of the virus [21, 22, 24, 42]. There are also reports on isolation, biological characterization and FPCS sequence based pathotyping of NDV from backyard chicken [6] and other species of birds such as pigeons [18, 24, 40, 42, 46] Japanese quail [21], Turkey [37], Duck [47], crow [31], Emu [23] and Ostrich [45]. In fact there is also an isolation of a lentogenic strain of NDV from backyard chicken that has been completely characterised with respect to its biological and sequence (both the fusion (F) and Haemagglutinin neuraminidase (HN) genes) characteristics [6, 32, 34, 43] and the same has been used in preparing an oral pellet vaccine.

There is only one report with respect to the genotyping of a NDV isolate from peacock in India employing the well characterized molecular determinant the FPCS [42]. This report points to the prevalence and circulation of genotype II virus in India. However, reports world-wide regarding the distribution of NDV indicate the circulation of multiple genotypes I to XI [29]. The repeated outbreaks of NDV in different commercial farms and reports on isolation of the virus from non chicken species of birds from India led us to hypothesize that there might be more than one genotype that is circulating in India that had not only resulted in several outbreaks but also suggests that the vaccination programmes are not optimized to deal the existing situation. In this study we have utilized the sequence information of the FPCS generated from the various NDV strains isolated from different sources by our laboratory (44 sequences) and downloaded from GenBank the sequence information generated by other Indian laboratories (24 sequences) and sequences belonging to different classical genotypes and vaccine strains (29 sequences) to analyze and determine the genotypes of the circulating Indian NDV strains.

The isolates used in this study from our laboratory (recovered since 1987) have been isolated from the pooled homogenate of spleen and brain samples or cloacal swabs in 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECE) using standard procedures [3], purified by triple terminal dilution and stored in our repository. The virus identity was re-confirmed by haemagglutination-inhibition assay with NDV antiserum and also by reverse transcription-polymerase chain reaction (RT-PCR) to amplify the FPCS sequence [36, 44]. The accepted biological characteristics namely, the mean death time (MDT) and intracerebral pathogenicity index (ICPI) were also determined by standard procedures [5, 44] for all the isolates. The FPCS sequence for the different isolates were generated by RT-PCR from the RNA that was extracted from two different lots of allantoic fluid employing superscript III RT mix (Invitrogen, CA) and high fidelity Taq DNA polymerase super mix (Invitrogen, CA). Sequencing

of the FPCS amplicons were done with Big-Dye terminator v 3.1 kit (Applied biosystems, USA) for at least three clones with both the forward and reverse primers to ensure a consensus and repeatable sequence data.

Lentogenic strains of NDV have been shown to depict the FPCS cleavage site motif $^{112}\text{G/E-K/R-Q-G/E-R-L}^{117}$ with few basic amino acid residues at carboxyl terminus of the F2 protein while the virulent strains (mesogenic and velogenic) possess $^{112}\text{R/K-R-Q-R/K-R-F}^{117}$ motif with two pairs of basic amino acids and a Phenylalanine in the N terminus of the F1 (residue 117) [36]. The variation in the amino acids contributing to the FPCS (112–117) in the different isolates from India is clearly evident (Table 1). To determine the genotype of the different isolates, the FPCS sequences were imported into MEGA 5 and aligned with the Clustal W programme. The Table 1 shows the details on the nucleotide sequence (GenBank Acc. Nos.), state of origin, and the species from which isolates were recovered. Following multiple alignment, the Bayesian Information Criterion (BIC), maximum likelihood values and Akaike Information Criterion corrected (AICc) scores were also determined for the maximum likelihood fits based on the data specific model to generate the phylogenetic tree and also to determine the genotype in MEGA 5 [38]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches (Fig. 1) with the tree drawn to scale. The phylogenetic tree grouping of the analyzed NDV strains from India revealed the existence of genotypes II, VI and VII (Table 1).

Among the 11 genotypes of Class II, the genotypes I, II, III, IV and IX are considered to be emerged during 1930–1960 and contain 15,186 nucleotides [11]. Most of the isolates from Tamil Nadu, three strains from Kerala (AF204752, AF204753, AF204754), one isolate from Karnataka (AF20475) and some of the isolates from north India (AJ245812 & AY09896 from UP; AF474243 from HP) grouped with the classical genotype II vaccine strain Lasota and the classical mesogenic strain Beaudette C. The whole genome sequence information generated by us recently on some of the isolates also indicated the genome size to be 15186 bases in Indian isolates (FJ986192, GU187941, HM357251 and HQ011508). The well known neurotropic virulent virus, Texas-GB isolated in 1948 and also used as a challenge virus for testing the vaccine efficiency in USA belongs to the genotype II. It is also interesting to note that three strains from south India (FJ986192, GU187941 and HQ011508) group themselves with the classical genotype IV viruses namely Herts/33 (AY741404) and Italian strain (EU293914). Reports indicate that genotype IV viruses have been the predominant viruses that were isolated from Europe before 1970 [11] and there has been no report of this genotype to GenBank since 1989 [29].

Table 1 Details of the sequences of Indian strains of *Newcastle disease virus* used in this study, their FPCS and predicted genotype

Name of the isolate	Year of isolation	Accession number	Species	Place (dist/state)	FPCS typing	Predicted genotype
NDV-R2B	1946	AJ249527	Chicken	–	RRQKRF	II
R2B	1946	AF204755	Chicken	–	RRQKRS	II
Komarov	Vaccine	EF440345	–	–	RRQKRF	II
Beaudette C	–	X04719	–	–	RRQKRF	II
Lasota	Vaccine	AY845400	–	–	GRQGRL	II
Fuller	–	AJ249516	Chicken	UP/India	GRQGRL	II
NDV TN1-87	1987	AJ249528	Chicken	UP/India	GRQARL	II
ND3	1987	AF204742	Chicken	Namakkal/Tamil Nadu	GRQGRF	II
2K4	1987	AY330719	Chicken	Chennai/Tamil Nadu	GRQGRL	II
ND9	1989	AF204744	Chicken	Namakkal/Tamil Nadu	GRQGRL	II
NDV-4	1987	HM357251	Chicken	Namakkal/Tamil Nadu	GRQGRL	II
UP2-92	1992	AJ245812	Chicken	UP/India	GRQGRL	II
D1	1995	AF204747	Chicken	Chennai/Tamil Nadu	GRQGRF	II
ND-KNBB/2k16	1996	AF204751	Chicken	Karnataka/India	GRQGRF	II
NDVCUL97	1997	AY359876	Parrot	Chennai/Tamil Nadu	GRQGRL	II
NDV-KPG	1998	AF204754	Pigeon	Kerala/India	RRQKRF	II
NDV-KQC	1998	AF204753	Japanese-quail	Kerala/India	RRQKRF	II
NDV-KQA (2K17)^a	1998	HQ902590	Japanese-quail	Kerala/India	RRQKRF	II
D8	–	AY365030	Chicken	Chennai/Tamil Nadu	RRQKRF	II
D58	–	AY365029	Chicken	Chennai/Tamil Nadu	GRQGRL	II
2K5	2000	AF474243	Layer Chicken	Palampur/HP	RRQKRF	II
NDV/TN/07	2000	AY069969	Emu	Chennai/Tamil Nadu	RRQKRF	II
2K9	2000	AY326432	Chicken	Namakkal/Tamil Nadu	GRQGRL	II
2K8	2000	AY326431	Chicken	Namakkal/Tamil Nadu	RRQGRF	II
2K15	2001	AY330722	Ostrich	Chennai/Tamil Nadu	GRQGRL	II
2K14	2001	AY330723	Chicken	Tirunelveli/Tamil Nadu	RRQKRF	II
2K12	2001	AY330721	Chicken	Tirunelveli/Tamil Nadu	GRQGRL	II
2K13	2001	AY330720	Turkey	Chennai/Tamil Nadu	RRQKRF	II
2K6	2001	AY324318	Pigeon	Chennai/Tamil Nadu	GRQGRL	II
2K20	2001	AY098987	Duck	Chennai/Tamil Nadu	RRQKRF	II
2K21	2001	AY098989	Turkey	Chennai/Tamil Nadu	RRQKRF	II
2K22	2001	AY098988	Japanese-quail	Chennai/Tamil Nadu	RRQKRF	II
2K19	2001	AY098986	Guinea fowl	UP/India	RRQKRF	II
2K7	2001	AF10738	Pigeon	Chennai/Tamil Nadu	RRQKRF	II
2K6	2002	AY249136	Pigeon	Chennai/Tamil Nadu	GRQGRL	II
2K28	2002	AY324317	Kadakhnath	Chennai/Tamil Nadu	GRQGRL	II
2K24	2002	AY339400	Crow	Chennai/Tamil Nadu	RRQKRF	II
Cell-Adapted virus	–	AY528454	Cell culture virus	Chennai/Tamil Nadu	RRQKRF	II
	–	AY528453	Cell culture virus	Chennai/Tamil Nadu	RRQKRF	II
NDV2^a	1987	GU187941	Chicken	Namakkal/Tamil Nadu	RRQRRF	IV
NDV 2K36^a	2008	HQ011508	Peacock	Chennai/Tamil Nadu	RRQRRF	IV
NDV 2K3^a	2000	FJ986192	Pigeon	Chennai/Tamil Nadu	RRQRRRL	IV
NDV/Pi01/AD	1991	AJ781070		Bareilly/Uttar Pradesh	RRQKRF	VI
NDV/Pi05/AD	1997	AJ781073		Bareilly/Uttar Pradesh	RRQKRF	VI
PiAD55	2004	AY581304	Pigeon	Bareilly/Uttar Pradesh	RRQKRF	VI
PiADPrd	–	AY581305	Pigeon	Bareilly/Uttar Pradesh	RRQKRF	VI
NDV/Pi04/AD	2004	AJ781072		Bareilly/Uttar Pradesh	RRQKRF	VI

Table 1 continued

Name of the isolate	Year of isolation	Accession number	Species	Place (dist/state)	FPCS typing	Predicted genotype
LCC9	1989	AY204510	Chicken	Chennai/Tamil Nadu	RRQKRF	VII
NDV/Pi02/AD	1991	AJ781071	–	Bareilly/Uttar Pradesh	RRQKRF	VII
UP1-93	1993	AJ249529	Chicken	UP/India	RRQKRF	VII
NDV-CCA	1993	AF204756	Jap. quail	Chennai/Tamil Nadu	RKQKRF	VII
RD-NPL-5	1993	AF204758	Chicken	Nepal	RRQKRF	VII
RD-BRLY-58	1993	AF204745	Chicken	Bareilly/Uttar Pradesh	RRQKRF	VII
WB1-94	1994	AJ249530	Chicken	UP/India	RRQRRF	VII
D4	1995	AF204750	Chicken	Chennai/Tamil Nadu	RRQGRL	VII
D3	1995	AF204749	Chicken	Chennai/Tamil Nadu	RRQKRF	VII
D2	1995	AF204748	Chicken	Chennai/Tamil Nadu	RRQKRF	VII
ND-PCK	1998	AF204759	Chicken	Chennai/Tamil Nadu	RRQKRF	VII
LB	1999	AF204757	Love bird	Chennai/Tamil Nadu	RRQKRF	VII
NDV/Pi06/AD	2001	AJ781074	–	Bareilly/Uttar Pradesh	RRQKRF	VII
2K23	2002	AY378325	Chicken-Aseel	Chennai/Tamil Nadu	RRQKRF	VII
2K29	2002	AY378323	Chicken	Chennai/Tamil Nadu	RRQKRF	VII
2K27-02	2002	AY378322	Pigeon	Chennai/Tamil Nadu	RRQKRF	VII
2K27	2002	AY236999	Pigeon	Chennai/Tamil Nadu	RRQKRF	VII
2K26	2002	AY339401	Aseel	Chennai/Tamil Nadu	RRQKRF	VII
2K31	2003	AY378319	Turkey	Chennai	RRQKRF	VII
HRR-03	2003	DQ340816	Chicken	Hissar/Haryana	Not avail	VII
2K32	2003	AY378320	Parrot	Chennai/Tamil Nadu	RRQKRF	VII
2K35	2003	AY378327	Jap. quail	Chennai/Tamil Nadu	RRQKRF	VII
2K34	2003	AY378326	Love bird	Chennai/Tamil Nadu	RRQKRF	VII
PiAD18	2004	AY581303	Guinea fowl	Bareilly/Uttar Pradesh	RRQRRF	VII
PiAD388	2004	AY581301	Quail	Bareilly/Uttar Pradesh	RRQRRF	VII
NDV/Chi/AP	2009	AB551367	Chicken	Andhra Pradesh	RRRKRF	VII
PiAD33	–	AY581302	Guinea fowl	Bareilly/Uttar Pradesh	RRQRRF	VII

The accession numbers of the well known classical NDV strains of different genotypes are mentioned in Fig. 1

Degenerate oligonucleotide primer pairs [36] Forward 5'-CCTTGGTGATCTATCCGIAG-3' and Reverse 5'-CTGCCACTGCTA GTTGIGA-TAATCC-3' (I standing for Inosine) were used to obtain the FPCS amplicon (254 bp) and the sequence information for the strains from our laboratory (bold faced)

The primer pairs used for generating the FPCS information by other Indian laboratories is not known

FPCS fusion protein cleavage site

^a The whole genome sequence has been generated for these strains from our laboratory

In the class II NDV, the other genotypes namely V, VI, VII and VIII have been shown to possess only the virulent viruses and indicated to be the predominant genotypes circulating world-wide. The genotypes V, VI, VII, VIII and X have been reported to have emerged after 1960 and contain 15,192 nucleotides. There has been no report of viruses from these genotypes as well the whole genome sequence information to confirm the same. The phylogenetic tree (Fig. 1) reveals that most of the isolates from North India (from UP), a Nepal strain (AF204758) and 12 from Tamil Nadu (AY373819, AY339401, AY378325, AY378320, AY378326, AY236999, AY378323—isolated after 2000 and AF204748, AF204749, AF204750, AF204756 and AF2047457—isolated before

2000) grouped themselves with the classical Genotype VIIb strains (AF293350 (IT-1/2000) and AF218127 (IT-112/84) from Italy and AF136768 from Hungary). Genotype VII has been divided into two sub-genotypes VIIa (emerged in 1990s in Far East and spread to Europe and Asia) and VIIb (emerged in Far East and spread to South Africa) [2]. IT-112/84 is a classical strain of genotype VIIb that was introduced in Italy in mid 1980s and responsible for the majority of the outbreaks in early 1990s in the southern part of Africa [13].

Five of the strains from Uttar Pradesh (AY581304, AJ781070, AJ781072, AY581305 & AJ781073) grouped themselves in the same clade with known genotype VI

Fig. 1 Phylogenetic relationship of various Indian isolates of *Newcastle disease virus* based on the 374-nucleotide sequence of the fusion protein gene. A rooted consensus tree drawn to scale using neighbour-joining method was obtained with the evolutionary distances inferred by using the based a Kimura-2 parameter method and gamma distribution model with evolutionary rate differences among sites, that also allowed some sites to be evolutionarily invariable (K2 + G + I). Bootstrap consensus value was inferred from 1000 replications. There were 389 positions in the final dataset. Evolutionary analysis was conducted in MEGA5. The Indian isolates of NDV were found to be of three major genotypes namely II, VII and VI. Most of the isolates from North India (from UP) are found to group with the genotype VI strains (IT-1/2000-AF293350, IT-112/04-AF218127 & ZA-25/93-AF136758) while the strains from South India (from Tamil Nadu and Kerala) grouped with the genotype II strains (Lasota, Beaudette-C)

strains (AF001110 from Lebanon & Z12111 from Warwick) that emerged in the 1960s and was the predominant genotype circulating in Asia until 1985 [26]. Most of these strains from North India (UP) have been reported to be isolated between 1991 and 1997 when the genotype VII became more common genotype world-wide during this time.

The vaccines that are being currently used in India as well as world-wide to control NDV utilize mostly the genotype II viruses (Lasota, B1, Komarov) that were isolated in the 1940s. It is also a recognized fact that all the NDV isolates belong to one serotype and any lineage of NDV incorporated in the vaccine and administered correctly would protect poultry not only from clinical disease but also from mortality from virulent ND challenge [7–9]. However, based on the phylogenetic tree it is clear that there are not only multiple genotypes circulating in India but also some of the strains belonging to genotype VI that have been replaced by the genotype VIIb in other parts of the world. Recent reports indicate the inefficiency of current vaccination programs in commercial chickens in preventing clinical disease and virus shedding after experimental challenge with recent virulent strains [15, 17, 27]. In countries like Asia, Africa and Central America the recurrent outbreaks of virulent NDV (vNDV) inspite of intensive vaccination is puzzling [4, 11]. However, evolution of virulent NDV in the field may have been largely facilitated due to the greater phylogenetic and antigenic distances between the used vaccine and circulating virulent strains [27]. However, there is no proof with controlled studies to prove the above phenomenon. Reports also demonstrate that the current vaccines only prevent disease but cannot stop the viral shedding [17, 28, 41]. Antigenically matched vaccines in recent studies (genotype VII and genotype V) have not only reported higher haemagglutination inhibition titres but also proved to prevent viral shedding [14, 28]. There are also contradictory reports to the above observation in that the current vaccines could offer protective efficacy against circulating viruses [15, 25].

Evidence does point out that due to sub-optimal vaccination, and circulation of different pathotypes NDV variants may be created in poultry. Thus with the preliminary information on existence of different genotypes, planned studies with vaccines incorporating the different genotypes have to be carried out to determine their efficacy in controlling vNDV outbreaks in India.

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