Detection and molecular characterization of Newcastle disease virus in peafowl (*Pavo cristatus*) in Haryana State, India

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Abstract Present study was undertaken to investigate the cause of deaths of peafowls in Haryana State. In total, 145 birds were sick and 28 birds were reported dead during July to September 2012. Some of the sick birds were showing signs of shaking of heads, torticollis and paresis. Blood and cloacal swab samples from sick birds along with brain and intestinal tissues from dead birds were collected for further investigation. Although post-mortem examination showed no typical lesions of Newcastle disease virus (NDV) yet raised HI titres against NDV in some serum samples and clinical signs indicated the presence of NDV. One of the brain tissues (NDV/IND2012/01) from the field case was processed and adapted to Vero cell line for virus isolation. The fusion (F) gene based nested RT-PCR (RT-nPCR) confirmed the presence of NDV in all field samples and cell culture isolate. Sequencing of the partial F gene amplicons (216 bp) using the PCR primers as sequencing primers confirmed the PCR results. The deduced amino acid

sequences of partial F gene were found to have the amino acid motif ¹¹¹GRRQKR/F¹¹⁷ in the fusion protein cleavage site (FPCS). This amino acid motif is indicative of the velogenic nature of these NDVs. Phylogenetic studies have shown that the virus belonged to class II genotype VII very closely related to virus isolates originated from outbreaks in Western Europe, Israel, Indonesia, Taiwan and India. Phylogenetic grouping of the virus and sequence of FPCS is indicative of pathogenic potential of virus strain circulating in peacocks in Haryana.

Keywords *Avulavirus* · Fusion protein cleavage site · Peacock · Velogenic · NDV · FPCS · Phylogenetic analysis

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Newcastle disease (ND) is an acute, rapidly spreading, contagious, nervous and respiratory disease of domestic and wild birds caused by an Avian Paramyxovirus 1 (APMV-1) also called as the Newcastle disease virus (NDV). About 250 species of birds of all age groups seem to be susceptible to infection with NDV [6]. Wild birds are

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natural reservoir of the virus [4, 25]. Many species of birds suffer from the natural infection from APMV-1 including the peafowls [14].

NDV is classified under the genus *Avulavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae* and order *Mononegavirales* [5]. It is an enveloped virus and has a negative-sense, non-segmented, single stranded RNA genome of approximately 15 kb in length [5, 10, 19]. The genome encodes six structural and two non-structural proteins [19]. According to their virulence in poultry, APMV-1 isolates can be classified as lentogenic, mesogenic or velogenic. The lentogenic strains are the least virulent while the mesogenic strains are moderately virulent and cause moderate respiratory signs with occasional nervous signs [5]. The lentogenic strains have been detected in both domestic poultry [15, 18] and wild bird populations [13, 17, 18]. Velogenic strains are the most virulent, infecting birds of all age groups with clinical findings of nervous signs or extensive hemorrhagic lesions in the gastrointestinal tract.

The pathogenicity determinants of NDV are based on the intracerebral pathogenicity index (ICPI) in day-old chicks, the intravenous pathogenicity index (IVPI) in 6-week-old chickens, or the mean death time in chicken embryos [3]. However, these tests are labour intensive and time consuming [1, 2]. Modern molecular techniques like polymerase chain reaction (PCR) and the amino acid sequence at the F0 protein cleavage site (111–117 amino acid) have been frequently used all over the world to detect and type NDV in the field samples [9, 12, 16, 21, 22]. According to the current World Organization for Animal Health definition, the assessment of virus virulence is based on the ICPI test and the amino acid sequence at the F0 protein cleavage site. Multiple basic amino acids have been demonstrated in the virus at the C terminus of the F2 protein and phenylalanine at residue 117, which is the N terminus of the F1 protein. NDV strains that are virulent for chickens contain at least three basic residues (lysine and arginine [K and R]) between positions 113 and 116 at the C terminus of the F2 protein (cleavage site motif ¹¹¹GRRQKR/F¹¹⁷) and phenylalanine (F) at the N terminus of the F1 protein (residue 117). As a result, the precursor protein of virulent viruses is more susceptible to cleavage by the ubiquitous proteases present in different tissues of the host [8]. Strains of low virulence have fewer basic amino acid residues at the C terminus of the F2 protein, leucine (L) at position 117 (cleavage site motif 112G/E-K/R-Q-G/E-R-L117), and trypsin-like proteases are required to cleave their precursor. The mesogenic and velogenic viruses cannot be differentiated on the basis of the nucleotide sequence of the F0 proteolytic cleavage site.

During July to September 2012, there were several deaths of peafowl in Gurgaon Faridabad and Jhajjar

districts of Haryana. In total, 145 birds were reported sick (45 from Gurgaon, 36 from Jhajjar and 64 from Faridabad) while 28 birds were reported dead (12 from Gurgaon, 7 from Jhajjar and 9 from Faridabad). From these outbreaks samples were collected for this study as detailed in Table 1 of Supplementary material. Similar outbreaks were also reported later on from Sonipat and Bahadurgarh districts of Haryana, however no samples were collected from these outbreaks. Some of the sick birds were showing signs of nervous system involvement including shaking of heads, torticollis and paresis. Necropsy findings included congestion in intestine and brain.

Since the postmortem lesions were not pointing towards a specific infection in birds therefore tissue, blood and cloacal swab samples from sick birds, brain and intestinal tissues from dead birds were collected for further investigation. This study describes the diagnosis, isolation and molecular characterization of NDV in samples collected from peacocks of Haryana.

A total of 20 tissue and swab samples were collected in 50 % buffered glycerin from peafowls which included dead and sick birds (Table 1 of Supplementary material). Of these, 15 were tissue samples (brain, trachea and lung) and 5 were oral and cloacal swab samples. Also five blood samples were collected from clinically sick birds for serological examination (Table 1 of Supplementary material). The tissue samples after collection, were kept at -20°C till processed. The HI test was performed on five sera samples (as listed in Table 1 of Supplementary material) using 1 % chicken RBCs using standard protocol [7].

A sample of brain tissue (NDV/IND2012/01) was triturated and homogenized in PBS with antibiotics (penicillin 1,000 IU/ml and streptomycin 1,000 $\mu\text{g/ml}$) making up a 10 % (w/v) solution. The 10 % suspension was clarified by centrifugation at $2,500\times g$ for 15 min. The supernatant was collected and stored at -20°C until further use. Part of this suspension was used for RNA isolation for RT-PCR and part was inoculated onto Vero cell culture after filtration through 0.22 micron filter for virus isolation. Monolayers of African green monkey kidney (Vero) cell line grown in 25 cm^2 tissue culture flasks were inoculated with sample supernatant. Following incubation at 37°C for 1 h maintenance media (Eagle MEM) without fetal calf serum (FCS) and with antibiotics (penicillin 10,000 UI/ml, streptomycin 100 $\mu\text{g/ml}$) was added. The flasks were incubated at 37°C and were observed daily for the appearance of cytopathic effects (CPE) for up to 7 days post-inoculation. When 100 % CPE was observed, the flasks were frozen at -20°C . The virus was harvested after two freeze–thaw cycles. Five blind passages were carried out for this sample.

Total RNA from the suspected samples was extracted using TRIzol reagent (Life Technologies, USA) as per the manufacturer's protocol. In brief, about 100 mg of the

suspected tissue was homogenized in pestle and mortar with 1.0 ml PBS buffer to make 10 % w/v suspension. The 10 % suspension was clarified by centrifugation at $2,500\times g$ for 15 min. The supernatant (600 μ l) was collected and mixed vigorously with 1.0 ml of TRIzol. 0.2 ml of chloroform was then added per 1 ml of TRIzol reagent. Samples were vortexed vigorously for 15 s and incubated at room temperature for 2–3 min. The samples were centrifuged at no more than $12,000\times g$ for 15 min. After phase separation, RNA remains exclusively in the upper aqueous phase. Upper aqueous phase was transferred carefully into fresh tube without disturbing the interphase. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5 ml of isopropyl alcohol per 1 ml of TRIzol reagent was used for the initial homogenization. Samples were incubated at 15–30 °C for 10 min and centrifuged at not more than $12,000\times g$ for 10 min. The RNA pellet was washed with 75 % ethanol, by adding at least 1 ml of 75 % ethanol per 1 ml of TRIzol reagent. The samples were mix by vortexing and centrifuged at $7,500\times g$ for 5 min. The RNA pellet was air dried and dissolved in 30 μ l RNase free water and then stored at –20 °C till used. The same protocol was followed for RNA extraction from the cloacal swab samples with initial differences in reconstitution of swab in 600 μ l of PBS.

RT-PCR was performed on purified RNA samples using Super script III one step RT-PCR platinum Taq Hifi Kit (Invitrogen). Fusion protein cleavage site (FPCS) specific primers [24] were used in the PCR reaction. One step RT-PCR cycling parameters were kept as per the manufacturers guidelines i.e. one cycle of cDNA synthesis at 55 °C for 30 min followed by pre-denaturation at 94 °C for 2 min and further amplification was carried out for 40 cycles of 94 °C for 15 s, 53 °C for 30 s, 68 °C for 1 min with final extension at 68 °C for 5 min. Nested PCR was performed using 6 μ l of amplicon from previous step and 10 pmol each of the forward and reverse primers (ND3 & ND4) in a total volume of 50 μ l reaction mix containing 25 μ l 2X Top Taq PCR Master Mix Kit (Qiagen). The incubation temperature and duration of each cycle of the PCR were 94 °C $\times$ 1 min for denaturation, 52 °C $\times$ 45 s for annealing and 72 °C $\times$ 45 s for extension. The amplification was carried out for 35 cycles with final extension at 72 °C for 10 min. The PCR products (6 μ l aliquots) were separated on 1.5 % agarose gel with ethidium bromide. For determining the DNA segment size, the 1 kb DNA marker (Fermentas) was used.

RT-nPCR amplified products were gel purified using Qiaquick gel extraction kit (Qiagen) and sequenced from both direction in Automated genetic analyser (ABI 3130XL) using BigDye terminator v3.1 cycle sequencing kit from Applied Biosystems. Cycle sequencing PCR was performed using standard protocol. The sequence data

generated was assembled using SeqMan programme of the Lasergene software ver 5.0 and was subjected to BLAST analysis for comparison with other available F gene sequences in Genbank. Sequences were aligned CLUSTAL W programme implemented in BioEdit (7.0.5.3) programme. Aligned sequences were used for phylogenetic analysis using MEGA 5.0 programme [26]. The nucleotide sequence identities were calculated using MEGA 5.0 programme.

This study was conducted to isolate and characterize NDV virus from sick/dead peafowls in Haryana. Sick birds were showing nervous sign including shaking of heads, torticollis, incoordination and inability to fly (Fig. 1). Clinical nervous signs in sick peafowls were suggestive of NDV infection. Mortality in peacock was observed with congestion of lungs and intestinal mucosa. NDV outbreaks in peafowls have been reported earlier from India and other part of the world [14, 28]. Contrary to our findings Vijayarani et al. [28] reported the absence of characteristics NDV signs in birds. Postmortem lesions that included congestion in lungs and intestinal mucosa were similar to those observed previously by Vijayarani et al. [28] and were also indicative of NDV infection.

HI test revealed that three out of the 5 sera samples (60 %), were negative for HI antibodies to NDV. However, one of the serum samples (NDV/IND2012/21) was strong positive for anti NDV antibodies with a titer of 1:32, while the other serum sample was weak positive (NDV/IND2012/23) with a HI titer of 1:8 only.

Since the peacocks are generally not vaccinated against NDV, the detection of HI antibodies against NDV indicates that these peacocks had NDV infection. This correlates with the history and RT-nPCR results. The result of this study partially agreed with the findings of Manin et al. [20], who successfully detected and differentiated NDV from the field samples by HI test using NDV specific polyclonal serum.

In order to adapt NDV on African green monkey kidney (Vero) cell line, five consecutive passages (designated as

Fig. 1 Peacocks showing paresis of wings, inability to fly

P1 to P5) were done. During first and second passage, wild NDV didn't produce any clear evidence of cytopathic effect (CPE), but in third passage characteristics changes in the cell monolayer were observed. During fourth and fifth passages, clear and consistent CPE was observed within 48–72 h of infection (Fig. 1 of Supplementary material). CPE was characterized by formation of syncytium, giant cell, dendritic-shaped cell and finally plaque. The P3, P4 and P5 were tested for the NDV RNA by RT-nPCR. Presence of correct sized amplicon (216 bp) in all three passages confirmed the presence of NDV (data not shown).

RT-nPCR on all 20 samples resulted in amplification of expected sized F gene amplicon (216 bp amplicon) indicating the presence of NDV genome in all the samples. Figure 2 shows amplification in five samples. The amplicons from NDV/IND2012/01 and NDV/IND2012/02 were sequenced directly using nested PCR primers as sequencing primers. BLAST analysis of sequences from these samples showed a maximum of 98 % nucleotide identity to published sequence of F gene of NDV, confirming the presence of NDV in these samples.

Virulence determinants of NDV are controlled by multiple genetic factors which are not completely understood, however, multibasic amino acid sequence at FPCS has been postulated as an absolute pre-requisite and major determinant of NDV virulence [11]. The fusion gene of virulent NDVs is characterized by the presence of a pair of dibasic amino acids at the cleavage site while in lentogenic strains it is characterized by the presence of monobasic amino acids. Velogenic/mesogenic strains possess a multibasic FPCS followed by phenylalanine at position 117.

The deduced amino acid sequence from nucleotide sequences of peacock samples (NDV/IND2012/01 and NDV/IND2012/02) carried the characteristic amino acid motif (¹¹¹GRRQKR/F¹¹⁷) in FPCS site which is indicative of the presence of virulent NDV in peafowls. Earlier, Vijayarani et al. [28] reported amino acid motif (¹¹¹GRRQRRF¹¹⁷) in FPCS site of NDV isolated from peacock from southern part (Chennai) of India. The FPCS

sequences obtained in the current study and that reported by Vijayarani et al. [28], differed at position 115 where arginine was replaced by lysine. Both Indian strains as reported by Vijayarani et al. [28], and the one used in this study had two cleavage-site sequences that were each consistent with a virulent pathotype and valogenic nature of the virus. Circulation of multiple genotypes of NDV in India has been recently reported based on the FPCS [27].

Phylogenetic analysis of the partial F gene nucleotide sequences (216 bp) of NDV/IND2012/01 and NDV/IND2012/02, was done by comparing them to already published sequences of both class I and class II categories of NDVs, using neighbor-joining methods and p-distance matrices implemented in MEGA software version 5.0 [26]. These analyses have shown that the virus belongs to Class II genotype VII closely related to virus isolates originated from outbreaks in Western Europe, Israel, Indonesia and Taiwan with nucleotide identity levels up to 98 % (Fig. 3a, b). NDV/IND2012/01 and NDV/IND2012/02 are very highly closely related with 99 % nucleotide identities among them. Phylogenetic grouping of the virus and sequence of FPCS is indicative of pathogenic potential of virus strain circulating in peacocks in Haryana. These results also indicated that these NDV strains from peacock are genotypically related to isolates recovered from a variety of avian species and few velogenic (Iowa/Salsbury, Beaudette) and mesogenic strains (Michigan) of exotic origin within class II, genotype VII.

After Vijayarani et al. [28], this is the 2nd report of NDV from peacocks. Vijayarani et al. [28] reported the prevalence and circulation of genotype II virus in Southern India in peacocks. To the best of our knowledge perhaps is the first confirmatory report of presence of genotype VII from Peacocks in India based on F gene phylogeny and FPCS sequence. Although Tirumurugaan et al. [27] has recently described the presence of genotype VII in different bird species (other than peacocks) in India employing the FPCS sequence classification system. In the class II NDV, the 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 [23]. The genotypes V, VI, VII, VIII and X have been reported to have emerged after 1960 [27, 29].

The nucleotide similarity in the fusion gene fragment of NDVs studied here to other NDV lineages was in the range of 70–98 %, with highest sequence identity with outbreak strains from Western Europe, Israel, Indonesia, Taiwan and other Indian strains. This is suggestive of wide distribution of pathogenic NDV of genotype VII. These circulating field strains also differed from vaccine strains. The wild birds are considered to be the natural reservoir of NDV and are responsible for ND outbreaks in susceptible host. The isolation of virulent strain of NDV with morbidity and

Fig. 2 Correct sized F gene amplicon (216 bp) generated in RT-nPCR from five field samples indicating the presence of NDV genome. Lanes 1 and 8 1 Kb DNA ladder. Lane 2 +ve control (R2B vaccine strain). Lanes 3–7 samples NDV/IND2012/01 to NDV/IND2012/05

Fig. 3 **a** Unrooted neighbour-joining phylogenetic tree using partial F gene sequences (282–478 bp). **b** Unrooted neighbour-joining phylogenetic tree using partial F gene sequences (282–478 bp) of genotype VII. The trees were constructed using a p-distance algorithm and pairwise deletion parameters. Texas show GenBank accession numbers and geographic origin of strains that were compared. Bootstrap values (%) are represented at each tree node. Node support was assessed with 500 bootstrap pseudo-replicates. The NDV/IND2012/01 and NDV/IND2012/02 strains from Haryana characterized in this study are indicated in *blue* colour and *bold* font

mortality in peacock in the study is suggestive of peacock being the natural host for NDV.

In conclusion the data presented here provide evidence of divergence in circulating virulent strains within an

outbreak in peacock in Haryana, India. These circulating field strains also differed from vaccine strains. The presence of virulent NDV in wild birds which are causing a series of ND outbreaks (as reported in this study) is

indicative of the threat posed by reservoir hosts in domesticated birds. It would be wise for any control programme of ND to address this problem with a view to control NDV infections in wild birds.

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References

1. Aldous EW, Collins MS, McGoldrick A, Alexander DJ. Rapid pathotyping of Newcastle disease virus (NDV) using fluorogenic probes in a PCR assay. *Vet Microbiol.* 2001;80:201–12.
2. Aldous EW, Mynn JK, Banks J, Alexander DJ. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. *Avian Pathol.* 2003;32:239–56.
3. Alexander DJ. Newcastle disease. In: Purchase HG, Arp LH, Domermuth CH, Pearson JE, editors. *A laboratory manual for the isolation and identification of avian pathogens*. 3rd ed. AAAP: Kennet Square; 1989. p. 114–20.
4. Alexander DJ. Gordon memorial lecture. Newcastle disease. *Br Poult Sci.* 2001;42:5–22.
5. Alexander DJ. Newcastle disease in the European Union 2000 to 2009. *Avian Pathol.* 2011;40:547–58.
6. Alexander DJ, Manvell RJ, Lowings JP, Frost KM, Collins MS, Russell PH, Smith JE. Antigenic diversity and similarities detected in avian paramyxovirus type 1 (Newcastle disease virus) isolates using monoclonal antibodies. *Avian Pathol.* 1997;26:399–418.
7. Anon. Newcastle disease. In: *Methods for examining poultry biology and for identifying and quantifying avian pathogens*. National Academy of Science: Washington DC; 1971. p. 66.
8. Collins MS, Bashiruddin JB, Alexander DJ. Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. *Arch Virol.* 1993;128:363–70.
9. Creelan JL, Graham DA, McCullough SJ. Detection and differentiation of pathogenicity of avian paramyxovirus serotype 1 from field cases using one-step reverse transcriptase-polymerase chain reaction. *Avian Pathol.* 2002;31:493–9.
10. Czegledi A, Ujvari D, Somogyi E, Wehmann E, Werner O, Lomniczi B. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. *Virus Res.* 2006;120:36–48.
11. Dortmans JC, Koch G, Rottier PJ, Peeters BP. Virulence of Newcastle disease virus: what is known so far? *Vet Res.* 2011;42:122.
12. Gohm DS, Thur B, Hofmann MA. Detection of Newcastle disease virus in organs and faeces of experimentally infected chickens using RT-PCR. *Avian Pathol.* 2000;29:143–52.
13. Huovilainen A, Ek-Kommone C, Manvell R, Kinnunen L. Phylogenetic analysis of avian paramyxovirus 1 strains isolated in Finland. *Arch Virol.* 2001;146:1775–85.
14. Jansen J, Kunst H. Newcastle disease in peacocks. *J Am Vet Med Assoc.* 1952;120:201–2.
15. Jorgensen PH, Handberg KJ, Ahrens P, Therkildsen OR, Manvell RJ, Alexander DJ. Strains of avian paramyxovirus type 1 of low pathogenicity for chickens isolated from poultry and wild birds in Denmark. *Vet Rec.* 2004;154:497–500.
16. Kant A, Koch G, Van Roozelaar DJ, Balk F, Huurne AT. Differentiation of virulent and non-virulent strains of Newcastle disease virus within 24 hours by polymerase chain reaction. *Avian Pathol.* 1997;26:837–49.
17. Kim LM, King DJ, Suarez DL, Wong CW, Afonso CL. Characterization of class I Newcastle disease virus isolates from Hong Kong live bird markets and detection using real-time reverse transcription-PCR. *J Clin Microbiol.* 2007;45:1310–4.
18. King DJ, Seal BS. Biological and molecular characterization of Newcastle disease virus isolates from surveillance of live bird markets in the northeastern United States. *Avian Dis.* 1997;41:683–9.
19. Kolakofsky D, Roux L, Garcin D, Ruigrok RW. Paramyxovirus mRNA editing, the “rule of six” and error catastrophe: a hypothesis. *J Gen Virol.* 2005;86:1869–77.
20. Manin TB, Shcherbakova LO, Bochkov IuA, El'nikov VV, Pchelkina IP, Starov SK, Drygin VV. Characteristics of field isolates of Newcastle disease virus isolated in the course of outbreaks in the poultry plant in the Leningrad region in 2000. *Vopr Virusol.* 2002;47:41–3.
21. Mathivanan B, Kumanan K, Mahalinga Nainar A. Characterization of a Newcastle disease virus isolated from apparently normal guinea fowl (*Numida melagris*). *Vet Res Commun.* 2004;28:171–7.
22. Meulemans G, Van den Berg TP, Decaesstecker M, Boschmans M. Evolution of pigeon Newcastle disease virus strains. *Avian Pathol.* 2002;31:515–9.
23. Miller PJ, Decanini EL, Afonso CL. Newcastle disease: evolution of genotypes and the related diagnostic challenges. *Infect Genet Evol.* 2010;10:26–35.
24. Nanthakumar T, Kataria RS, Tiwari AK, Butchiaiah G, Kataria JM. Pathotyping of Newcastle disease viruses by RT-PCR and restriction enzyme analysis. *Vet Res Commun.* 2000;24:275–86.
25. Takakuwa H, Ito T, Takada A, Okazaki K, Kida H. Potentially virulent Newcastle disease viruses are maintained in migratory waterfowl populations. *Jpn J Vet Res.* 1998;45:207–15.
26. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol.* 2011;28(10):2731–9.
27. Tirumurugaan KG, Vinupriya MK, Vijayarani K, Kumanan K. 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. *Indian J Virol.* 2011;22:131–7.
28. Vijayarani K, Muthusamy S, Tirumurugaan KG, Sakthivelan SM, Kumanan K. Pathotyping of a Newcastle disease virus isolated from peacock (*Pavo cristatus*). *Trop Anim Health Prod.* 2010;42:415–9.
29. Yi J, Liu C, Chen B, Wu S. Molecular characterization of a virulent genotype VIII strain of Newcastle disease virus from farmed chickens in Shanghai. *Avian Dis.* 2011;55:279–84.