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Short Communication

Molecular Characterization and Epitope Mapping of Fusion (F) and Hemagglutinin (HN) Genes of Avian Paramyxovirus Serotype I from Peacocks in Pakistan

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SA, MAM, KM, MYT and IH conceived and designed the experiment, SA, MA, IR and RR performed the laboratory procedures and analyzed the data, SA and IH wrote the manuscript.

Key words
Avian Paramyxovirus type 1 (APMV-1), Newcastle disease virus (NDV), Peacock, Pakistan.

ABSTRACT

Effective diagnosis and control measures for Newcastle disease virus (NDV) requires continuous disease surveillance and understanding of field NDV strains. The NDVs, a prototype of Avian Paramyxoviruses (APMV-1), have been reported previously both in chicken and feral birds (natural reservoirs) in Pakistan; however, their molecular characterization is largely inadequate and requires continuous evaluation. In this study, sequencing and molecular analysis of fusion (F) and hemagglutinin (HN) genes of NDV strain isolated from an outbreak in a peacock flock was undertaken. Proteolytic cleavage site of F_0 showed a cleavage motif ($_{112}$RRQKR↓F$_{117}$), representative of a velogenic serotype. Moreover, based on F and HN gene nucleotide sequence analysis, the isolate clustered as genotype VII closely associated to those reported from Indonesia. Further sequence analysis of hyper-variable region within F gene revealed its clustering to sub-genotype VIIe. The inferred residue analysis of the virus revealed a number of substitution mutations in the structural and functional domains when compared to the representative strains of each genotype including the vaccine strains (genotype II and III). Interestingly, some of these mutations were found exclusive to the study isolate. Not only do these prime findings improve our understanding about currently circulating strains of NDVs but they also help us to envisage potential efforts to avoid future outbreaks in wild birds, as well as in commercial and backyard poultry.

Throughout the world, Newcastle disease is one of the most important diseases of poultry having significant economic implications. The disease occurs both in intensively-raised commercial flocks as well as backyard poultry (Kattenbelt *et al*., 2006). Newcastle disease virus is categorised as a member of the family *Paramyxoviridae* in the genus *Avulavirus*. It is an enveloped virus, having a negative sense and single stranded RNA genome of approximately 15.2 Kb (Mayo, 2002). The cleavage site of the velogenic and/or mesogeneic strains of NDVs has amino acid sequence motif $_{112}$R/KRQR/KR↓F$_{117}$ in the F protein, whereas the lentogenic viruses have amino acid sequence of $_{112}$G/EK/RQG/ER↓L$_{117}$ (Wei *et al*., 2008). The clinical manifestations of NDV in susceptible birds depend on the age, host immune status, environmental stress, virulence and tissue tropism of the isolate (Dortmans *et al*., 2011; OIE, 2012). Clinical signs vary from in-apparent to severe; the latter is associated with respiratory, enteric and neurological signs. The hosts targeted by NDVs are more than 200 bird species that include wild, domestic and cage birds (Alexander, 2003). It is interesting to note that an avirulent virus isolated from wild birds when subjected to passages in chicken embryos transforms to a virulent form after 20 passages for chickens (Zanetti *et al*., 2008). Moreover, just a two- nucleotide change at the F protein cleavage site is enough to change the virus from a low virulent to a virulent strain (Gould *et al*., 2001).

A number of epidemics of ND in commercial poultry and wildlife have been recorded since the initiation of high density, captive husbandry system in Pakistan. Compared to isolates originating from natural reservoirs such as wild-birds, NDVs from chickens have been isolated and well-characterized previously (Munir *et al*., 2012; Shabbir *et al*., 2012, 2013a, b). In this study, however, analysis of

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complete F and HN genes of an isolate recovered from ND-suspected outbreak in a peacock flock was done. The obtained sequences were processed for phylogeny and residue analyses. The study of the isolate has added to our understanding of genetic diversity among indigenous strains of NDVs originating from wild-birds in Pakistan.

Materials and methods

A ND outbreak occurred in a flock of peacock (n = 128) in district Lahore, Pakistan during 2014. Four days post appearance of clinical symptoms, 13 peacocks were dead whereas 29 were morbid. The affected birds showed circling movements and tremors while some birds were also presenting mild respiratory symptoms such as sneezing, coughing and nasal discharge. Necropsy was performed and samples (trachea, lungs, and brain) were collected and processed for isolation of NDV in 9-11 day-old embryonated chicken eggs (ECE).

Agglutinating virus in harvested allantoic fluid was identified by spot agglutination assay using 10% chicken red blood cells (RBCs) and the identity of the virus was determined by NDV specific antisera through standard haemagglutination inhibition (HI) assay. Further identification was confirmed by F-gene based reverse transcriptase polymerase chain reaction (RT-PCR) (Ling, 1997) as per optimized protocols available at Quality Operation Laboratory, University of Veterinary and Animal Sciences, Lahore.

Total RNA was extracted from allantoic fluid using commercially available RNA extraction kit as per manufacturer's instruction (QIAamp Viral RNA Mini Kit, QIAGEN, Hilden, Germany). The quantity and quality of extracted RNA was determined by (Qubit Flourometer, USA). Reverse transcription polymerase chain reaction was performed for complete F and HN genes spanning the genomic region from 4498-6330 nucleotides. Primers and protocol used for this amplification were the same as described previously (Munir *et al*., 2010) (Supplementary Table I). Briefly, using QIAGEN OneStep RT-PCR Kit (QIAGEN, Hilden, Germany), a total reaction volume (25µl) comprising of 3 µl of template RNA, 5µl of 5X buffer, 5µl of Q buffer, 1 µl of 2.5 mM dNTPs, 1 µl of enzyme mix, I µl of each forward and reverse primers (10 pm/µl each) and 8 µl of nuclease free water were incubated at 50°C for 30 and 95°C for 15 minutes for cDNA preparation followed by 40 cycles each of 95°C for 30 sec, 52°C for 60 sec and 72°C for 90 sec. The reaction was terminated by final extension at 72°C for 10 min. Amplicons were purified using GeneJET PCR Purification Kit (ThermoFisher Scientific, USA) and sequenced in both forward and reverse directions (First BASE Lab., Malaysia) employing the same set of primers used for amplification of study genes. Sequence files were viewed in BioEdit and consensus sequence was established. The obtained sequence was blasted and matching top-hit nodes were downloaded. The obtained sequences were aligned using ClustalW programs and cut to previously known equal lengths for F and HN genes. Using MEGA6.0, phylogenetic relationships of complete F and HN genes of the study isolate were elucidated to the corresponding regions of previously characterized NDVs around the globe. The evolutionary distances were inferred and expressed based on the number of nucleotide substitutions per site. The codon positions included in the analysis were the 1st, 2nd, 3rd and noncoding. All positions containing gaps and missing data were eliminated from the data set (the "complete deletion" option). Amino acid residue analysis of representative strains of each known genotype including the vaccinal ones was compared by BioEdit.

Results and discussion

Harvested virus from allantoic fluid agglutinated chicken RBCs with a titer of 1:256 and also reacted with NDV specific antiserum confirming that the virus belonged to AMPV-1 group of NDVs. The identity of the virus was further confirmed by nucleic acid based PCR technique using NDV specific primers that targeted F gene. The fact that a number of functionally important structures such as signal peptide, cleavage activation site, hydrophobic and hydrophilic regions, and transmembrane domains are located in F and HN genes of NDVs, and that the same very genes are also used for phylogenetic analysis/comparison to determine the geographic relationship of circulating NDV strains/genotypes (Toyoda *et al*., 1988; Yussof *et al*., 2001; Aldous *et al*., 2003), we characterized the virus with respect to these features. Thus, epitope mapping of biologically important regions of F and HN genes of the study isolate was carried out to see as to how our isolate differed from previously reported NDV isolates available at GenBank. Since most of the sequences available in GeneBank from Pakistan comprise of 247bp of hyper-variable region spanning 4674 to 4921nt of viral genome and most of nucleotide and subsequent amino acid substitutions are located in that particular region, we used the aligned sequence of the same length while comparing study isolate to those reported previously.

Sequence comparison of the complete F and HN genes revealed that the study isolate belonged to genotype VII. Further phylogenetic analysis of hyper-variable region of F gene demonstrated that the isolate belonged to sub-genotype VIIe (Supplementary Fig. 1). Nucleotide comparison of F gene of the study isolate with that of the representative strains of NDV exhibited maximum per cent similarity with genotype VII (92.8%)

Table I.- Percentage nucleotide and residue identity of Fusion and Hemagglutinin gene of study isolate to representative strains of each genotype reported worldwide.

Study isolate	Ulster:67 Ireland AY562991 Genotype-I		LaSota China AY845400 Genotype-II		Mukteswar China EF201805 Genotype-III		Herts/33 Netherlands AY741404 Genotype-IV		14698/90 Indonesia AY562985 Genotype-V		IT-227/82 Italy AJ880277 Genotype-VI		NA-1 China AJ880277 Genotype-VII	
Peacock / MZS2-UVAS/2014	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Fusion gene	87.1	90.7	83.9	88.4	86.7	90.7	88.5	94.0	86.6	92.2	90.4	94.7	92.8	96.7
Hemagglutinin gene	85.0	89.5	81.3	86.5	85.1	88.2	87.4	89.6	85.8	89.3	89.5	92.6	92.1	94.0

nt, nucleotide; aa, amino acid.

whereas it was observed to vary from 84 -90 for genotype of vaccine strains; it was 83.9% for genotype II and 86.7% for genotype III. Similarly for HN gene, the per cent similarity for VII was found to be 92.1; for genotype II, 81.3; and for genotype III, 85.1 (Table I). Based upon complete F and HN gene phylogeny, peacock isolate was found to be closely related to isolates of Cockatoo/Indonesia/14698/90, Ch/Sukorejo/019/10, and Ch/Banjarmasin/010/10 that were previously reported from wild cockatoo and chickens in Indonesia. However, comparative nucleotide sequence analysis of hyper-variable region of F gene revealed close association of the study isolate with previously reported Pakistan's NDV isolates as sub-genotype VIIe. Lineage 5/genotype VII is thought to be originated from the Far East with the first isolation from Taiwan in 80s (Yang *et al*., 1999). Since then, the presence of this genotype has been indicated from various parts of the globe (Aldous *et al*., 2003). Varying at sub-lineage level, a number of virulent NDVs of class II have been reported from many Asian countries including those that share border with Pakistan (Aldous *et al*., 2003; Tirumurugaan *et al*., 2011; Zhang *et al*., 2011). Furthermore, genetically related NDVs to sub-genotype VIIa have been previously reported from wild birds while sub-genotype VIc, VIIb, VIIe and VIIf were reported from backyard and commercial poultry (Munir *et al*., 2012; Siddique *et al*., 2013; Rehmani *et al*., 2015). However, it is the first time that this genotype (VIIe) has also been identified from wild-bird (peacock) indicating that the genotype has potential to be transmitted between the two species.

Amino acid residue analysis and a comparison with previously published public database of NDVs were also undertaken for F and HN genes of the study isolate. Complete F gene spanned nucleotide sequences from 4550 – 6211 totalling 1662 bp which coded for 553 amino acid residues (Supplementary Fig. 2). Likewise, HN region comprised of DNA sequences from 6418 – 8133 with 1716 bp giving rise to 571 aa residues in total (Supplementary Fig. 3). Within the hyper-variable region of F-gene (47-421 nt), we observed multiple basic amino acid residues at proteolytic cleavage site (F_0) considered typical of velogenic strains. The corresponding residues at the F_2 protein and the N-terminus of F_1 protein were found to be $_{112}$RRQKR$_{116}$ and phenylalanine ($_{116}$↓F$_{117}$), respectively. Analysis of the deduced residues at the cleavage site for the isolate in the current study and previously-reported isolates from Pakistan indicated co-circulation of different genotypes of NDVs in the country with similar pathogenicity (based on the motif in the cleavage site). This is of much importance since invariability in residue pattern has been observed for vNDVs in a given geographical location (Samuel *et al*., 2013).

The sites for glycosylation and cysteine residues are believed to be conserved for F and HN proteins. However, in comparison to each other and to the representative genotype particularly of the vaccine strain, we found differences in the composition of residues for a given glycosylation site and variations in both the number and position of cysteine residues. Further, comparison of functional domains of F and HN protein to other genotypes and vaccine strain revealed several substitutions that were more often in F protein than in the HN protein. Six potential glycosylation sites [Asp(N)-X-Ser(S)/Thr(T), where, X could be any residue except proline (P) and aspartic acid (D) including $_{85}$NRT$_{87}$ $_{191}$NNT$_{193}$, $_{366}$NTS$_{368}$, $_{447}$NIS$_{449}$, $_{471}$NNS$_{473}$, $_{541}$NNT$_{543}$ were identified in the F protein. Furthermore, 12 cysteine (C) residues located at position 27, 76, 199, 338, 347, 362, 370, 394, 399, 401, 424 and 523 were observed. Compared to the consensus sequence of vaccinal strains used in Pakistan and other representative strains, a number of substitution mutations in residue sequences were recorded such as the fusion peptide (117-142aa) at position 117 (L→F), 121 (I→V) and 124 (G→S), 139 S→A, hydrophilic region-a (143-185aa) at position 145 (K→N),170 (D/G→N),176(A→S) hydrophilic region-b (268-299aa) at position 272 (N→Y),

275(F→L), 288 (T→N) and hydrophilic region-c (471-500aa) at position 472 (H→N), 479 (N→D), 482(E/A/V/S→T), 486 (R→S) 494(K→R). Mutation at position 506(V→A), 509(I→V), 513(V→F) 514(L→F) and 516(I/M→V) 520(V/I→G) of the major trans-membrane region (501-521aa) further indicated lack of conserveness of this particular part of genome (Supplementary Fig. 2). On the other hand, HN gene analysis of predicted amino acid sequences revealed a conserved pattern of amino acid residues when compared with previous NDV strains. For example, cysteine residues at positions 123, 172, 186, 196, 238, 247, 251, 455, 461, 465, 531 and 542 were found to be conserved. Other biologically active key residues included residues for receptor (sialic acid) binding at positions R174, E401, R416, Y526; residues for the hydrophobic core of the stalk 4HB at positions, Y85, V88, S92, L96, T99, I103, I107, L110 and I114; and stalk residues at positions R83, A89, L90, L94 and L97 involved in direct interaction with F protein (Ke *et al*., 2010; Yuan *et al*., 2011) were found to be conserved. Furthermore, five N-glycosylation sites at positions $_{119}$NNS$_{121}$, $_{341}$NDT$_{343}$, $_{433}$NKT$_{435}$, $_{481}$NHT$_{483}$, and $_{538}$NKT$_{540}$ were identified as has been previously reported (Umali *et al*., 2014). When compared to most common vaccine strain used in Pakistan (genotype II and III), beside substitutions at several point in total length of HN gene, we observed substitution mutations at (trans-membrane domain) including hydrophilic region a 75(G→S), 77(N→S), 81(V→I), and hydrophilic region b 98(K→N),101(T→S)102 (T→I), (antigenic sites) at site 23, 203 (Y→H), at site 1 and 14, 347(E→K), at site 12, 494(G→D), at site 2 and 12, 514(I→V) at site 2, 569(D→G), and neutralization epitope 263(N→K). Other substitutions exclusive to the study isolate were found at positions 52(I→V),107(T/S→A), 445(Q→L) for F protein and 2(D→S),9(A/V→M) 34(V→I), 50(A→T), 57(V/A→T), 62(T→V), 71(T→A), 218(R→K),290(V/T→A) 308(I→V) 310(N/D/G/S→E) 431(V→I) for HN protein (Supplementary Fig. 3). The substitutions particularly in the fusion peptide, hydrophobic regions and trans-membrane region of F protein and neutralizing epitopes of HN protein could result in altered fusion activity and neutralizing escape variants (Hu *et al*., 2010; Wang *et al*., 2015). Taken together, variation in nucleotide and subsequent substitutions/alternations in amino acid profile as observed in this study is consistent with previously described theories of evolution of RNA viruses particularly the APMVs (Yu *et al*., 2001; Umali *et al*., 2014).

We found amino acid substitutions with dissimilar properties and a scattered pattern of distribution. For example, 36th amino acid residue (isoleucine) of HN gene in our study isolate is hydrophobic but it is polar (threonine) in the representative NDVs. On the other hand, 50th aa in our isolate is polar but it is hydrophobic in previously reported isolates. Similar substitutions could be appreciated at various regions of the genes. It is interesting to note that such substitutions with amino acids having dissimilar properties have also been found, albeit at lower rate, in representative strains of NDVs. The implications of these changes in the critical regions of F and HN genes could be serious as these changes may mean escape of the virus from the established immunity. Since the peacock flock had the history of vaccination with lentogenic strain (LaSota), identification of cleavage motif similar to velogenic strain raises concerns for type of vaccine used to protect the flock and need for post-vaccine evaluation. Substitution and subsequent mutations at fusion peptide, HR regions and trans-membrane domain could affect the fusion activity of NDV (Umali *et al*., 2014) and alteration in antigenic epitopes particularly those that are involved in virus attachment could result in escape variants and subsequent vaccine failure (Cho *et al*., 2007; Wang *et al*., 2015).

Conclusion

We have characterized a circulating genotype of peacock which was closely related to previously known clades of VII and VIIe sub-genotype. Based on the deduced amino acid analysis, various mutations were found throughout F and HN genes of the isolate, some were exclusive to the isolate. Whether these changes have any real impact on the immunity and disease dynamics of the virus, studies ascertaining transmission of these viruses in vaccinated as well as immunologically naïve flock and their subsequent shedding are needed for understanding their potential to cause disease in the wild birds as well as in the commercial and backyard poultry.

Statement of conflict of interest

The authors declare no conflict of interest regarding this paper.

Supplementary Material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2017.49.2.sc9

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