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Biological characterization and phylogenetic analysis of a novel genetic group of Newcastle disease virus isolated from outbreaks in commercial poultry and from backyard poultry flocks in Pakistan

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ABSTRACT

Newcastle disease (ND) is a contagious viral disease of many avian species particularly domestic poultry, and is responsible for devastating outbreaks in the poultry industries around the globe. In spite of its importance and endemicity in Southern Asia, data on the genetic nature of the viruses and epizootiological information of the disease is scarce. In this study, six isolates from an emerging wave of ND outbreaks in the north of Pakistan and two isolates from healthy poultry flocks were biologically and genetically characterized. Based on pathogenicity indices such as intracerebral pathogenicity index (ICPI), mean death time (MDT) and cleavage motifs in the fusion protein, all these isolates were classified as virulent. Phylogenetic analysis of the fusion (F), hemagglutinin-neuraminidase (HN) and matrix (M) genes indicated the emergence of a novel genetic group within lineage 5, distinct from isolates previously reported in the region. Several mutations in the neutralizing epitopes and functionally important motifs of the F and HN genes pose a need for re-evaluation of the currently used vaccine and vaccination practices. The characteristics of Newcastle disease virus (NDV) as virulent (F protein cleavage site, ICPI and MDT) in apparently healthy backyard poultry (BYP) explain that BYP can play crucial role in the epizootiology and spread of the disease. The present investigation provides essential information on the genetic nature of NDV circulating in Pakistan and its implication on disease diagnosis and control. Furthermore, these investigations emphasize the importance of continuous surveillance of ND in developing countries.

1. Introduction

Newcastle disease virus (NDV), a specific form of avian paramyxovirus type 1 (APMV-1), cause highly contagious viral infection in a wide range of bird species that can result in significant economic losses in domestic poultry around the globe (Alexander, 2003). It is a negative sense single stranded RNA virus classified into genus *Avulavirus*, order *Mononegavirales* within family *Paramyxoviridae* (ICTV, 2009). The genome of APMV-1 is always either 15186, 15192 or 15198 nucleotides in length and follow the so-called "rule of six" which is essential for viral replication (Kolakofsky et al., 2005). The genome, from 5′ to 3′ terminus, encodes for nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F) and hemagglutinin-neuraminidase (HN) and large RNA-dependent polymerase protein (L) (Alexander, 2003).

Based on pathogenicity in embryonated eggs, the NDV is classified into four categories: velogenic [MDT (mean death time) <60 h], mesogenic (MDT 60–90 h), lentogenic (MDT >90 h) and avirulent (does not kill the embryos) (Beared and Hanson, 1984). The underlying molecular mechanism behind this variable level of pathogenicity lies in the amino acid sequence motif present in the protease cleavage site of the precursor fusion protein (F0), and subsequent abilities of the cellular proteases to cleave this F0 protein. For the successful infection and replication of NDV in host cells, the F0 precursor glycoprotein has to be cleaved into F1 and F2. The amino acid sequence for the velogenic and mesogenic NDV strains is ^{112}R/K-R-Q-R/K-R↓F^{117}, which is cleavable by a wide range of proteases, resulting in systemic infection. The sequence

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for lentogenic strains is ^{112}G/E-K/R-Q-G/E-R↓L^{117}, and is cleaved by trypsin-like proteases present only in respiratory and gastrointestinal systems (de Leeuw et al., 2005; Miller et al., 2010; Panda et al., 2004; Maminiaina et al., 2010).

Based on phylogenetic analysis of the nucleotide sequence of the F gene, NDV strains can be classified into six lineages designated as 1–6. Each lineage can further be subdivided into sublineages (Aldous et al., 2003). Using an alternative method, the NDV strains can be classified into one of two classes, based on size of the genome where class I carries strains of NDV with large genomes (15198 nt), which are avirulent in chickens, and class II strains with shorter genomes which carry lentogenic, mesogenic and velogenic strains of NDV. Class II viruses can then be divided into 11 genotypes (I–XI) again based on the partial sequence of the F gene, and genotypes VI and VII, which are genetically diverse are further classified into eight (a–h) and five (a–e) subgenotypes, respectively (Aldous et al., 2003; Herczeg et al., 1999).

ND is endemic in Pakistan, and as such associated with large economical losses for the national poultry sector. After textile, the poultry industry is biggest commercial sector in the country and contributes considerably to the national economy. In addition, Pakistan has a very large and locally very important domestic poultry sector and it has been estimated that every rural family and every fifth urban family breed poultry (Sadiq, 2004). In the past few decades, implementation of extensive vaccination programs in commercial poultry farms, and to some extent in small rural poultry farms have reduced the number of epizootics outbreaks of ND in Pakistan. However, instead the disease appears in an endemic form throughout the year. This has raised concerns regarding the efficacy of implemented vaccination programmes; are the apparent vaccination failures due to incompatibility between field and vaccine strains, or due to inappropriate vaccination procedures, or to generation of novel genotypes under high immune pressure (Miller et al., 2009). The role of backyard poultry in this epizootiological situation of ND in the country is also unclear.

To understand this situation in regards to vaccine failure and the role of backyard versus commercial poultry in the endemicity of ND, this study attempts a comparison of NDV strains circulating in backyard and commercial poultry industries in Pakistan. To this end, samples from outbreaks in six commercial poultry farms and from two healthy backyard poultry flocks having history of personnel contacts with the commercial farms, were included. Isolates from both poultry sectors were biologically characterized by MDT and ICPI and genetically characterized by the sequence analysis of NP, F, HN and M genes.

2. Materials and methods

2.1. Sample collection, virus isolation and titration

The six isolates reported in this study were obtained from different outbreaks (June to December 2010) in commercial poultry farms, in and around the areas of the capital (sample collection was permitted on the condition to not to disclose the name of the farm, hence only city names are mentioned in Table 1). Upon reports from local authorities, several cloacal and tracheal swabs and blood samples from different live birds were collected along with complete history and postmortem examination. Likewise, samples were collected from two healthy backyard poultry flocks from the homes of attendants working at these commercial poultry farms with frequent visits to and from the farms.

Initially, samples were inoculated in 10-day-old specific pathogen free (SPF) embryonated chicken eggs. The isolates were identified by standard hemagglutination inhibition (HI) tests using specific antisera to the reference strains of NDV (avian paramyxovirus type I). Allantoic fluids from the sample showing high HI titers were divided into working stocks and stored at −20 °C. These allantoic fluids were used as working stocks for pathogenicity assessments and for sequence analysis. For genome detection and characterization, allantoic fluids from each outbreak were stored on QIAcard FTA Indicator Four Spots (Qiagen, Hilden, Germany), which preserve nucleic acids and inactivate the virus. The samples were shipped at ambient temperature from Pakistan to the Department of Biomedical Sciences and Veterinary Public Health at the Swedish University of Agricultural Sciences (SLU, Uppsala, Sweden), for processing.

2.2. Pathogenicity assessments

To assess the pathogenicity of the viruses, mean death time (MDT) and intracerebral pathogenicity index (ICPI) were performed from representative isolates of each outbreak. For MDT, the allantoic fluid carrying virus was 10-fold diluted in PBS (pH 7.2) for inoculations into embryonated chicken eggs. The MDT induced by minimal lethal dose was determined by the procedure described before (Alexander, 2003; OIE, 2004). The isolates with MDT of up to 60 h, from 61 to 90 h and more than 90 h were designated as velogenic, mesogenic and lentogenic, respectively. ICPI was performed in ten 1-day old chicks by inoculation of 50 μl of allantoic fluid with HA titer of more than 16 HA units/50 μl and diluted 10-fold in PBS without antibiotics. The birds were kept under observation for 1 week and examined daily. The virus isolates that scored an ICPI <0.7 were declared as lentogenic and those with an ICPI >1.5 were considered as velogenic strains of NDV. The NDV strains with intermediate ICPI values were designated as mesogenic (Alexander, 2003; OIE, 2004).

2.3. Screening of the samples using real-time PCR

The RNA was extracted from Qiacard FTA Indicator (Qiagen) impregnated with allantoic fluid as recommended by the manufacturer (preparation of isolated RNA from FTA_Cards, Rev 1 10/17/07; Whatman, Hilden, Germany) with the following modifications. Using a 2.0-mm-diameter Harris micropunch (Whatman), one punch for each sample was removed according to manufacturer's protocol (BD09; Whatman) and placed in separate 1.5 ml micro-

Table 1
History, pathogenicity and cleavage site of the samples included in this study.

Sample name	Age of birds	Number of birds at farm	Location	Date of outbreak/collection	HA titer	MDT (h)	ICPI	Cleavage site
Chicken/CP1/Islamabad/2010	16 days	17000	Islamabad	15-12-2010	1024	53.6	1.5	^{112}RRQKR↓F^{117}
Chicken/CP2/Islamabad/2010	1 year	5000	Islamabad	03-11-2010	256	44.6	1.6	^{112}RRQKR↓F^{117}
Chicken/CP3/Islamabad/2010	100 weeks	4000	Islamabad	12-10-2010	1024	46.4	1.72	^{112}RRQKR↓F^{117}
Chicken/CP/Attock/2010	18 days	3000	Attock	20-09-2010	1024	53.6	1.75	^{112}RRQKR↓F^{117}
Chicken/CP1/Rawalpindi/2010	22 days	28000	Rawalpindi	24-08-2010	1024	45.6	1.72	^{112}RRQKR↓F^{117}
Chicken/CP2/Rawalpindi/2010	9 weeks	3350	Rawalpindi	01-06-2010	512	52.8	1.67	^{112}RRQKR↓F^{117}
Chicken/BYP/Lahore/2010	6 months	18	Lahore	02-07-2010	256	49.6	1.5	^{112}RRQKR↓F^{117}
Chicken/BYP/Rawalpindi/2010	3 months	07	Rawalpindi	02-08-2010	1024	52.0	1.5	^{112}RRQKR↓F^{117}

fuge tubes; 200 μl Tris–EDTA buffer (10 mm Tris–HCl, pH 8.0, 0.1 mm EDTA) was used instead of RNA processing buffer and incubated for 15 min (flicking tubes three times over the course of incubation) on ice. The detection of nucleic acid for NDV was performed using real-time PCR for M and F genes, as described (Wise et al., 2004). The reaction was carried out in a Rotor-Gene 6000 real-time analyzer (Qiagen).

2.4. PCR amplification, TOPO cloning and sequencing

One sample from each outbreak and backyard poultry flocks was selected for sequence analysis. The degenerate primers for the amplification of complete NP, F, M and HN gene were designed as previously described (Munir et al., 2011a, 2010) (see Supplementary Table 1). A 3 l of eluted RNA extract (the same as used in real-time PCR) was supplemented in a 25 μl reaction of One-Step RT-PCR kit (Qiagen) for the amplification of F, M, HN and NP genes (Munir et al., 2010, 2011b). The amplified PCR products were gel extracted and cloned in pCR3.0 using TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. At least three recombinant plasmids were processed with Miniprep (Promega, Madison, WI, USA) for DNA extraction and processed for sequencing using ABI PRISM BigDye Terminator version 3.1 (Applied Biosystems), according to the manufacturer's instructions. Sequences were analyzed with an automated nucleic acid analyzer (ABI PRISM 3100; Applied Biosystems). Each DNA fragment was sequenced at least twice in both directions. Sequence assembly and editing were performed using the SEQMAN program from DNASTAR Lasergene suite 9 (version 9.0.4 39; DNASTAR, Inc., Madison, WI, USA). All the sequences used in this study were submitted to GenBank under accession numbers JN682184–JN682191 (F genes), JN682192–JN682199 (M genes), JN682200–JN682207 (HN genes) and JN682208–JN682209 (NP genes).

2.5. Recombination among NDV sequences

To detect putative recombination breakpoints in the NDV genes and to identify sequences possibly originated from a recombination event, six methods implemented in the RDP v3 program (Martin et al., 2010) were evaluated (RDP, GeneConv, BootScan, MaxChi, Chimaera and SiScan). The six methods used the following general settings: window size = 20, highest acceptable *P*-value = 0.001 and Bonferroni correction. Only putative recombination events detected by more than one method were considered.

2.6. Phylogenetic analysis

To determine the phylogenetic relationships between APMV-1 viruses previously characterized from Asia and other parts of the world, the sequences of the 373 bases at the 3′ hypervariable region in the F gene were compared to the corresponding region of NDV strains available in the GenBank (http://www.ncbi.nlm.nih.gov/), for each known genotype (Aldous et al., 2003; Cattoli et al., 2010). All the sequences were aligned in BioEdit version 5.0.6 (Hall, 1999) using ClustalW and cut to equal length. A phylogenetic tree was then constructed using Bayesian Inference with the program MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). Two independent Monte Carlo Markov (MCM) chains were executed and sampled every 1000 generations using the default parameters of the priors' panel. Once chains reached convergence (standard deviation values below 0.01), four million additional generations of the MCM were run. Trees saved in this last step were used to construct a majority rule consensus tree. The analysis was based on the GTR + I + G model, which allow significantly changed posterior probability estimates. The nomenclature, based on lineages, was used in this study as described by Aldous et al. (2003).

To confirm the genetic pattern demonstrated in tree based on partial sequences of the F genes, the complete sequences for F, HN and M genes were determined and trees were constructed using the neighbor-joining method (Kimura 2 parameter) with 2000 bootstrap replicates in MEGA4 software (CEMI, Tempe, AZ, USA) (Tamura et al., 2007). Neighbor-joining (NJ), maximum likelihood (ML) and Bayesian analysis were applied for the tree construction. However, the tree of only one approach was shown. NJ is one of the distance-based methods and has been advocated for analysis of large datasets (Tamura et al., 2007). ML is a character-based method that has been used for phylogenetic analysis and the most probable tree is found by an optimality criterion based on the character (nucleotide) at each position of a set of sequences. The Bayesian approach has been recently developed for inferring phylogeny and has received rapid acceptance in phylogenetic studies (Alfaro and Holder, 2006). In contrast to the traditional ML method that only gives the topology of a tree, the Bayesian analysis produces both a tree estimate and a measurement of uncertainty for the groups on the tree, thus providing a measure of support faster than ML bootstrapping. The NP sequence alignment was created by graphic view option in BioEdit version 5.0.6 and processed in Adobe Illustrator CS5 (version 15.0.0).

2.7. Pairwise sequence comparisons (PASC) and distance estimations

In order to test the reliability of the lineages defined for NDV, PAirwise Sequence Comparisons (PASC) were performed using 509 F gene sequences of all the lineages of NDV strains available in GenBank. PASC is a widely accepted method in virology (Biagini et al., 1999), which is based on the histogram of pairwise differences among sequences, also known as mismatch distribution (Rogers and Harpending, 1992). Mean distances among and within lineages were calculated using PASC in MEGA4 software.

3. Results

The Qiacard FTA Indicator (Qiagen) functioned as an appropriate sampling system for this study, and all allantoic fluid samples tested positive using the F gene based real-time PCR, with Ct values $\leqslant$15 indicating high viral load. On the other hand, a USDA validated real-time PCR based on the M gene failed to detect even a single PAK isolates, as described previously (Khan et al., 2010). Using the small volume of eluted RNA (2 μl), we were also able to amplify the complete NP, F, HN and M genes using conventional PCR.

3.1. Biological characterization of the viruses

The eight viral isolates from commercial (CP) and backyard poultry (BYP) are listed in Table 1. First, we attempted to evaluate the biological properties of these isolates using hemagglutination (HA) activity and *in vivo* pathogenicity tests. The HA titers of eight chicken isolates were determined against chicken RBCs in V-form microtitration plates. All the isolates had an HA titer higher than or equal to 256 HA units/50 μl (Table 1). Five isolates showed HA activity with HA titer of 1024 HA units/50 μl, two isolates showed HA titer of 256 HA units/50 μl and one isolate showed HA activity with an HA titer of 512 HA units/50 μl. All the isolates exhibited MDT of less than 60 h and ICPI of $\geqslant$1.5, both these characteristics are typical for velogenic NDVs (Table 1). Notably, two isolates named as Chicken/BYP/Lahore/2010 and Chicken/BYP/Rawalpindi/2010, initially isolated from healthy flocks were shown to be virulent for chickens by both *in vivo* pathogenicity tests (MDT of <60 h and ICPI of 1.5).

3.2. Recombination among NDV sequences

Evidence of recombination among poultry and ostrich NDV has been reported (Miller et al., 2009; Qin et al., 2008; Yin et al., 2011). Recombination analyses performed on the F genes of the NDV isolates belonging to all six lineages and PAK strains, did not demonstrate any recombination events among PAK strains or the sequences downloaded from GenBank (data not shown), and has been used for phylogenetic inference.

3.3. Phylogenetic relationship and PASC analysis

The Bayesian consensus tree based on 509 NDV sequences, described by Aldous et al. (2003) and Cattoli et al. (2010), identified the six lineages and the newly proposed seventh lineage (Fig. 1). The PAK strains are grouped together and clustered within lineage 5, close to the newly proposed lineage 7. The PAK strains sequenced in this study showed nucleotide divergence of 5.8%, whereas the reported lineage 7 showed nucleotide divergence of 4.7% when compared to lineage 5 (Table 2). Furthermore, the PAK strains showed a maximum of 30.4% genetic divergence to lineage 6, which is classified as Class I, according to the alternative method of classification of NDV strains (Czegledi et al., 2006; Lomniczi et al., 1998).

The PASC analysis identified several cut-off values when F genes were compared (Fig. 2A). The lowest percentage identity (PI) reported (<70%) are those between the lineage 6 (Class I) and the remaining lineages (Class II). Between 81% and 88% PI fall several cut-off values that differentiate the Class II lineages. However, it is not possible to define a single cut-off value, which is valid for all the lineages. As expected, applying this range criterion for lineage definition all the strains of NDV can clearly be differentiated into six lineages with an exception of lineage 3 which appeared to be a polygenic branch and can be divided into different lineages (Fig. 2B), as has been divided into several subgenotypes (in an alternative classification system) (Maminiaina et al., 2010). A higher resolution of the phylogenetic tree revealed that the PAK strains of NDV clustered distinctly within lineage 5 (Fig. 2B). Notably, the sequences presented in this study and previously reported as a new lineage (lineage 7; Cattoli et al., 2010) showed significantly lower genetic divergence and hence should not be considered a lineage. Moreover, the novel NDV strains (genotype VIII–XI) described after 2003 in the Aldous nomenclature were grouped in lineage 3, more specifically in lineage 3d–3g, respectively.

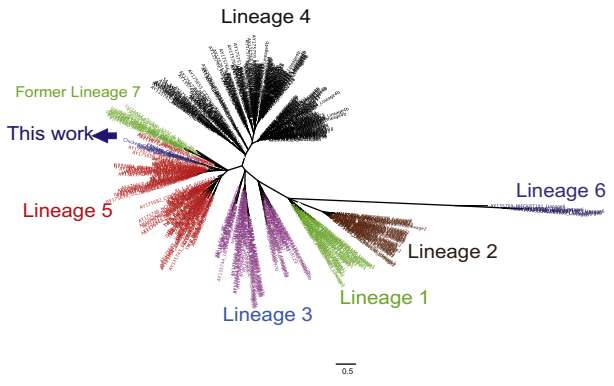

Fig. 1. A Bayesian tree analysis of the 373 bases at the 3′ hypervariable region in the fusion genes NDV isolates belonging to six lineages identified by Aldous et al. (2003) and former novel lineage (lineage 7) reported by Cattoli et al. (2010). To differentiate each lineage, different color were used. Isolates presented in this study are colored blue (this work) between lineage 5 (red) and former lineage 7 (green). The scale indicates the number of substitutions per site. Trees based on neighbor-joining and maximum likelihood approaches shown same branching pattern. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Pairwise sequence comparison of the F gene sequences belonging to all the lineages.

	p-Distance (%)							
	1	2	3	4	5	6	7	5i
Lineage 1	0							
Lineage 2	8.3	0						
Lineage 3	6.2	10.5	0					
Lineage 4	9.3	13.0	4.5	0				
Lineage 5	10.0	14.2	5.1	5.5	0			
Lineage 6	27.2	29.8	26.0	27.6	27.6	0		
Former lineage 7	11.4	14.3	6.7	8.5	4.7	27.7	0	
Sublineage 5i	14.1	16.9	8.3	9.3	5.8	30.4	8.7	0

Using MEGA4 programme the data was generated and the values indicate % nucleotide sequence distances.

In consistency with the phylogenetic tree pattern, PASC analysis of the F gene sequences of the NDV strains within lineage 5 identified a single and clear cut-off percentage identity of 95% for sublineage differentiation (Fig. 3A). Since the under-study isolates fell into lineage 5, the PASC analysis was applied to the F gene sequences of the NDV strains within lineage 5. The analysis of the genetic divergence within lineage 5 indicated that PAK strains show a genetic distance of 9.3% from previously characterized lineage 7 (Table 3). The nucleotide divergence of PAK strains when compared with sublineages 5a and 5d was higher than that of lineage 7; lineage 7 was more divergent when compared with 5b, and similar distance estimations were reported when PAK and lineage 7 were compared with sublineage 5c.

In consistency with the PASC analysis, the phylogenetic analysis within lineage 5 (Fig. 3B) showed that PAK strains, characterized in this study or reported previously, clustered distinct enough to constitute a new sublineage, proposed here as sublineage 5i. The sequences presented before as a separate lineage (Cattoli et al., 2010) showed a percentage identity with the remaining sequences of lineage 5 as high as that of the PAK strains, and it would consequently be more appropriate to designate them as four new sublineages (5e–5h), instead of a separate lineage. Following PASC evaluation, the sublineages within lineage 5 could provisionally be named as 5a, 5b, 5c (previously defined), 5e, 5f, 5g, 5h (former lineage 7) and 5i (this work) (Fig. 3A).

3.4. Nucleotide analysis of F, M, and HN genes

To confirm the robustness of the genetic groupings and topology of the phylogenetic tree obtained by the partial F gene sequences, phylogenetic analysis was conducted based on the complete open reading frames of F, M and HN genes (Fig. 4A–C). The tree was constructed using selective sequences representing all lineages and for parallel comparison of F, M and HN genes, only complete genomes were chosen for analysis. Based on the neighbor-joining tree, it was possible to confirm that PAK strains of NDV, characterized in this study or reported previously, clustered apart to be considered a new sublineage and the branching pattern remained comparable on F M and HN genes analysis (Fig. 4A–C). Since HN gene sequences for previously characterized Pakistani isolates were not available, the comparison of all PAK strains was not possible (Fig. 4C).

Among the F, M and HN gene sequences, the HN gene showed highest nucleotide similarity (99.2–100%) between PAK strains, followed by M gene (98.5–100%) and F gene (97.1–100%). Moreover, there were four mismatches in the probe-binding site whereas sequence at the forward and reverse primer binding sites showed two and three nucleotide mismatches, respectively. Since

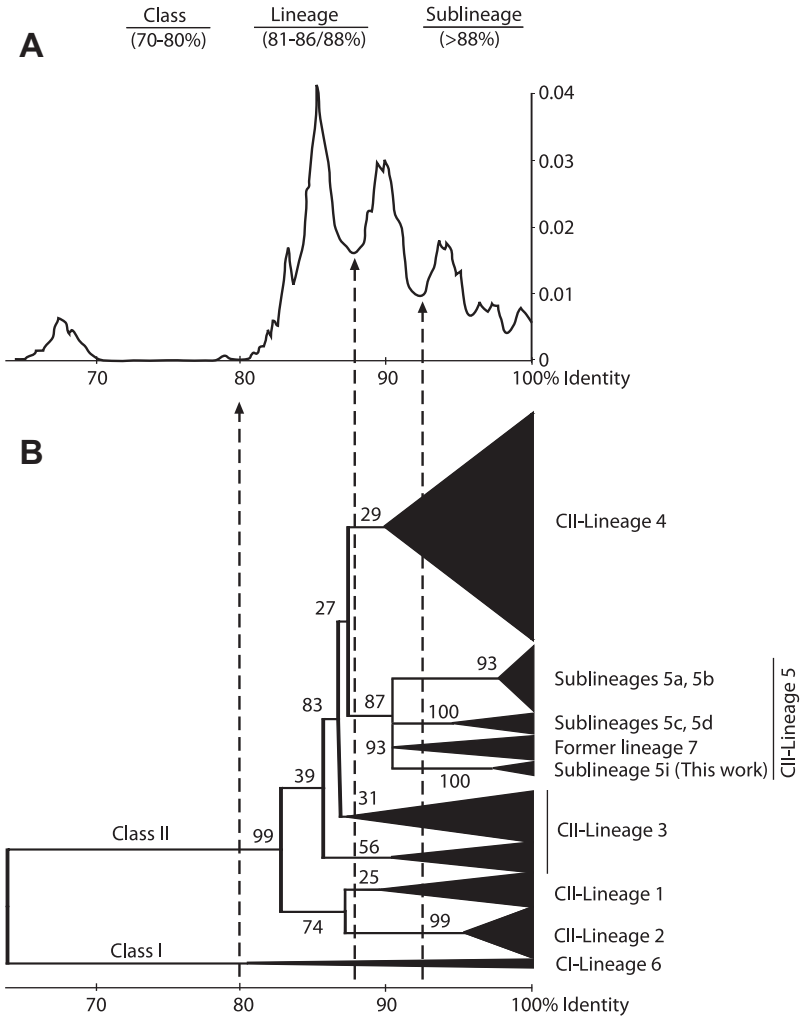

Fig. 2. Frequency distribution of pairwise distance between all the lineages using F gene sequences as demonstrated in Fig. 1. (A) The cut-off values for class, lineage and sublineage differentiation appeared to be 70–80%, 81–86/88% and >88%, marked with up head arrows. The sequences in the current study (this work) and former lineage (lineage 7) are presented. (B) Simplified tree deduced from the comparison of F gene sequences belonging to all the six lineages. For clarification, the larger sequences in each lineage were grouped.

the mismatches were located at the 5′ end of the site, which is crucial for binding, it is likely that lack of probe binding failed to generate fluorescent signals in a validated real-time PCR. However, the primers seemed to tolerate the mismatch and amplified the product as seen on the electrophoresis gel (data not shown). The analysis of the non-synonymous to synonymous substitution (d*N*/d*S*) was performed among PAK strains and other full sequences of APMV-1 of all lineages (1–6). Ratios of 2.18, 0.38 and 0.31 were observed for F, HN and M genes, respectively. This indicates that the F gene is under positive selection, and HN and M gene are under negative pressure. All PAK strains have an insertion of six nucleotides in the 5′ noncoding region of the NP gene (Czegledi et al., 2002). With this insertion, the genome length increase to 15192 nucleotides and this character is considered typical for the recent lineages (Fig. 5).

3.5. Analysis of the amino-acid sequences of F protein

Proteolytic cleavage site motifs (amino acids 112–117) for the F0 protein of the eight isolates were analyzed. The pathogenicity prediction, based on the cleavage site of the fusion protein, indicated that all the isolates were to be considered velogenic with the motif ^{112}RRQKR↓F^{117} (Table 1). Of particular interest, two NDV strains isolated from apparently healthy flocks of local breeds were also shown to have the same motif associated to virulent strains of NDV (^{112}RRQKR↓F^{117}). This motif is generally identified in the strains of NDV that are highly virulent in chickens.

Comparison of the predicted amino acid sequences of the complete F gene showed that the seven neutralizing epitopes (D^{72}, E^{74}, A^{75}, K^{78}, A^{79}, L^{343}), believed to be critical for structure and function of F protein, were conserved and identical in six PAK isolates except for a E74Q substitution. Another stretch of the sequence (^{151}ILRLKESIAATNEAVHEVTDG^{171}) with similar functionality was also seen conserved in PAK strains of NDV (Liu et al., 2003; Toyoda et al., 1987; Yusoff et al., 1989). The potential N-glycosylation sites in F-glycoprotein (Asn-X-Ser/Thr or N-X-S/T, where X present any amino acid except aspartic acid or proline) were found at position ^{85}NRT^{87}, ^{191}NNT^{193}, ^{366}NTS^{368}, ^{447}NIS^{449}, ^{471}NNS^{473} and ^{541}NNT^{543} in all the PAK strains. Comparison of the complete F gene sequences among all the lineages showed that the PAK strains shared highest nucleotide and predicted amino acid (95.7% and 91.3%) with lineage 5 whereas lowest (86.5% and 90.9%) with lineage 1.

3.6. Analysis of the amino-acid sequences of HN protein

The HN protein of NDV strains exists in different amino acid lengths: 571, 577, 581, and 616 (Munir et al., 2010; Romer-Oberdorfer et al., 2003). The HN protein in PAK strains composed

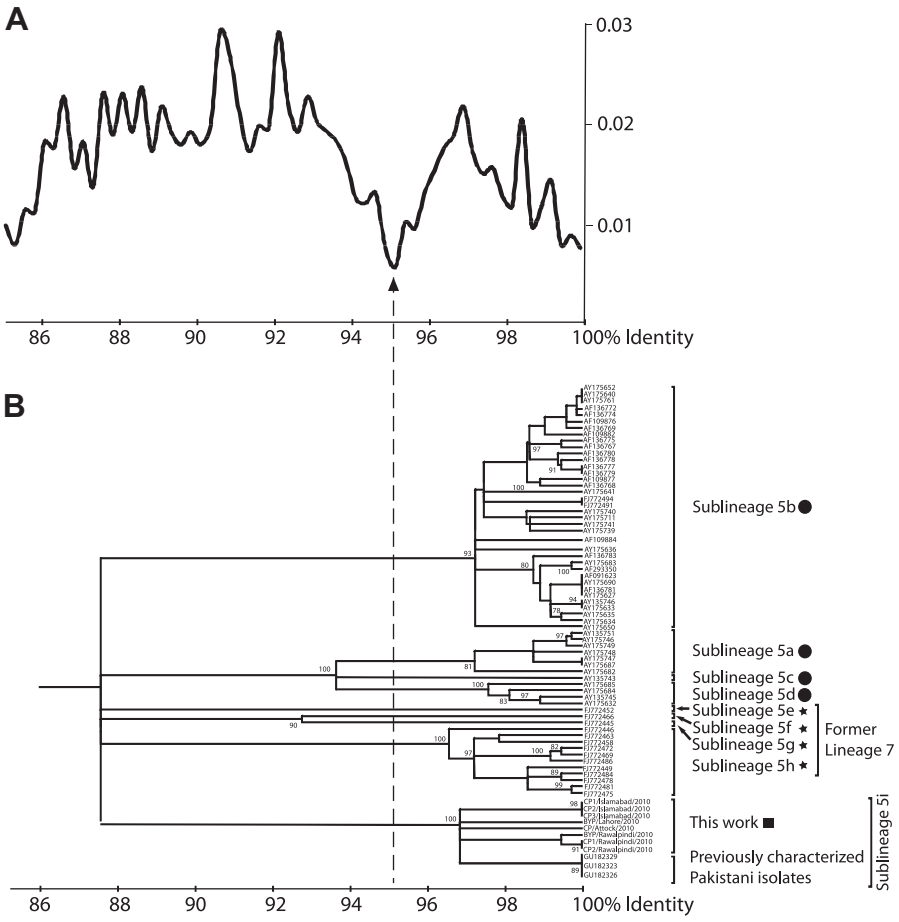

Fig. 3. Clustering pattern and frequency distribution of pairwise distance between the sequences belonging to lineage 5. (A) Isolates belonging to distinct sublineages based on the genetic distance more than 95%, marked with dotted up head arrow. (B) Simplified tree deduced from the comparison of F gene sequences belonging to lineage 5. The distinct sublineages in current study (this work) are marked with black square (■) and former lineage (lineage 7) is marked with black star (✱). The previously characterized NDV strains are labeled with black circle (●).

Table 3
Pairwise sequence comparison of the F gene sequences belonging to all the sublineages within lineage 5.

	p-Distance (%)					
Group	5a	5b	5c	5d	7	5i
Sublineage 5a	0					
Sublineage 5b	5.3	0				
Sublineage 5c	5.2	7.5	0			
Sublineage 5d	4.7	7.6	5.5	0		
Former lineage 7	6.9	6.6	10.0	8.9	0	
Sublineage 5i	8.9	5.9	10.0	10.2	9.3	0

Using MEGA4 programme the data was generated and the values indicate % nucleotide sequence distances.

of 571 amino acids, which is a feature of virulent NDV strains (Maminiaina et al., 2010). The complete HN gene of PAK strains was aligned with NDV strains pertaining to all the lineages with variable lengths. The results showed that the HN protein of PAK strains carry highest nucleotide and amino acid similarity (89.4% and 93.5%) with lineage 5 whereas lowest nucleotide (84.3%) and amino acid (88.8%) with lineages 3 and 2, respectively. The results also showed that the previously described neutralizing epitope ^{193}LSGC**K**DHSH^{201} (with R197K substitution in PAK strains), ^{263}R (with R263K substitution in PAK strains), ^{287}D, ^{321}K, ^{332}G**R**^{333} (with R333K substitution in PAK strains), ^{346}D**D**QDYQI**Q**^{353} (with E347D and R353Q substitutions in PAK strains), ^{356}K, ^{481}N, ^{494}D, ^{513}R**V**TRVSSSS^{521} (with I514V substitution in PAK strains), ^{569}E (with G/D569E substitution) remained the same in all the PAK strain analyzed here including the isolates from healthy backyard poultry flocks. Three amino acid, ^{401}E, ^{416}R and ^{526}Y, essential for receptor binding to the host are not modified in PAK strains of NDV. The amino acids recognized as essential for the neuraminidase activity, ^{401}E, ^{416}R, ^{526}Y which are characterized by functional triarginyl cluster remained same for all PAK strains isolated from commercial poultry as described for other NDV strains. Notably, a Y526Q substitution was observed only in the isolates from backyard poultry. It has been demonstrated that mutation at this position leads to reduce the neuraminidase, receptor binding, and fusion activities of NDV and subsequently caused attenuation of virus *in vivo* (Khattar et al., 2009). There are three regions within HN protein, which are responsible for the hemagglutinating activity: ^{234}NRKSCSV/I/L^{240}, ^{314}FPVYGGL/V/M^{320} and ^{399}GAEGRIL/V/I^{405}, which remained identical to other NDV strains. The N-glycosylation sites at residues 119, 341, 433, 481 and 508 are conserved within APMV-1. The PAK strains contain all the glycosylation sites except at position 119 and contained an additional site at position 538. However, studies have reported that position 508 and 538 are not true glycosylation sites (McGinnes and Morrison, 1995) despite the additional site has already been reported common among novel strains of NDV (Cattoli et al., 2010).

4. Discussion

Newcastle disease is one of the most devastating diseases in poultry in many Asian countries. In 2010 only, 25 Asian countries

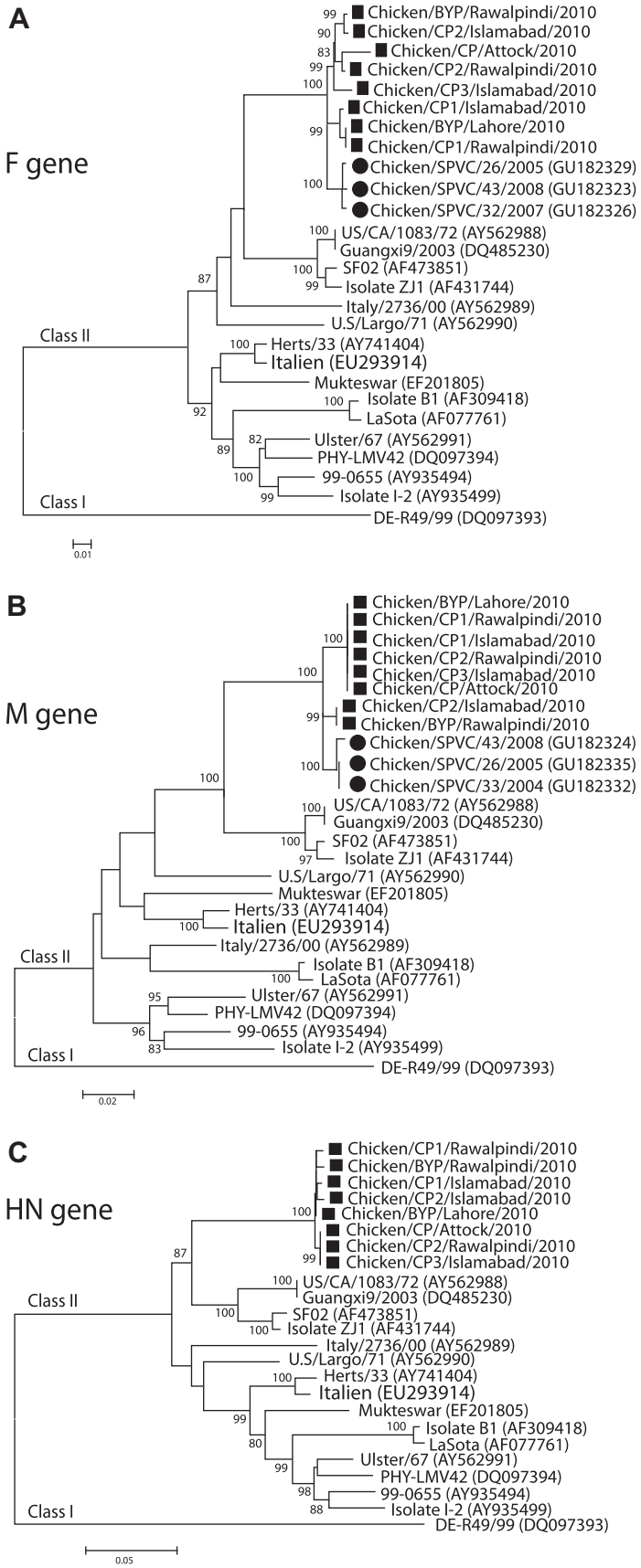

Fig. 4. The neighbor-joining phylogenetic trees constructed based on the complete coding region of the F gene (A), M gene (B) and HN gene (C) and compared with the representative of each lineage. The scale indicates the number of substitutions per site. The sequences used in this study are labeled with black square (■), whereas previously characterized Pakistani NDV strains are labeled with black circle (●). Trees based on Bayesian and maximum likelihood approaches shown same branching pattern.

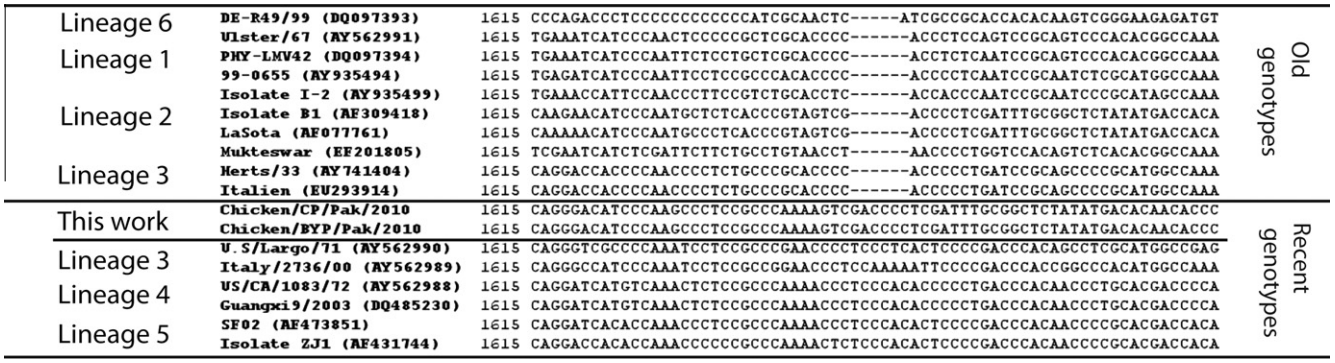

Fig. 5. An alignment of the NP genes from representative of each lineage where insertion of six nucleotides are shown in the 5′ non-coding region. Due to sequence identity, only one representative isolate from commercial and backyard poultry is shown (this work).

notified 3998 outbreaks of ND to the World Organization for Animal Health (OIE-WAHID interface, available at: http://www.oie.int/wahis/public.php, accessed on 7th September 2011). Among south Asian countries, the highest number of outbreaks was reported from India (n = 497) and Iran (n = 904), both bordering Pakistan. A large number of ND outbreaks (n = 561) were also reported from China. Pakistan shares not only borders with China, but also has huge ongoing free bilateral trade. By these facts, it is clear that ND is widespread in the region. Unfortunately, very limited information is available on NDV in Pakistan, and no outbreak was reported to OIE neither in 2010 nor in 2011 so far. However, the disease is endemic in Pakistan since its first appearance in 1926 in Southeast Asia and is continuously posing economical threat to the poultry sector (Alexander, 2003). Strategically, Pakistan is located in a position between the important regions of South Asia, Central Asia and the greater Middle East. Hence, it is of importance to characterize the viruses circulating in this part of the continent.

In the present study, PAK strains of NDV were characterized at the molecular level. The salient findings of this work include the strong evidence for the existence of a distinct genetic group, which may have implication on disease diagnosis and control by vaccination. Recently, a study of similar nature has proposed the emergence of a novel lineage (lineage 7) in West and Central Africa (Cattoli et al., 2010). The PASC analysis, a useful method to plot the frequency distribution of pairwise nucleotide sequence differences, was employed to ascertain the criteria for sublineage classification (Bao et al., 2008). Based on the genetic divergence of the F gene fragment in this analysis, the percentage of maximum intralineage nucleotide variations within former lineage 7 was lower than the nucleotide distance found between PAK strains and lineage 5. This analysis clearly demonstrated that PAK strains and former lineage 7 are closely related to lineage 5, and that these are not distinct enough to be regarded as new lineages. The classification of the NDV strains reported by Cattoli et al. (2010) as new lineage would make any attempt to define lineages on the basis of genetic distances in this genomic region more difficult. Moreover, this would lead to emergence of countless new lineages out of lineage 5, which would complicate the classification of NDV. However, the clear branching of lineage 5, including former lineage 7 and PAK strains (Fig. 3A) and the PASC analysis (Fig. 3B), clearly suggests that these isolates belong to a new sublineage within lineage 5. The cluster topology of the PAK strains based on the partial sequence of the F gene was further confirmed by the phylogenetic analysis based on full-length sequences of F, HN and M genes and their comparison with the representative of each lineage. The Pakistani NDV isolates, previously characterized from Karachi (in the other side of the country), also grouped distinctly with the isolates of this study, thereby confirming the continuous existence of this genetic cluster of NDV (Khan et al., 2010). It is to mention that the tree construction methods for the isolates presented here and reported in Cattoli et al. (2010) was the same. Therefore, it is likely that the characterization of the novel NDV isolates after 2010 has filled the gap between isolates of Cattoli et al. (2010) and lineage 5. It is clear to observe in Fig. 1 after presenting the isolates in this study. However, some of newly identified strains of NDV clustered in the lineage 3, which make it polygenic group and therefore demand an urgent need of setting a definite criterion for the classification of NDV strains.

The sequence analysis of the NP gene revealed the insertion of six nucleotides in the 5′ non-coding region of the NP gene of PAK isolates, which is considered characteristic for the recent genotypes (Maminiaina et al., 2010). In addition, PAK strains carry a molecular substitution (E104G) in the F protein, which is considered characteristics for the old genotypes. Yu et al. (2001), have speculated that the $^{104}E \rightarrow G$ substitution causes conformational changes in the F gene, and triggers the emergence of recent lineages from the old lineages by the process of evolution. In contrast, arginine at position 114 (^{114}R) and V to I substitution ($^{118}V \rightarrow I$) at position 118, are only described in recent lineages. However, these changes were missing in PAK strains of NDV investigated in this study.

All the PAK strains show an identical motif in the cleavage site of the F protein ($^{112}RRQKR\downarrow F^{117}$) including the isolates from healthy backyard poultry flocks, which is typical for virulent strains of NDV. Previously, it has been described that the phenylalanine at position 117 ($\downarrow F^{117}$) is associated with the neurological form of the disease (Kattenbelt et al., 2006). Complimentary to this, the length of the HN protein (571 aa) is also characteristic for virulent strains of NDV (OIE, 2004; Romer-Oberdorfer et al., 2003; Ujvari et al., 2003). Thereafter, the virulence prediction, based on the F protein cleavage site and the HN protein length, was confirmed by the *in vivo* pathogenicity tests, which revealed a high ICPI (1.5) and MDT (49.6–52.0). Interestingly, NDV isolates recovered from healthy and unvaccinated BYP flocks showed pathogenicity indices and of high genetic resemblance to that of virulent isolates from CP. The possibility that BYP were sampled during the incubation period or attenuation of the NDV with prior immunization with ND infection cannot be ruled-out. However, and as it has been stated, ND is endemic in Pakistan, Hence, local BYP breeds have been in contact with the disease for a long period, with chicks being infected by NDV and selected for resistance continuously. Accordingly, sampled BYP may also acquire resistance to the disease in this way, and explain why virulent isolates were isolated from diseased CP and healthy BYP.

It was previously reported that due to presence of only one single serotype for APMV-1, the genetic variability of viruses should in principle not lead to vaccine failure (Alexander et al., 1997). However, due to the differential level of cross-strain protection,

field virus infection of vaccinated birds may result in subclinical infections due to escape of immune responses, and thus provoke the emergence of novel strains (Liu et al., 2003; Qin et al., 2008). It is tempting to hypothesize that the presence of mutations in the neutralizing epitopes in the F and HN proteins of the PAK strains, targeted by the protective immune response, may lead to increase in virus shedding and emergence of escape mutants and subsequently weaken the vaccine efficacy. Similarly, a study conducted by Miller et al. (2007) demonstrated that the genetic diversity within APMV-1 compromises the efficacy of vaccine by increase in virus shedding which result in increase in virus spread, in spite of apparent protection in term of clinical signs. Currently, the LaSota strain of NDV is in use as vaccine strain in Pakistan (Numan et al., 2005; Sadiq, 2004). The high genetic and phylogenetic gaps between field and vaccine strains may also facilitate the evolution of virulent NDV strains (Miller et al., 2007). Although the origin of newly emerging NDV strains is complicated, it has been demonstrated by Czegledi et al. (2006) that this rate has increased significantly in recent years and the role of vaccination in this scenario cannot be ignored. However, this phenomenon is currently at the stage of infancy and warrants further investigations. Moreover, these novel genetic groups have a high level of PCR false-negative results, which not only have serious consequences in the detection of these viruses but may also impose a hurdle in efficacy of the vaccine (Cattoli et al., 2010; Maminiaina et al., 2010; Snoeck et al., 2009). The results in our study revealed a positive selection in the F gene, which is involved in the immune response against NDV. It is tempting to postulate that F gene may escape from the immune system of the host in contrast to two other genes analyzed (HN and M) which showed negative selection pressure.

In conclusion, analysis of the six isolates from commercial poultry farms and two isolates from backyard poultry flocks provide valuable data on characterization, epidemiology and diagnosis of NDV circulating in Pakistan. The study results thus emphasize the importance of continuous surveillance of this disease and of sharing the information to the global scientific community, which would help to fill the epidemiological gaps in the regions and to validate the robustness of diagnostic screening. It is tempting to postulate that the import of live poultry and extensive use of live vaccines in Pakistan can pose a huge risk for the emergence of novel genetic lineages by continuous evolution of APMV-1. This study also provided new information regarding the degree of variability and the classification of novel lineages into sublineages, which will not only improve the phylogenetic analysis of the NDV but also provide information to reconsider the classification of NDV strains as it has been practiced for avian influenza viruses (WHO/OIE/FAO H5N1 Evolution Working Group, 2008).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2012.02.015.

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