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Biological and genotypic characterization of the Newcastle disease virus isolated from disease outbreaks in commercial poultry farms in northern Punjab, Pakistan

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ABSTRACT

Newcastle disease (ND) is a highly contagious disease of many avian species and is particularly responsible for devastating disease outbreaks in commercial poultry flocks in Pakistan that incur huge economic losses to the national poultry industry annually. Despite implementation of an extensive vaccination program for poultry birds, the disease appears in an endemic form in commercial broiler and layer poultry farms. This study was conducted to identify the prevalent velogenic NDV strain responsible for disease outbreaks in commercial poultry farms in Punjab, Pakistan. The NDV strains isolated from pathological specimens through inoculation in embryonated chicken eggs were characterized biologically through the intracerebral pathogenicity index (ICPI), and genetically on the basis of the fusion (F) protein cleavage site. Among these, six NDV isolates showed an F protein cleavage site motif (^{112}RRQKRF^{117}) and an ICPI value ranging between 1.5 and 1.88, both are characteristics for velogenic strains of NDV. In addition, phylogenetic analysis based on a partial sequence of the F protein gene clustered these isolates within class II, genotype VII and specifically within genotype VII-e. This is the first report that demonstrated the presence of

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such NDV strains in commercial poultry farms in northern Punjab of Pakistan.

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1. Introduction

Newcastle disease (ND) is an economically important and highly infectious disease of both wild and captive birds (Saif et al., 2003). The disease manifestation in chickens occurs in three well-defined forms viz. viscerotropic velogenic (highly virulent), neurotropic velogenic (highly virulent), and mesogenic (moderate virulent). These pathotypes cause variable respiratory and neurologic signs along with high morbidity (Miller et al., 2010). ND was first identified in 1926 in Java, Indonesia, and subsequently in Newcastle Upon-Tyne, UK (Alexander, 1991). Since then, three major panzootics of ND have been recorded and it is currently present in all continents except Antarctica (Alexander, 2003).

ND is considered as a major infection of commercial poultry in Pakistan among all the viral, bacterial and parasitic diseases (Rehman et al., 2013). Although the prevalence of ND in broiler and layer birds remains higher throughout the year, it reaches its plateau during seasonal stress (January–February, June–July). During September 2011–January 2012, ND has killed 45 million chickens that resulted in a loss of approximately 65 million US $ to the poultry industry only in Punjab, Pakistan (Unknown, n.d.). However, under same climatic conditions, breeder flocks remain healthy highlighting the importance of biosecurity measures and good management exercise that are practiced at breeder farms (Rehman et al., 2013). The role of exotic and wild birds in the epizootics of ND is not fully understood; however, it is assumed that the wild birds play a critical role in the dissemination of virus from the affected poultry farms to the non-affected farms within the country. Accordingly, an outbreak of ND hit seven southern districts of the Sindh province of Pakistan resulting in the death of approximately 167 wild peacocks in just three weeks (Munir et al., 2012a).

The genome of avian paramyxovirus serotype 1 (APMV-1), causative agent of ND, is approximately 16 kb in length that encodes for nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and large RNA-dependent polymerase protein (L) (Alexander, 2003; ICTV, 2009). The virulence of NDV is dependent on various factors; however, the cleavage site of the F protein is the critical site responsible for major changes in virulence. The velogenic and mesogenic strains of NDV have a ^{112}R/K-R-Q-R/K-R↓F^{117} F protein cleavage site motif that is cleaved by a variety of proteases, resulting in systemic infection, while lentogenic strains have a ^{112}G/E-K/R-Q-G/E-R↓L^{117} motif, which is cleaved by trypsin like proteases present in respiratory and gastrointestinal systems only (Morrison, 2003). The pathogenicity of NDV can also be determined on the basis of various biological properties including mean death time (MDT) in 9–10 day old embryonated chicken eggs, intravenous pathogenicity index (IVPI) in six-week old chickens and intracerebral pathogenicity index (ICPI) in day old chickens (Alexander, 1988).

On the basis of the partial hypervariable region of the F protein gene, NDV strains are classified into two major divisions represented by class I and class II, with class I being further divided into nine (I–IX) and class II into sixteen (I–XVI) genotypes (Ballagi-Pordany et al., 1996; Kim et al., 2007; Courtney et al., 2013). Owing to their more pathogenic nature, NDV strains of class II have been studied comprehensively. Previous reports from different parts of Pakistan in commercial, backyard and wild poultry birds indicated the prevalence of multiple genotypes (Munir et al., 2012a,b,c). However, the nature of circulating NDV has not been investigated in northern Punjab, Pakistan. This is of particular interest since the disease remains endemic in these regions despite extensive vaccination. This study was conducted to ascertain the genetics of the circulating NDV that causes disease outbreaks in northern Punjab, which is considered the hub of poultry due to environmentally favorable conditions.

2. Results

2.1. Isolation and pathogenicity assessment of NDV

NDV was isolated from respective samples through inoculation of triturated tissue specimens from morbid organs of affected birds in specific pathogen free (SPF) eggs. Subsequently, biological properties of each isolate

were studied using HA and ICPI tests. Out of 35 samples, hemagglutination activity was observed in 74.3% (n = 26) of the total samples. Among these, 10 samples showed specific inhibition of agglutination when treated with ND antisera and 16 samples showed inhibition of agglutination with H9 antisera. In addition, one sample showed hemagglutination inhibition with both ND and H9 antisera that indicated the presence of mixed infection. However, none of the samples showed reactivity against the H7 or H5 strain of avian influenza virus (Table 1).

Only NDV isolates were further propagated in embryonated chicken eggs to enhance viral copy number. The complete history, initial HA titer (after 1st passage) and final HA titer (after 3rd passage) of each isolate are shown in Table 2. The hosts for these isolates include broiler, layer and desi birds of different ages reared in commercial poultry farms located at Rawat, Pindbagwal, Shamsabad, Barakoh, Mandra, ChakBeli, Chakri and Chakwal. Passaging of virus in embryonated chicken eggs resulted in an increase in the hemagglutination activity of all isolates that is an indication of an increase in viral copy number.

2.2. F protein cleavage site and bioinformatics analysis

The F protein cleavage site sequence is the major determinant of NDV virulence (Morrison, 2003). A partial region (~700 bp) of the F protein of NDV isolates that contained the cleavage site sequence was amplified from respective samples and directly sequenced at Macrogen Inc., Korea using Sanger's dideoxy chain-termination

Table 1
HA activities and mean $\log^2$ HI titers of NDV isolates used in this study. Mean $\log^2$ titers were obtained using infective allantoic fluid from isolates (as antigen) and specific antisera against Newcastle disease and Avian Influenza H9, H7 and H5.

Sample ID	HA activity	HI titers ($\log^2$)			
		ND	H9	H7	H5
Unknown-1	+	5	0	0	0
Unknown-2	+	6	0	0	0
Unknown-3	+	0	7	0	0
Unknown-4	+	7	4	0	0
Unknown-5	+	0	6	0	0
Unknown-6	−	0	0	0	0
Unknown-7	+	5	0	0	0
Unknown-8	+	0	7	0	0
Sihala-9	−	0	0	0	0
Ch.Beli-10	+	0	4	0	0
Mandra-11	−	0	0	0	0
Mandra-12	+	5	0	0	0
Barakoh-13	+	0	7	0	0
Islamabad-14	+	0	6	0	0
Rawat-15	−	0	0	0	0
Rawat-16	+	4	0	0	0
Karor-17	−	0	0	0	0
Tumair-18	+	0	6	0	0
Alipur-19	+	0	7	0	0
Ch.Beli-20	+	0	3	0	0
Pindbagwal-21	+	0	7	0	0
Alipur-22	+	0	0	0	0
Rawat-23	+	0	0	0	0
Pindbagwal-24	+	4	0	0	0
Shamsabad-25	+	0	5	0	0
Barakoh-26	+	0	7	0	0
Mandra-27	+	0	5	0	0
Ch.Beli-28	−	0	0	0	0
Chakri-29	+	6	0	0	0
Mandra-30	+	0	6	0	0
Chakwal-31	+	0	7	0	0
Islamabad-32	+	6	0	0	0
GFPMultan-33	−	0	0	0	0
Terlai-34	+	6	0	0	0
Pindbagwal-35	+	0	5	0	0

Table 2
Complete history and HA titers of each sample.

Sample ID	Type of birds	Flock size	Age of birds (days)	Average mortality per day (over last 4 days)	HA titers (after 1st passage)
Rawat-16	Broiler	30,000	25	40	64
Ch.Beli-20	Layer	2500	189	80	64
Rawat-23	Desi	10	200	3	128
Pindbagwal-24	Broiler	28,000	27	18	128
Shamsabad-25	Desi	20	56	48	32
Barakoh-26	Broiler	15,000	15	24	64
Mandra-27	Broiler	38,000	25	20	32
Chakri-29	Broiler	28	21	15	64
Mandra-30	Layer	32,000	175	100	128
Chakwal-31	Broiler	21,000	28	14	32

method. Raw sequence data was received and annotated for the deduction of the F protein cleavage site sequence of each isolate. The sequences obtained were then analyzed by using a basic local alignment tool (BLASTn). Out of 10 samples, 7 showed 99% identity with the F protein gene sequence of previously reported NDV isolates. However, 3 isolates did not show similarity with any NDV genotypes due to poor sequence quality. Processed nucleotide sequences were submitted to NCBI GenBank and are available under accession numbers from KF687959 to KF687965. The complete details of each isolate including the sequence ID, name of the isolate, F protein cleavage site sequence (amino acid residues 112–117) and ICPI value are shown in Table 3.Six isolates (NIBGE-1861 to 1869) with NCBI accession numbers (KF687959–KF687964) revealed a ^{112}RRQKRF^{117} cleavage site motif. However, one isolate (NIBGE-1881) revealed a sequence at the F protein cleavage site (^{112}GRQGRL^{117}) that is identical to the cleavage site of the LaSota vaccine strain (Fig. 1).

2.3. Phylogenetic analysis

To predict the genetic relatedness and phylogenetic distribution of these viral isolates, a phylogenetic tree was constructed. A set of sequences, representing all reported genotypes around the globe and previously reported from Pakistan, were aligned with the sequences presented in this study using the ClustalW algorithm in BioEdit. The tree was constructed using the neighbor-joining method in MEGA5 as reported earlier (Munir et al., 2012b). The tree topology indicated the distribution of 6 isolates within genotype VII (Fig. 2), specifically with subgenotype VIIe (Fig. 3), along with previously reported strains. However, one isolate clustered with the NDV isolates belonging to genotype II, where vaccine strains of NDV are predominantly grouped.

3. Discussion

Newcastle disease is one of the most prevalent viral infections of poultry birds in Pakistan (Rehman et al., 2013). NDV is a group of diverse and continuously evolving genotypes that are classified into two major classes on the basis of nucleotide sequences of the fusion (F) protein gene (Ballagi-Pordany et al., 1996; Kim et al., 2007). Currently, NDV viruses of class II, genotypes V, VI, VII and VIII are most the predominant genotypes and are causing disease outbreaks worldwide. Among these, genotype VI viruses emerged in 1960 and remained

Table 3
ICPI and F protein cleavage site sequence of NDV isolates.

Sample ID	Sequence ID	Isolates	Cleavage site sequence (112–117)	NCBI accession number	ICPI
Rawat-16	NIBGE1861	1861-Rawat	R-R-Q-K-R-F	KF687959	1.50
Pindbagwal-24	NIBGE1862	1862-Pindbagwal	R-R-Q-K-R-F	KF687960	1.88
Shamsabad-25	NIBGE1863	1863- Shamsabad	R-R-Q-K-R-F	KF687961	1.64
Ch.Beli-20	NIBGE1866	1866-Ch.Beli	R-R-Q-K-R-F	KF687962	1.83
Mandra-27	NIBGE1868	1868-Mandra	R-R-Q-K-R-F	KF687963	1.50
Chakwal-31	NIBGE1869	1869-Chakwal	R-R-Q-K-R-F	KF687964	1.62
Rawat-23	NIBGE1881	1881-Rawat	G-R-Q-G-R-L	KF687965	0.35

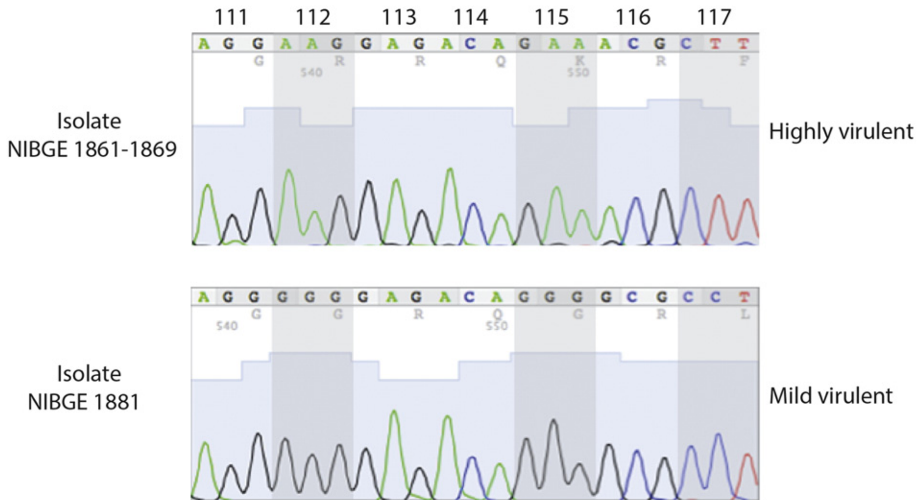


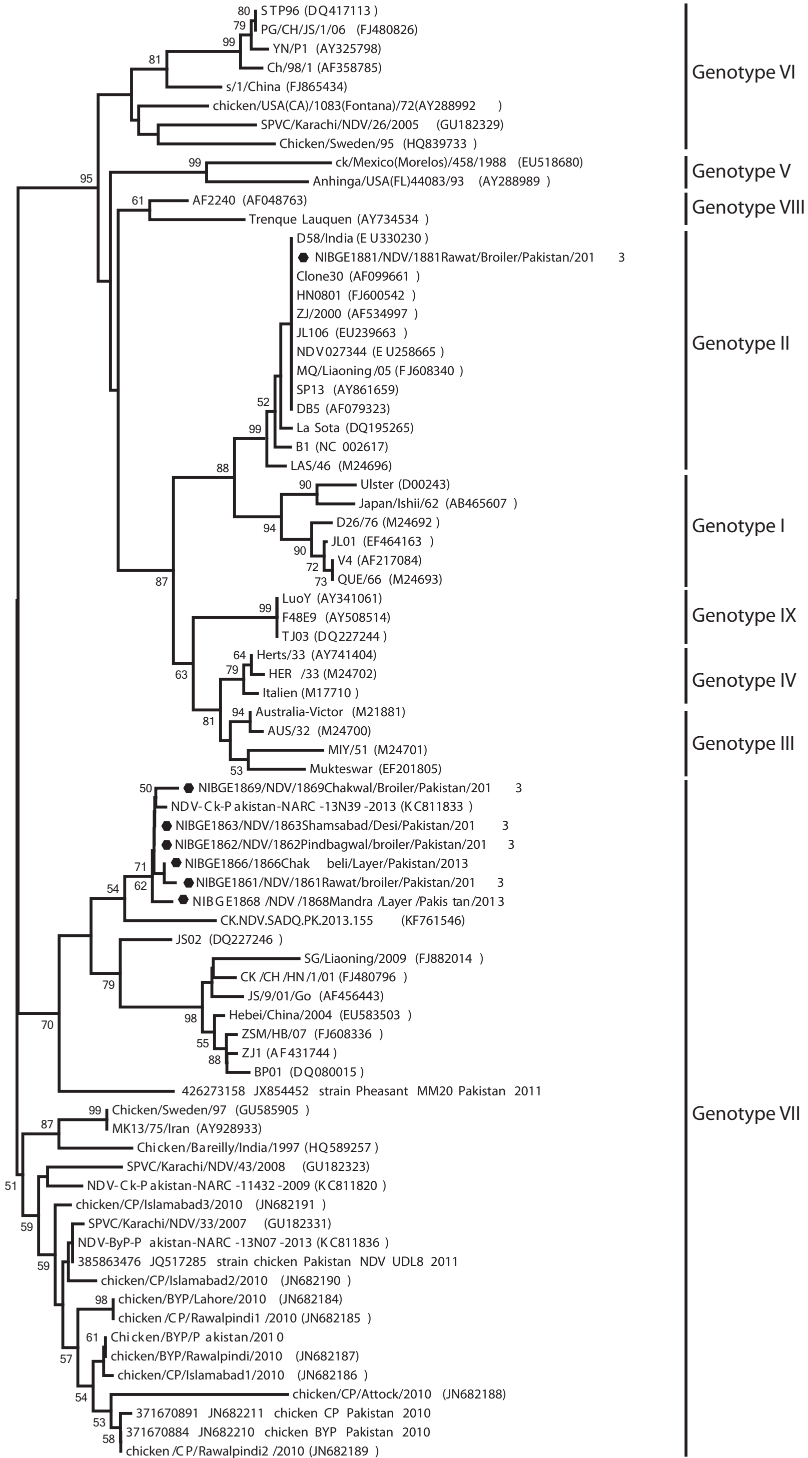
Fig. 1. Sequence analysis of NDV cleavage site. Nucleic acid that encodes F protein cleavage site of NDV isolated from different disease outbreaks in Punjab, Pakistan was subjected to DNA sequence analysis using Sanger's dideoxy chain termination method. (Upper panel) A representative F protein cleavage site sequence of highly virulent NDV that encodes amino acid residues ^{112}R-R-Q-K-R-F^{117}. (Lower panel) A representative F protein cleavage site sequence of less virulent NDV that encodes amino acid residues ^{112}G-R-Q-G-R-L^{117}.

as the most predominant viruses in Asia until 1985 (Mase et al., 2002). Subsequently, genotype VII became more prevalent in this region, which is further divided into eight subgenotypes (VIIa–VIIh). Subgenotypes, VIIa–VIIe represent isolates from China, Malaysia, Kazakhstan and Kyrgyzstan (Bogoyavlenskiy et al., 2009; Wang et al., 2006) and VIIf–VIIh represent African isolates (Snoeck et al., 2009).

In the present study, a total of 35 samples were collected during the period of December 2012 to July 2013. Keeping in view the pantropic nature of velogenic and mesogenic strains of NDV, tissue samples collected from the suspected birds included the liver, trachea, spleen and proventriculus (Krzysztof et al., 2006). The serological analysis of these samples revealed that the birds with a maximum NDV antibody titer of 4–5 $\log^2$ were not able to withstand the natural exposure of NDV (Table 1). However, HI antibody titers of $\geq 6 \log^2$ at the third week of age are considered better protection levels against subsequent viral exposure (Siddique et al., 2013). Some samples also indicated a variable HI titer against the H9 strain of avian influenza A virus. However, no antibodies were detected against either the H7 or H5 strain of AI. This was presumably due to the fact that most of the commercial flocks in Pakistan are vaccinated against AI using the H9 strain. In addition, the H9N2 genotype of AI is endemic in many Asian countries including Pakistan (Iqbal et al., 2009). As the main focus of this study was characterization of NDV, therefore, no further effort was made to characterize avian influenza virus from clinical samples.

NDV was isolated from pathological specimens using embryonated chicken eggs. Among the four types of tissue specimens, the rate of virus isolation was found to be higher in the spleen compared to those of the trachea, liver and proventriculus. Accordingly, pathological specimens from the spleen were considered the best source of virus isolation. Seven NDV isolates of this study were further characterized both biologically using ICPI, and genotypically based on the F protein cleavage site motif (amino acid residues 112–117). Biological and genotypic pathotyping revealed that six out of seven isolates (1861Rawat–1869Chakwal) are of velogenic nature. In addition, phylogenetic analysis based on the partial sequence (nucleotides 1–450) of the F protein gene clustered these isolates with class II, genotype VII and specifically with genotype VIIe isolates. The prevalence of this strain is greater in Asia specifically Indonesia and Pakistan (Xiao et al., 2012).

Fig. 2. Phylogenetic analysis of F gene of NDV isolates reported here and characterized previously.Phylogenetic tree was constructed by neighbor-joining method as implemented in MEGA5. Numbers at the nodes indicate confidence level of bootstrap analysis with 2000 replications as percentage value. Values less than 50% are not shown. Horizontal lengths are proportional to distance. Sequences reported in this study are labeled with hexagonal.


STP96 (DQ417113)
PG/CH/JS/1/06 (FJ480826)
YN/P1 (AY325798)
Ch/98/1 (AF358785)
s/1/China (FJ865434)
chicken/USA(CA)/1083(Fontana)/72(AY288992)
SPVC/Karachi/NDV/26/2005 (GU182329)
Chicken/Sweden/95 (HQ839733)
ck/Mexico(Morelos)/458/1988 (EU518680)
Anhinga/USA(FL)44083/93 (AY288989)
AF2240 (AF048763)
Trenque Lauquen (AY734534)
D58/India (EU330230)
NIBGE1881/NDV/1881Rawat/Broiler/Pakistan/2013
Clone30 (AF099661)
HN0801 (FJ600542)
ZJ/2000 (AF534997)
JL106 (EU239663)
NDV027344 (EU258665)
MQ/Liaoning/05 (FJ608340)
SP13 (AY861659)
DB5 (AF079323)
La Sota (DQ195265)
B1 (NC 002617)
LAS/46 (M24696)
Ulster (D00243)
Japan/Ishii/62 (AB465607)
D26/76 (M24692)
JL01 (EF464163)
V4 (AF217084)
QUE/66 (M24693)
LuoY (AY341061)
F48E9 (AY508514)
TJ03 (DQ227244)
Herts/33 (AY741404)
HER /33 (M24702)
Italien (M17710)
Australia-Victor (M21881)
AUS/32 (M24700)
MIY/51 (M24701)
Mukteswar (EF201805)
NIBGE1869/NDV/1869Chakwal/Broiler/Pakistan/2013
NDV-Ck-Pakistan-NARC-13N39-2013 (KC811833)
NIBGE1863/NDV/1863Shamsabad/Desi/Pakistan/2013
NIBGE1862/NDV/1862Pindbagwal/broiler/Pakistan/2013
NIBGE1866/1866Chak beli/Layer/Pakistan/2013
NIBGE1861/NDV/1861Rawat/broiler/Pakistan/2013
NIBGE1868/NDV/1868Mandra/Layer/Pakistan/2013
CK.NDV.SADQ.PK.2013.155 (KF761546)
JS02 (DQ227246)
SG/Liaoning/2009 (FJ882014)
CK/CH/HN/1/01 (FJ480796)
JS/9/01/Go (AF456443)
Hebei/China/2004 (EU583503)
ZSM/HB/07 (FJ608336)
ZJ1 (AF431744)
BP01 (DQ080015)
426273158 JX854452 strain Pheasant MM20 Pakistan 2011
Chicken/Sweden/97 (GU585905)
MK13/75/Iran (AY928933)
Chicken/Bareilly/India/1997 (HQ589257)
SPVC/Karachi/NDV/43/2008 (GU182323)
NDV-Ck-Pakistan-NARC-11432-2009 (KC811820)
chicken/CP/Islamabad3/2010 (JN682191)
SPVC/Karachi/NDV/33/2007 (GU182331)
NDV-ByP-Pakistan-NARC-13N07-2013 (KC811836)
385863476 JQ517285 strain chicken Pakistan NDV UDL8 2011
chicken/CP/Islamabad2/2010 (JN682190)
chicken/BYP/Lahore/2010 (JN682184)
chicken/CP/Rawalpindi1/2010 (JN682185)
Chicken/BYP/Pakistan/2010
chicken/BYP/Rawalpindi/2010 (JN682187)
chicken/CP/Islamabad1/2010 (JN682186)
chicken/CP/Attock/2010 (JN682188)
371670891 JN682211 chicken CP Pakistan 2010
371670884 JN682210 chicken BYP Pakistan 2010
chicken/CP/Rawalpindi2/2010 (JN682189)
Genotype VI
Genotype V
Genotype VIII
Genotype II
Genotype I
Genotype IX
Genotype IV
Genotype III
Genotype VII
0.01

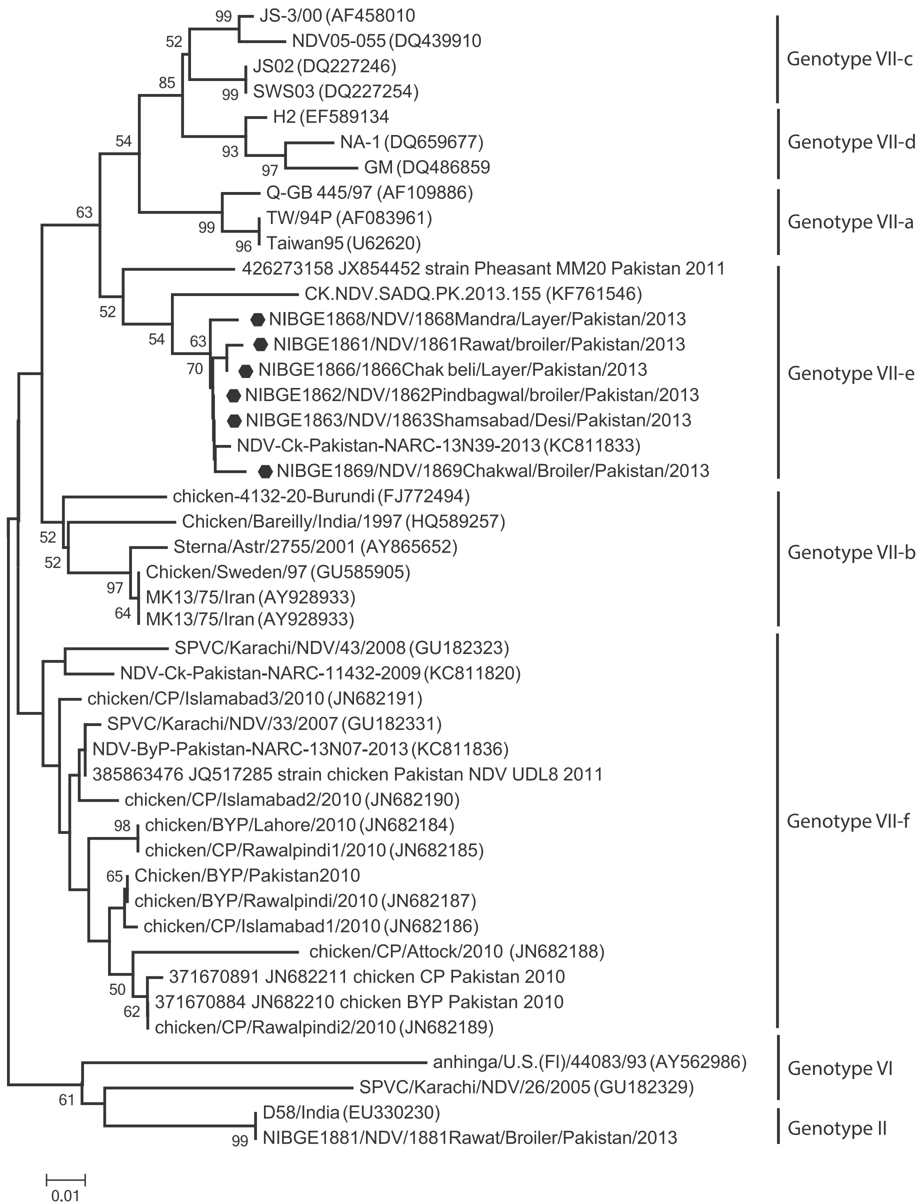


Fig. 3. Clustering pattern of genotype VII based on partial sequence of F protein gene of NDV isolates. Phylogenetic tree was constructed by neighbor-joining method as implemented in MEGA5. Numbers at the nodes indicate confidence level of bootstrap analysis with 2000 replications as percentage value. Values less than 50% are not shown. Horizontal lengths are proportional to distance. Sequences reported in this study are labeled with hexagonal.

However, one NDV isolate (1881Rawat) clustered with class II, genotype II isolates with an ICPI value of 0.35, although the F protein cleavage site of the 1881Rawat isolate resembles the LaSota strain. However, the reason for higher ICPI value of this strain remains unknown. This might be either due to the secondary infection or due to the emergence of novel mutation in the genome of NDV that transformed apparently avirulent strain

into a moderate pathogenic form. Previous work has demonstrated that virulence of NDV does not solely rely on the F protein cleavage site sequence; however, the HN protein also contributes to the virulence (Diel et al., 2012; de Leeuw et al., 2005).

The findings of this study and those of previously reported studies (Munir et al., 2012a,b,c; Siddique et al., 2013; Shabbir et al., 2012a,b) revealed the existence of heterotypic NDV strains circulating in commercial poultry flocks and wild birds in Pakistan. The divergence in NDV strains in such environment is potentially serving as a reservoir for the evolution of new pathogenic genotypes of NDV. These facts emphasize the need for continuous surveillance of NDV at the country level in both commercial and wild birds.

4. Materials and methods

4.1. Collection of samples and virus isolation

A total of 35 samples, consisting of cloacal swabs, tracheal swabs, and tissue specimens, were collected from ND suspected outbreaks in several commercial poultry farms located in Punjab during December 2012–July 2013. For the preparation of inoculum of morbid organs, each organ was triturated separately in sterile phosphate buffer saline and sterile sand in an autoclaved pestle and mortar. The homogenized material was centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was collected and filtered through 0.45 and 0.2 micron porosity. Subsequently, virus suspected filtrate was inoculated in five 10-day-old embryonated chicken eggs for NDV propagation (Rehman et al., 2013). After 48 h of inoculation, all eggs were transferred to a refrigerator for 16 h to facilitate the harvesting of allanto-amniotic fluid (AAF). Allanto-amniotic fluid (AAF) was collected from each egg and spot test was performed to check hemagglutinating activity with 25% RBC suspension. The aliquots of sterile AAF were made and stored at −20 °C until further use.

4.2. Hemagglutination (HA) test

The HA test was conducted according to the procedure described by Alexander and Chettle (1977) using polystyrene 96-well round bottomed microtitration plates (GIBCO, USA). Positive HA activity indicated the presence of hemagglutinating agents such as NDV or Avian InfluenzaViruses (AIV).

4.3. Hemagglutination inhibition (HI) test

The HI test was performed with NDV positive antiserum to confirm the presence of the virus. The assay was performed following the OIE guidelines (OIE, 2000). HI was also performed with positive H9, H5 and H7 antiseras to rule out the cross-reactivity or coinfection of NDV and AIV.

4.4. Pathogenicity testing

The pathogenicity of HI-positive NDV isolates was evaluated by determining the intracerebral pathogenicity index (ICPI) in day-old chicks according to the OIE (2000) manual.

4.5. RNA extraction

Viral RNA was extracted from HI-positive ND samples using TRIzol®. After thawing, 200 μl of allantoic fluid was mixed with 800 μl of TRIzol reagent and incubated at room temperature for 5 min. 200 μl of chloroform was added and tubes were shaken vigorously. Debris was removed by centrifugation at 12,000 *g* for 15 min at 4 °C to retrieve the supernatant. The upper aqueous phase was carefully transferred to a new tube and 500 μl of isopropyl alcohol was added per ml of TRIzol to precipitate the RNA. The mixture was incubated for 10 min at 15 to 30 °C. Centrifugation was done at 14,000 *g* for 10 min and the supernatant was discarded. Washing of RNA was carried out with 1 ml of 75% ethanol and centrifuged at 7500 *g* for 5 min. The RNA pellet was air dried for 5 to 10 min and dissolved in 25 μl of DEPC treated water.

4.6. cDNA synthesis

Viral RNA was reverse transcribed to cDNA by random primers using a two-step reverse transcription kit (Thermo Scientific, UK) following the manufacturer's instructions. Reactions were carried out in an Eppendorf mastercycler using the following conditions: reverse transcription at 42 °C for 15 min, heat inactivation of reverse transcriptase at 85 °C for 5 min and samples held at 4 °C for 5 min for one PCR cycle. The synthesized cDNA samples were stored at −20 °C until further use.

4.7. Reverse transcription polymerase chain reaction (RT-PCR)

DNA encoding the partial open reading frame of the F protein gene of NDV was generated by PCR using cDNA of each virus and oligonucleotide primers 4306F 5′ GACCGCTGACCACGAGGTTA 3′ and 5005R 5′ AGTCGGAGGATGTTGGCAGC 3′ (Aldous et al., 2003) in the presence of Pfu DNA polymerase (Fermentas). A total of 50 μl reaction mixture that consisted of 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 1 mM $MgCl_2$, 0.5 μM of each primer, 200 μM of each deoxyribonucleotide triphosphate, 0.5 U of Pfu DNA polymerase and 0.5 μl of cDNA was prepared in 0.2 ml PCR tubes. The PCR reaction conditions consisted of an initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min, an extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. Amplified DNA products were examined on 1.5% (w/v) agarose gel in 0.5× TAE (Tris base, glacial acetic acid, 0.5 M EDTA (pH 8.0)) buffer containing ethidium bromide. Fragment sizes were estimated by comparing with the 100 bp DNA ladder (Fermentas). The 6× DNA loading dye (0.4% bromophenol blue, 25% ficoll 400 and 0.4% xylene cyanol FF) by MBI Fermentas, Graiciunau 8, Vilmius 2028, Lithuania, was used as tracking dye. Finally the gel was observed under UV light and photographed.

Author's contributions

MF conceived the study, carried out genetic characterization and drafted the manuscript. US carried out sample collection and biological characterization of viral isolates and helped to draft the manuscript. QMK participated in the design and coordination of study. MM carried out phylogenetic analysis and critically reviewed the manuscript. All authors read and approved the final manuscript.

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