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COMPLETE GENOMIC ANALYSIS OF NEWCASTLE DISEASE VIRUS OF A RECENT PANZOOTIC ISOLATED FROM VACCINATED POULTRY FLOCK IN 2014 IN PAKISTAN

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ABSTRACT

Newcastle disease (ND) is one of the most significant OIE listed disease that causes a huge economic losses in the poultry sector worldwide. ND is endemic in Pakistan and continues outbreaks have been reported in vaccinated and non-vaccinated birds. Sequence analysis revealed several amino acids substitutions at the functional domains of fusion (F) and hemagglutinin-neuroaminidase (HN) protein. The current study proposed that the vaccination is leading to pressure on the NDV to mutate into more virulent strains. We noticed that the host range of NDV is extending to pet and other species and suggest it could be due more virulent strains emerging. The NDV strain Chicken/NDV/Pak/AW-14 was isolated from the vaccinated chicken from a poultry farm located near Lahore, Punjab. The isolated was biologically and genetically characterized through intra cerebral pathogenicity index (ICPI) and real time PCR (rRT-PCR). To better understand the epidemiology of ND outbreak, the full genome was sequenced and phylogenetic analysis was performed on the basis of full F and genome sequencing. AW-14 isolate was categorized as a velogenicNDV strain assessed biologically by the ICPI (1.8) and polybasic amino acid residues at the F protein cleavage site. The complete genome was 15,192 nucleotides (nt) in length with six genes in order of 3`-NP-P-M-F-HN-L-5`. Several polymorphisms were identified at the functional domains of surface glycoprotein F and HN, including signal peptide, transmembrane domain, hepad repeat region and N-glycosylation site, transmembrane domain, neutralizing epitope respectively. Phylogenetic analysis showed that the AW-14 belongs to sub-genotype VIIi, the newly emerging NDV strain has been implicated in the recent outbreaks in Pakistan, Indonesia and Israel and becoming established in poultry sector through Asia and Middle East. Here, we provide a summary of the genetic evolution and molecular epidemiology of the vNDV strain suggesting responsible of a fifth panzootic.

Key words: Newcastle disease, complete genome, vaccinated birds, phylogenetic analysis, Pakistan

INTRODUCTION

Newcastle disease is caused by Newcastle disease virus (NDV), also known as avian paramyxovirus type-1 (APMV-1) is highly infectious disease of birds and has a significant adverse impact in the poultry industry worldwide. It remains to be one of the major threats to the poultry producers for under developed countries including Pakistan but now developed countries are also included as a victim of threats to the poultry industry. The national poultry sector of the country due to concurrent outbreaks of ND faces huge economic losses. Apart from the fact that much advancement has been made in the field of diagnosis and vaccinology, the damage to the commercial poultry due to ND is enormous. The NDV has the ability to cause severe disease in more than 250 species of birds and can spread through various routes. Newcastle disease virus is a single stranded RNA with negative sense withat least three genomic lengths, 15186 (Paldurai *et al*., 2010), 15189 (Yurchenko *et al*., 2015)15192 (Wajid *et al*., 2015, Umali *et al*., 2014) and 15198 (Kim *et al*., 2012) and encodes for nucleocapsid protein (NP), phosphor-protein (P), matrix protein (M), fusion protein (F), haemagglutini-neuroaminidase protein (HN), and large protein (L) (Shabbir*et al.,* 2013; Miller *et al.,* 2010). The NDV contains two surface glycoproteins F and HN mediate the role of host cell's sialic acid receptors and later one is responsible for virus penetration and virus-induced cell fusion and haemolysis (McGinnes *et al*., 2006).

The amino acid sequence of F protein proteolytic cleavage site is determinant of virus pathogenicity in birds. The F protein is produced as an inactive precursor F0, later cleft by enzymes like trypsin/subtilisin into two biologically active subunits F1 and F2. The F protein's cleavage is necessary for viral entry into host cells and cell-cell fusion.

The current classification of NDV grouped into two classes, class I and II, however, the earlier one has

one genotype whereas the later one has eighteen genotypes (Snoeck *et al.,* 2013; Courting *et al.,* 2013; Diel *et al.,* 2013). The genotypes I, II, are further divided into Ia, IIa and Ib and IIb, genotype VI is further divided into Via-VIf and genotype VII into VIIa to VIIi (Wajid *et al.,* 2015; Miller *et al.,* 2015; Miller *et al.,* 2010; Kim *et al.,* 2007; Yu *et al.,* 2001). Different genotypes of NDV contains in class II are distributed worldwide since first time identified in 1926 (Miller *et al.,* 2010). Detection of NDV first time in Indonesia and at the same time at Newcastle-on-tyne, England since 1926 the various genotypes were documented for different panzootic, the NDV genotypes I, II, III and IV contains in class II were responsible for first ND panzootic that last since 1920s to 1960s (Alexander, 2001). The second panzootic of ND was occurred in late 1960s in various regions of Europe, the genotypes V and VI strains were responsible for ND (Alexander, 2003). The subtype of genotype VI (VIb) was originated from the Middle East was named as third panzootic and erupted from the Racing Pigeons in 1980s (Kaleta *et al.,* 1985). The fourth panzootic of ND was occurred in 1980s, caused several outbreaks in Europe, Africa, South America and Asia including Pakistan, the NDV strains of genotype VII and their sub-genotypes were responsible for the outbreaks (Miller and Koch, 2013; Perozo *et al.,* 2012).

New sub-genotype (VIIi) of vNDV from genotype VII is affecting the poultry industry in Asia including Middle East leading severe outbreaks of ND, named as an existence of fifth panzootic (Miller *et al*., 2015). However, the main concern of these viruses has demonstrated causing higher mortality in vaccinated commercial chickens (Wajid *et al*., 2015, Rehmani *et al*., 2015). However, ND is endemic in Pakistan, the epidemiology, the mechanism of maintenance and evolution of the new genotypes are not well understood. Despite the vigorous vaccination in the region, ND outbreaks have been reported from all areas of Pakistan since 1963 (Khan and Huq, 1963), affecting not only the commercial poultry but also wild birds in zoos and backyards. The emergence new sub-genotype in Middle East represents a continuous threat to the poultry industry and backyard poultry farming in Asia.

The epidemiological and clinical findings demonstrated that the birds affected during the outbreaks occurred in 2011-12, is still causing disease to susceptible birds either vaccinated or non-vaccinated. A continuous outbreak of ND in Pakistan needs nonstop research work to find the various reasons either the usage of un-matched vaccine strain, or any immunological stress using various vaccines with short span or selection of breeds, highly susceptible to NDV. This study would assist us to compare the NDV full genome described during the period of outbreak and the changes after two years period its effect on the economy of poultry industry.

MATERIALS AND METHODS

Isolate: The chicken/NDV/Pak/AW-14, a virulent NDV strain, was isolated in 2014 from a commercial poultry farm previously vaccinated against ND. The farm was located in Lahore Punjab, Pakistan.The isolate was propagated in 9-11-day old embryonated SPF (33 incubated at 37 °C and monitored for viability up to 72 hours. The allantoic fluid was extracted and screened for hemagglutination assay (HA). The HI was performed using four HA units of antigen and anti LaSota chicken serum according to standard protocol (OIE, 2008). All the positive fluids were collected and virus stocks were kept at -80°C for further use.

Characterization: The pathogenicity of the isolated strain was evaluated through Intracerebral Pathogenicity Index (ICPI). It was calculated as described in the International OIE standards (OIE, 2008). Day-old chicks were injected with 50 uL of virulent NDV virus 10^{-1} dilution, in phosphate buffer saline (PBS) by an intra-cerebral route. Two chickens from the same batch were injected with 50 uL of PBS treated as control. The chickens were observed and recorded every 24 h for 8 days, scoring 0, 1, 2 for normal, sick and dead respectively.

RNA extraction & sequencing: RNA was extracted from allantoic fluid through TriZol LS according to manufacturer's instructions (Invitrogen, Carlsbad, CA). cDNA was synthesized using Reverse transcription PCR (RT-PCR) using cDNA synthesis kit (Thermo Scientific, USA). For amplification to have a complete genome the oligonucleotide primers were designed as overlapping fragments. Complete genome sequence of chicken/NDV/Pak/AW-14 isolate was determined using high fidelity Platinum ®supermix PCR kit (Invitrogen, Carlsbad, CA,USA).The PCR amplicons were prepared for sequencing after running on 1% agarose gel for electrophoresis and purified through Thermo Gel extraction kit according to manufacturer's instructions (Thermo Scientific, USA). The purified PCR products were cloned into pTZ57R/T vector (Thermo Scientific, USA). For sequencing fluorescent di deoxyribonucleotide terminators were applied in an automated sequencer ABI 3700 (Applied Bio Systems, USA).

Nucleotide sequencing and Phylogenetic Analysis: Assembly and editing of sequencing of chicken/NDV/Pak/AW-14 isolate was performed using the BioEdit software v 7.2.5 (Hall, 1999) and compared to the published strains by using the software Clustalw 2.2. MEGA software version 6 was used for the phylogenetic analysis inferred by the Maximum Liklihood method with standard error being calculated based on the 1000 bootstrap replicates (Tamura *et al*., 2013). The chicken/NDV/Pak/AW-14 genome sequence

was compared against 64 complete genome sequences from class I and II, having genotypes I-XVIII available at Genbank. To analyze through full fusion gene sequences, the tree was constructed with 96 sequences of NDV strains belong to class I and II.

Nucleotide Sequence Accession Number: NDV isolate chicken/NDV/Pak/AW-14 genome sequence is available in GenBank under accession number KP776462.

RESULTS

Biological characteristics: The strain chicken/ NDV/Pak /AW-14 was found pathogenic as assessed by the biological standard test, ICPI 1.8 andthe amino acid sequence of F protein cleavage site (^{112}-R-R-Q-K-R-F-^{117}), revealed the presence of the three basic amino acid residues at position 113, 115, and 116 and a phenylalanine at position 117 (Miller *et al.,* 2015; Rehmani *et al.,* 2015).

Genomic analysis and deduced protein: Complete genomic feature of NDV strain chicken/NDV/Pak/AW-14 is summarized in Table 1. The isolate has similar characteristics as presented for other virulent APMV-1. The nucleotide (nt) and amino acid (aa) sequences of chicken/NDV/Pak/AW-14 isolate was compared with selected strains of class II reference strains representing genotype I-XIII and XVIII are presented in Table 2. Nucleotide sequences comparison of this newly emerging strain (chicken/NDV/Pak/AW-14) showing 99% homology to Indonesian NDV strains chicken/Banjarmasin/010/2010 (genotype VIIi), contrary to that, chicken/NDV/Pak/AW-14 showed lowest nucleotide homology with LaSota strain i.e 83%; genotype II.

The chicken/NDV/Pak/AW-14 had full genome nucleotides 15,192bp in length with GC level of 55% comprised of six ORFs, complete nt sequence of nucleoprotein (NP) was 1470 bp, phophoprotein had 1188bp, matrix protein (M) 1095 bp, fusion protein had 1662 bp, hemagglutinin-neuroaminidase (HN) 1716 bp and large protein (L) contained 6615 bp.

Analysis of the functional domain of the fusion protein cleavage site showed three basic aa residues at positions 113, 115 and 116 and a phenylalanine at position 117 (^{112}-R-R-Q-K-R-F^{117}), as generally identified in highly virulent strains of NDV in chicken. Comparing with consensus sequence the seven neutralizing epitopes (D^{72}, E^{74}, A^{75}, K^{78}, A^{79}, L^{343}, ^{151}ILRLKESIAATNEAVHEVTDG^{171}), observed as critical for structure and function of F protein, and were conserved in chicken/NDV/Pak/AW-14 strain. The Signal and fusion peptides almost conserved in chicken/NDV/Pak/AW-14 isolate except a single substitution at position C^{25}→Yin signal peptide. Analysis of heptad repeat regions (HR; HRa, HRb, HRc) revealed a total of four substitutions in HRa (143-185 aa) with change A^{176}→S; HRb (268-299 aa) with change N^{272}→Y and HRc (471-500 aa) with two changes E^{482}→T; K^{494}→R.Analysis of chicken/NDV/Pak/AW-14 strain's transmembrane domain showed four substitutions at positions V^{506}→A; V^{513}→F; A^{516}→V and V^{520}→G.

The HN gene of NDV strain chicken/NDV/Pak/AW-14 is 1716 nt in length and encode for 571 aa, characteristics of the vNDV strain. Twelve aa (174, 175, 198, 236, 258, 299, 317, 401, 416,498, 526, and 547) constituting sialic acid binding site of the HN protein were completely conserved (Table 3). Cysteine residues (123, 172, 186, 196, 238, 247, 251, 344, 455, 461, 465, 531, and 542) and N-glycosylation sites (119, 341, 433, 481, 508, and 538) were almost conserved except for the loss of N-glycosylation at position 508 in chicken/NDV/Pak/AW-14 isolate. There were two substitutions at position V^{34}→I and T^{36}→I in transmembrane domain of HN protein of chicken/NDV/Pak/AW-14 strain, in comparison with the consensus aa sequences. Analysis of ten neutralizing epitopes of the HN protein identified a single aa change at position E^{347}→K, have been observed in region 14 of seven antigenic sites (1, 2, 3, 4, 12, 14, and 23) within HN protein of chicken/NDV/Pak/AW-14 isolate (Table 4).

Phylogenetic analysis: The phylogenetic analysis of the chicken/NDV/Pak/AW-14 isolate is presented in Figure 1, 2, this strain belong to newly emerged panzootic virus's sub-genotype VIIi genotype VII on the basis of complete coding sequence of F gene and full genome. The NDV /Pak/AW-14 strain was clustered together with recently isolated NDV viruses from poultry farms either commercial, backyard or pet birds throughout Pakistan, Indonesia and Israel. The genotype VIIi become the predominant sub-genotype causing ND outbreaks in vaccinated and non-vaccinated avian species related to poultry. The sub-genotype is circulating since 2011-12 NDV as an epidemic in this region. The VIIi strains are highly virulent in all type of species and have replaced the NDV isolates of genotype XIII, which were commonly isolated in 2009-11. The phylogenetic analysis also included the previously characterized NDV isolates to ascertain the genetic diversity with currently isolated ND strain. The phylogenetic tree inferred through complete genome of chicken/NDV/Pak/AW-14 with other NDV strains available from GenBank, revealed the same topology of the tree as constructed by F gene sequences. The degree variation of nucleic acid between the AW-14 strain and NDV strains in genotype VIIi was 0.3-1.6% for the F gene and 0.0-1.4% for protein. More than 99% homology of the said strain to Indonesian strain chicken/Banjarmasin/010/10 (HQ697254) has already mentioned above.

Table 1. Genome features and protein characteristics of NDV strain chicken/NDV/Pak/AW-14.

Protein	Intergenic sequence (IS)	Genomic Characteristics					Deduced protein	
		Nucleotide length (nt)	5UTR	ORF length (nt)	3UTR	%G+C	Size (aa)	MW (kDa)
NP	2	1752	66	1470	216	50.81	489	53.2
P	1	1451	83	1188	180	55.05	395	42.3
M	1	1241	34	1095	112	48.58	364	39.6
F	31	1792	46	1662	84	45.42	553	59.0
HN	47	2002	91	1716	195	45.86	571	67.6
L	-	6703	11	6615	77	44.47	2204	248.6
Genome	-	15,192	-	-	-	47.07	-	-

Table 2. Nucleotide and amino acid comparison between the vNDV isolate chicken/NDV/Pak/AW-14 and viruses representing other genotypes within class II.

Genotype	I		II		III		IV		V		VI		VII		VIII		IX		X		XI		XII		XIII		XVI		XVIII	
Strain	Ulster		LaSota		Muktaswar		Herts/33		Largo/71		Fontana/72		Banjarmasin/010/10		QH4		JS/1/2/Du		Mullard/US/4-411/04		MG-1992		Poultry/Peru 1918-03/08		Sterna/Astr 2755/2001		D-Republic/4993/2008		NDV/chick/Tog/Ak018	
Access No	AY 562991		AY 845400		EF 201805		AY 741404		AY 562990		AY 562988		HQ 697254		FJ 751919		FJ 436306		GQ 288377		HQ 266603		JN 800306		AY 865652		JX 119193		JX 390609	
	%		%		%		%		%		%		%		%		%		%		%		%		%		%		%	
	nt	aa	Nt	Aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	Aa	nt	aa	nt	aa	nt	aa	nt	aa
NP	88	94	85	92	87	95	89	97	89	96	91	97	99	99	89	96	88	94	86	93	86	95	90	97	90	97	86	95	90	96
P	84	83	83	82	84	86	86	84	87	85	88	88	99	99	86	84	84	83	82	81	84	81	89	86	89	86	83	82	88	88
M	85	90	84	88	86	90	88	93	88	95	92	96	99	99	88	93	85	90	83	91	85	92	91	96	91	97	86	93	89	95
F	87	91	84	89	87	92	89	94	88	93	92	96	99	99	89	94	87	92	86	92	84	88	87	95	91	95	85	91	90	95
HN	85	90	82	87	85	89	87	90	89	91	90	94	99	99	86	92	84	89	83	90	84	88	89	92	90	92	84	92	89	93
L	87	95	85	92	86	94	89	95	90	96	92	96	99	99	89	95	88	94	86	95	85	94	90	96	91	96	87	94	90	95
Genome	85		83		86		87		88		90		99		87		85		84		84		89		89		84		89	

Table 3. Amino acid substitution in the functional domain of HN protein.

Genotype	Trans membrane domain 25-45																				Receptor recognition 174, 175, 198, 236, 258, 299, 317, 401, 416,498, 526, 547													N-linked glycosylation sites 119, 341, 433, 481, 508, 538	Cysteine residues 123, 172, 186, 196, 238, 247, 251, 344, 455, 461, 465, 531, 542
Consensus	F	R	I	A	V	L	L	L	I	V	M	T	L	A	I	S	A	A	A	L	V	R	I	D	K	E	Y	Y	E	R	R	Y	E		-
I (AY562991)	-	-	-	-	I	-	-	-	T	-	V	-	-	-	-	-	-	-	-	-	A						-								-
II AY845400	-	-	-	-	I	-	F	-	T	-	V	-	-	-	-	-	V	-	S	-	L						-								-
III EF201805	-	-	-	-	A	-	-	-	M	-	I	-	-	-	V	-	-	V	-	-	A						-								-
IV AY741404	-	-	-	-	I	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	-						-								-
V AY562990	-	-	-	-	-	-	S	-	-	-	M	-	-	-	-	-	V	-	-	-	-						-							Lost at 508	-
VI AY562988	-	-	-	-	-	-	-	-	-	-	M	-	-	T	-	-	-	-	-	-	-						-								-
VII chicken/NDV /Pak/AW-14	-	-	-	-	-	-	-	-	M	I	M	I	-	-	-	-	-	-	-		A						-								-

Table 4. Amino acid constituting the neutralizing epitopes of the HN protein.

Sites	23										3			4				14								4	12	2 and 12								2
Genotype	193-201										263	287	321	332-333				346-353								356	494	513-521								569
Consensus	L	S	G	C	R	D	H	S	H	N	D	K	G	K	D	E	Q	D	Y	Q	I	R	K	G/D	R	I	T	R	V	S	S	S	S	D
I (AY562991)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-
II (AY845400)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	G	-	-	-	-	-	-	-	-	-	-
III (EF201805)	-	-	-	-	-	-	-	-	-	K	-	-	-	R	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-
IV (AY741404)	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-
V (AY562990)	-	-	-	-	-	-	-	-	-	K	-	R	-	-	-	-	-	-	-	-	V	-	-	N	-	-	-	-	-	-	-	-	-	N
VI (AY562988)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	V	-	-	-	-	-	-	-	D
VII(chicken/NDV/Pak/ AW-14)	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	K	-	-	-	-	-	-	-	D	-	I	-	-	-	-	-	-	-	-

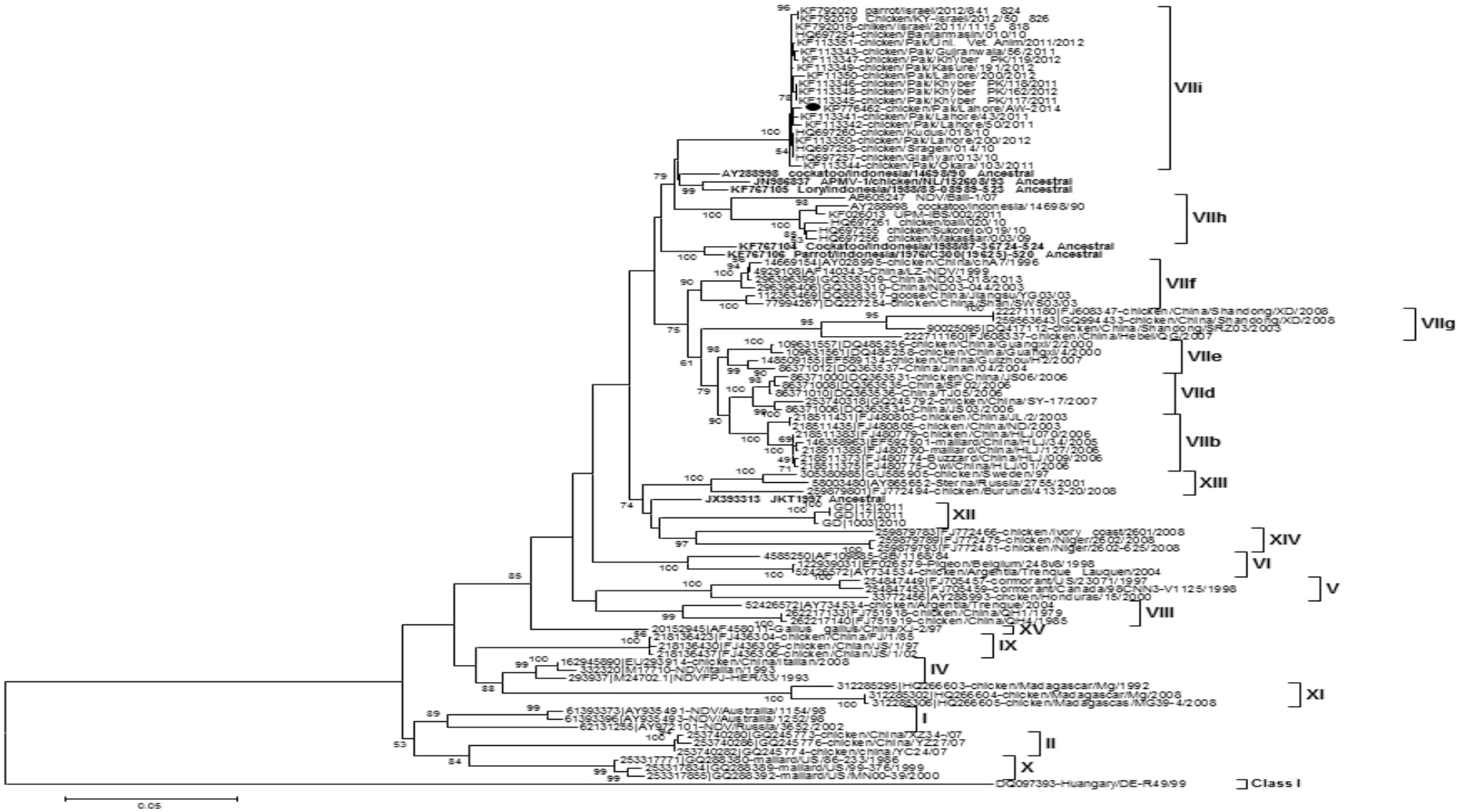

Figure 1. The phylogenetic analysis based on the complete fusion gene sequences of 96 isolates from class I and II available in GenBank. The new vNDV isolate (chicken/Pak/NDV/AW-2014) is denoted with a Black circle in the tree. The evolutionary history was inferred using the maximum likelihood method based on the General Reversible model (38). The optimal tree with the sum of branch length = 2.03170535 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (39) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 3). All positions containing gaps and missing data were eliminated. There were a total of 1662 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (13).

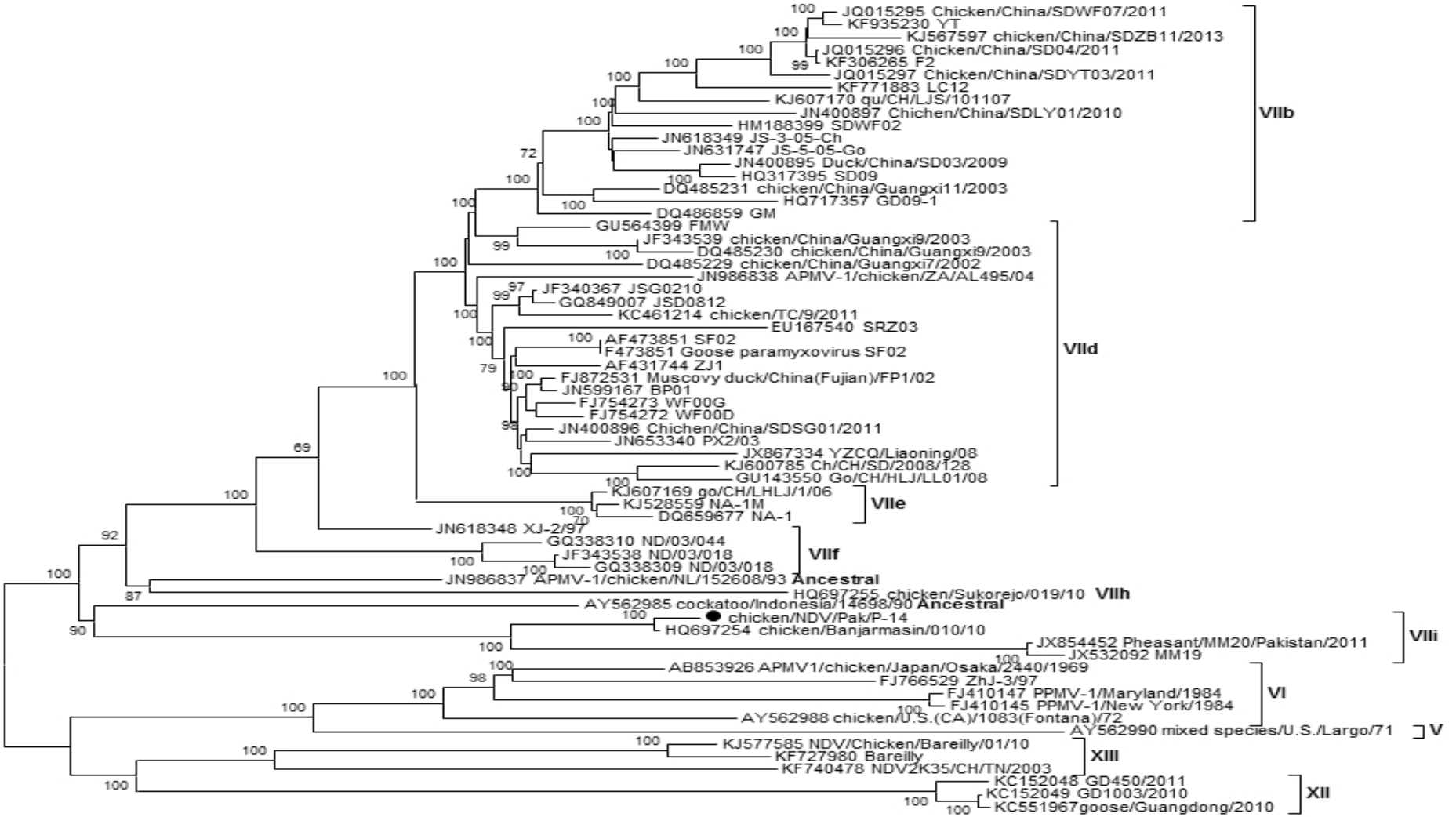

Figure 2. Molecular phylogenetic analysis of selected full genome isolates was inferred by using the maximum likelihood method based on the General Reversible model (38). A total of 64 nucleotide sequences of various genotypes were involved in the analysis. There are a total of ~15192 positions in the final data set. Evolutionary analysis was conducted through MEGA version6 (13). The new vNDV isolate (chicken/Pak/NDV/AW-2014) is denoted with a Black circle. The evolutionary history was inferred using the maximum likelihood method. The optimal tree with the sum of branch length = 0.94756395 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 3).

DISCUSSION

ND remains one of the most economically significant burdens on poultry production globally despite stringent vaccination by the commercial poultry producers. The disease is endemic in Pakistan, therefore, occasional outbreaks in commercial poultry, backyard chickens and recently in wild birds reported now and then. The presence of NDV in apparently healthy chicken in Pakistan is common with regard to low or moderate NDV strains existence. However, all of our isolates causing mortality have a fusion gene cleavage site of virulent NDV strains, some isolates were confirmed to be highly virulent when used as a challenged virus. Non-availability of data regarding the immune status prior to vaccination remains uncertain, therefore, it is hard to know whether disease onset was due to challenge by virulent NDV strains or due to failure of vaccination program. The second assumption in the current situation is that the viruses currently circulating in the region (Pakistan) belong to a new sub-genotype VIIi, whereas the vaccine used for preventive measure belonged to genotype II. This strain is used in the industry since 1970 that could raise a question the efficacy of vaccine as the evolutionary distance between chicken/NDV/Pak/AW-14and vaccine viruses of genotype II is the largest distance (17% divergence) observed between all genotypes.

Newcastle disease is associated with seasonal outbreaks in vaccinated and non-vaccinated chickens either from commercial or backyards farming. High prevalence of ND has been noted during winter season and moderate mortality during spring, however, the occasional outbreaks continue for the whole years both in commercial, backyard production facilities as well as in wild and pet birds (Wajid *et al.,* 2017). Stress associated with harsh conditions/environment has been suggested to exacerbate the outbreaks of NDV (Abdu*et al*., 1992). Frequent fluctuation in ambient temperature, humidity and high virus load act as stress factors, caused the immune status of the birds worse; make the room for vNDV infection. Due to booster dose of live attenuated vaccination against IB, IBD and NDV at the age of 2 to 3 weeks, when they are not immune competent to resist the viruses load and may lead to the reason of outbreaks of vNDV. Local hatcheries in Pakistan do not intimate the immune status of day old chicks, the vaccination schedule followed by the farmers as subscribed by the veterinary officer, who are hired by the owners of the farms. Little is known, whether these outbreaks are associated with a single or multiple viral genotypes and whether the quick changes/mutation in the genome evolves new genotypes during disease transmission.

Epidemiological and sequencing analysis reveals that five distinct genotypes (II, III, VI, VII, &XIII) circulating among birds in Pakistan since mid1990's. However,genotype XIII, which were commonly isolated in 2009-11 (Wajid*et al*., 2015, Miller *et al*., 2015, Rehmani*et al*., 2015) have replaced with novel sub-genotype VIIi.Previously conducted studies on virulent NDV, confirmed the genotype VI from Karachi, Sindh (Khan *et al*., 2010), and genotype II and III as a vaccine strains. Genotype II commonly used in commercial poultry LaSota NDV and the route of vaccination is either Eye drop or drinking water whereas, genotype III (Mukteswar strain) is manufactured locally and used by the backyard farmers. Furthermore, the extensive use of vaccines of different origin (imported) makes the situation more complex for genetic modification in virulent strains.

We report here the full genome sequencing of circulating field NDV isolate from recent outbreak in Pakistan. The virus belongs to genotype VII, always thought to be endemic in many Asian countries (Munir*et al*., 2012). However, a distinct sub-genotype VIIi has been circulating since a severe outbreak reported during 2011-12 suggesting a novel viral origin. The data indicate that the concurrent outbreaks in this region are genetically related to Indonesian and Israeli strains. F and HN glycoproteins are the critical virus neutralizing antigen and thus the major protective antigens (Taylor *et al*., 1990). Newcastle disease strains of NDV of different genotypes have functional domains of F and HN proteins with consensus sequences that recognized several amino acid substitutions. F gene has been mostly considered for the genetic characterization of NDV strains, major determinant of virulence particularly the variable region (47-421 nt), as it codes a signal peptide (aa 1-31), for cleavage activation sequence (aa 112-116),and hydrophobic region (aa 117-142) (Umali *et al*., 2013). Another standard criterion for NDV genotyping is using the variable region (nt 47-420) of F gene (Qin *et al*., 2008), however, its cleavage activation sequence (aa 112-116) at the C-terminus of the F2 protein and L (leucine) or F (phenylalnine) at the N-terminus of F1 protein (aa 117) are major determinants of NDV virulence (Alexander, 2008, Alexander, 2009). The cleavage site motif ^{112}-R-R-Q-K-R-F^{117} was present in AW-14 strain, which is typical of vNDV strain (Miller *et al*., 2015). Seven major neutralizing epitopes at position D, E, A, K, A, and L, and a stretch of aa from residues 151-171 believed to be critical for structure and function of F protein (Neyt *et al*., 1989, Yusoff *et al*., 1989), and all were conserved in AW-14 strain. Comparison of functional domain of F protein with consensus sequences identified several amino acid substitutions in signal peptide with a single substitution, total of four substitutions in heptad repeat regions (HRa,HRb,HRc) and transmembrane domain had four substitutions.

The HN gene sequence of the chicken/NDV/Pak/AW-14 strain was 571 aa, characteristics of the vNDV strain (Tsai *et al*., 2004,

Habib *et al*., 2015, Maminiaina *et al*., 2010). The sialic acid binding site and cysteine residues of HN protein were completely conserved as compared to consensus sequences, however loss of N-glycosylation at position 508 was observed in AW-14 strain (Table 3).Analysis of the ten Nneutralizing epitopes in the HN protein identified a single aa change at position 347 (E→K), observed in region 14 of seven antigenic sites within HN protein, useful marker of antigenic variant and enables the field virus to evade neutralizing by a specific MAbs (Gotoh *et al*., 1988) (Table 4).The earlier studies indicate that the neutralizing epitopes due to amino acid substitution and formation of antigenic epitope that may lead to an escape of neutralizing variants (Cho *et al*., 2007, Cho *et al*., 2008, Hu *et al*., 2010). Though the poultry industry has revolution during the last two decades in Pakistan, but the selection of vaccine strain and route of vaccination still remained the same. There are chances that a continuous use of LaSota strain (genotype II) unable to protect the chickens got stricken by the newly emerging virulent form of genotype VIIi. The reason might be the variation at the antigenic sites of newly emerging NDV strains. As compared to the vaccine strain used in the field and the past pandemic NDV viruses in Pakistan, recently identified viruses reveal amino acids substitutions at neutralizing epitopes, which in previous studies have been suggested that these variations may lead to antigenic change and have effect on viral attachment to the receptor on host cells (Iorio *et al*., 1989, Iorio *et al*., 1991). In such case, the antibody recognition its neutralizing activity may be altered, resulted the escaping of specific antigen and the release of virus as a virulent strains in the systemic circulation and developed as an outbreak.

The full genome of AW-14 strain was compared with the viruses from genotype 1-XVIII, the highest nucleotide identity (>99%) was found with chicken/Banjarmasin/010/2010 and chicken/Pak/QOL/SFR-611/13(genotype VIIi; GenBank accession number HQ697254 and KM670337 respectively) and the lowest similarity with LaSota strain (genotype II; GenBank accession number AY845400) (Table 2). Although the full fusion of AW-14 strain has highest nucleotide similarities (99%-100%) with NDV isolates from Middle East, Indonesian and previously characterized Pakistani NDV strains (genotype VIIi). These close identities were also figured out by phylogenetic analysis (Figure 1, 2). Over-all, the data confirmed that the commercial poultry, village chickens and pet birds in Pakistan, Indonesia and Middle East were also affected by identical vNDV strains since 2011-12, leading to fifth panzootic.

In summary, whole genome sequencing of NDV has enable us to study the dynamics of NDV transmission and evolution during a localized outbreak in Pakistan in 2014. Furthermore, whole genomic study is not only important for diagnosis and pathogenicity assessment but also important in vaccine strain selection as a master seed virus for the region.

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