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ANNOTATED SEQUENCE RECORD

Sequencing and analysis of the complete genome of Newcastle disease virus isolated from a commercial poultry farm in 2010

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Abstract Newcastle disease virus (NDV) infects wild and domestic birds but causes contagious and lethal disease in domestic poultry. ND is currently endemic in Pakistan, but no complete genome sequence of a Pakistani NDV isolate has been reported. An NDV strain isolated from a commercial poultry farm was completely sequenced. Phylogenetic analysis revealed that the isolate is closely related to genotype VII and, more specifically, to subgenotype VIIb, yet with substantial enough differences to be regarded as new subgenotype (VIIf). These findings provide insight into the genetic nature of NDV circulating in Pakistan and are useful for both laboratory diagnosis and vaccine development for NDV.

Newcastle disease virus (NDV) is the causative agent of Newcastle disease (ND), which is a fatal disease of a wide range of avian species. NDV is a member of the genus *Avulavirus*, family *Paramyxovirdae* in the order *Mononegavirales* [12]. The viral genome is approximately 15 kb long and encodes a nucelocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and large RNA-dependent polymerase protein (L) in the order 3′-NP-P-M-F-HN-L-5′ [1]. Each of these genes is flanked by 5′ and 3′ UTRs at their respective ends, and each gene starts with a conserved gene start (GS) and ends at a stretch of sequence termed gene end (GE). The pathogenicity of NDV is categorized as velogenic, mesogenic, or lentogenic in the order of most to least pathogenic [3]. NDV strains are classified into nine genotypes based on the partial hypervariable nucleotide sequence of the F gene. Genotype VI is further subdivided into seven subgenotypes (VIa-g), and genotype VII is subdivided into five subgenotypes (VIIa-e) [4, 9].

Due to its global distribution and the fact that it is a great economic threat, ND is regarded as one of the most devastating diseases in the poultry industry [13]. The seriousness of this disease is further complicated by the involvement of a diversity of domestic and wild bird populations in its epizootiology [14]. The disease has been endemic in Pakistan since its first appearance in 1926 in Southeast Asia and continuously poses an economic threat to poultry production [1]. There has been extensive use of vaccines on commercial poultry farms in Pakistan for the control of ND. These vaccines are imported from other countries regardless of their suitability and protection. The unavailability of NDV vaccine from local isolates is mainly due to a lack of research and an unavailability of data on the nature of the NDV isolates circulating in the region. Although there are a few reports of disease outbreaks in Pakistan, these focus entirely on serological diagnosis [5, 10]. Information regarding epidemiology and genome characterization of NDV is completely lacking. For the first

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time in the whole region (Iran, Afghanistan, Bangladesh, Nepal and Pakistan, except for India and China), an NDV isolate from an outbreak in a commercial poultry flock has been biologically characterized, and its complete genome has been sequenced and analyzed. Moreover, due to recent reports of new emerging strains of NDV [2, 17], it is extremely important to characterize NDV strains from areas that have not yet been investigated to fill in the gaps and to establish an epidemiological link to adjacent countries and those participating in free trade.

Provenance of the virus materials

In April 2010, an outbreak of NDV occurred in Rawalpindi, a city located in the Pothohar plateau in the north of the country that is considered a commercial hub for poultry production in Pakistan. Cloacal swabs, tracheal swabs and blood samples were collected from dead birds and inoculated into five 10-day-old specific-pathogen-free chicken embryos via the allantoic cavity, and their hemagglutination (HA) titer was determined. Allantoic fluid from the samples that showed a high HA titer was stored on QIAcard FTA Indicator Four Spots (QIAGEN, Hilden, Germany). These QIAcards preserve nucleic acids and inactivate the virus. The samples were shipped at ambient temperature from Pakistan to the Swedish University of Agricultural Sciences, Uppsala, Sweden, for processing. The same samples were also processed for intracerebral pathogenicity index (ICPI) and mean death time (MDT) calculations, as recommended [1].

The RNA was eluted from QIAcard FTA Indicator (QIAGEN) impregnated with allantoic fluid as recommended by the manufacturer and screened for the presence of viral nucleic acid using a validated real-time PCR for the M and F genes [16, 19]. The same samples were subjected to conventional PCR for complete genome amplification using 22 pairs of primers as already described [14], except that amplification was performed using a One-Step RT-PCR Kit (QIAGEN). Each fragment was sequenced, with the same primers that were used for amplification, using ABI PRISM BigDye Terminator version 3.1 (Applied Biosystems) according to the manufacturer's instructions.

Sequences were processed using the SEQMAN program from DNASTAR Lasergene Suite 9 (version 9.0.4 39; DNASTAR, Inc., Madison, WI, USA). The nucleotide and amino acid sequences were compared with published sequences of NDV strains. Phylogenetic trees were generated by the neighbor-joining method with 2000 bootstrap replications using the Molecular Evolutionary Genetics Analysis (MEGA) software program version 5.03 [18].

Biological properties

NDV isolates are characterized as velogenic, mesogenic, and lentogenic based on their mean death time (MDT) in chicken embryos, where death is caused in less than 60 hours, 60-90 hours, and more than 90 hours, respectively. Chicken/CP/Pakistan/2010 exhibited an MDT of 45.6 hours in embryonated chicken eggs. This demonstrated that the pathogenicity of Chicken/CP/Pakistan/2010 was similar to that of velogenic strains of NDV. Consistent with this result, the ICPI value of Chicken/CP/Pakistan/2010 was calculated to be 1.5. The amino acid sequence for the cleavage site was shown to be ^{112}RRQKRF^{117}, which corresponds to a cleavage site of a velogenic pathotype [1], as predicted from the high ICPI and MDT values.

Complete sequence properties

Three lengths of the NDV complete genome have been reported: 15186, 15192 and 15198 nucleotides. The complete genome sequence of NDV strain Chicken/CP/Pakistan/2010 (GenBank accession number JN682211) was 15192 nucleotides in length and hence follows the rule of six, a general prerequisite for efficient viral replication [7]. The predicted amino acid sequences of this isolate revealed six proteins NP-P-M-F-HN-L that are similar in structure to those of the known paramyxoviruses [15]. The properties of each gene are summarized in Table 1. The GS for NP, P, M, F and HN was

Table 1 Genome characteristics of Chicken/CP/Pakistan/2010 and the predicted molecular weight of the proteins

Gene	Gene length (nt)	ORF length (from-to = total nts)	Protein length (aa)	Predicted molecular protein weight (kDa)
NP	1753	122–1591 = 1470	489	53.4
P	1451	1893–3080 = 1188	395	41.9
V	–	–	239	25.3
W	–	–	227	24.6
M	1241	3296–4390 = 1095	364	39.8
F	1791	4550–6211 = 1662	553	59.0[a]
HN	2002	6418–8133 = 1716	571	62.8[a]
L	6703	8387–15001 = 6615	2204	248.9

[a] The predicted molecular weights are excluding phosphorylation

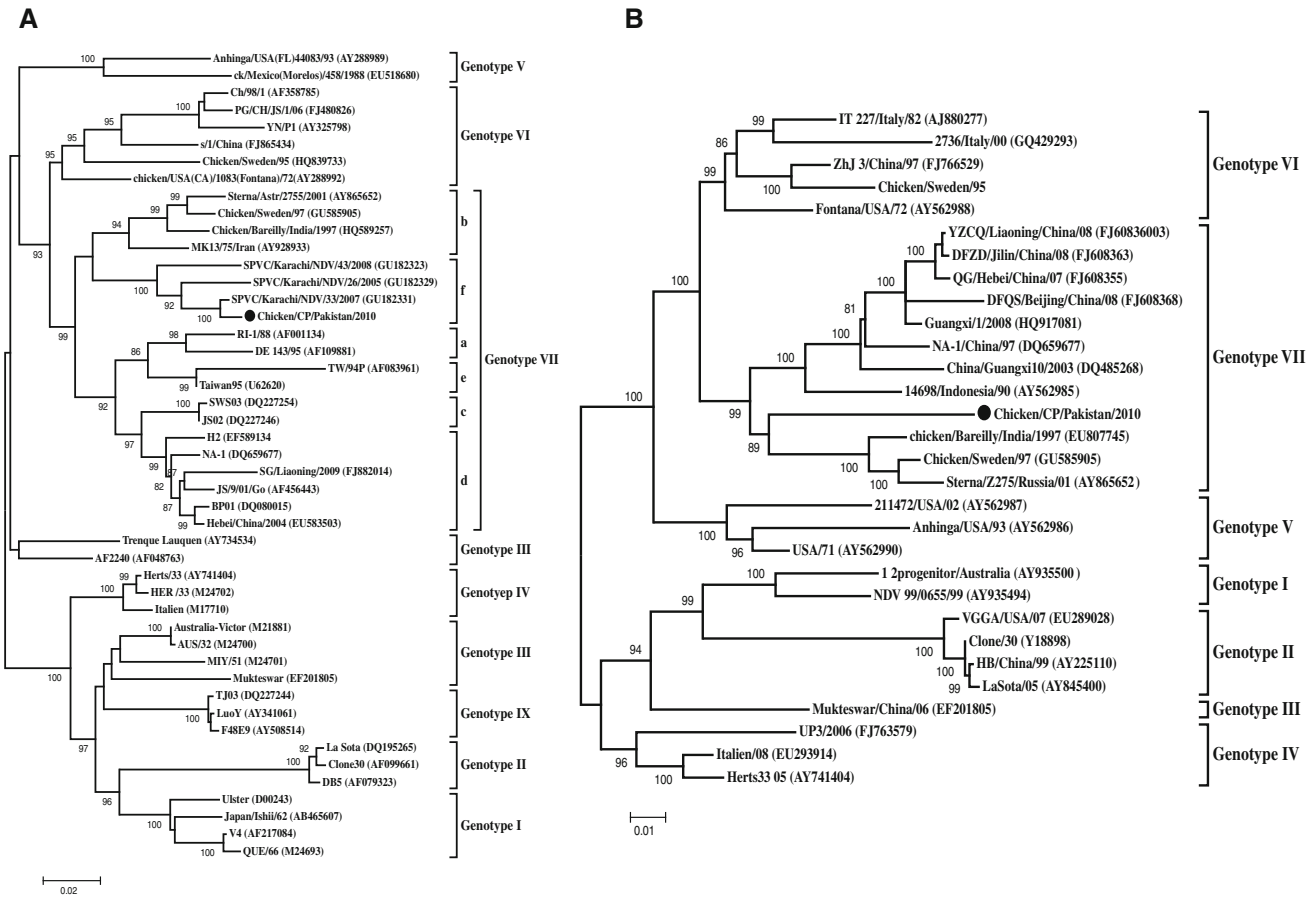

Fig. 1 Phylogenetic trees of Pakistani NDV strain Chicken/CP/Pakistan/2010 and other known NDV strains. Analysis was conducted in MEGA 5.03 using the neighbor-joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. Only values above 80% are shown. Chicken/CP/Pakistan/2010 is marked with block dot (●). (**A**) Phylogenetic tree of the complete open reading frame of the F gene. (**B**) Phylogenetic tree of the complete open reading frame of the HN gene

found to be ACGGGTAGAA, and for L, it was found to be ACGGGTAGGA. The GE for Chicken/CP/Pakistan/2010 was TTAGAAAAAA for NP, M, HN and L, whereas for P and F gene it was TTAAGAAAAAA. The complete genome sequence of Chicken/CP/Pakistan/2010 was highly similar (nucleotide identity of 90%) to that of chicken/Sweden/97 (GU585905) and Sterna/Astr/2755/2001 (AY865652). The chicken/Sweden/97 strain was reported from a commercial poultry farm in Skåne, in the south of Sweden [8], whereas Sterna/Astr/2755/2001 was isolated from a little tern (*Sterna albifrons Pallas*) in the Volga river delta in Russia. The identity to non-Asian isolates may be due to the unavailability of data from countries bordering Pakistan. In line with this, two Chinese isolates ND/03/018 (JF343538) and JSD0812 (GQ849007) showed a nucleotide identity of 89%.

Phylogenetic analysis

A phylogenetic tree was constructed based on the sequence of open reading frames of the F and HN genes of Chicken/CP/Pakistan/2010. The branching patterns of both trees clearly distinguished all of the genotypes of NDV (Fig. 1A, B). The analysis revealed that Chicken/CP/Pakistan/2010 grouped in genotype VII along with other strains of NDV previously characterized from Pakistan, and it was found to be closely related to the Indian, Iranian, Russian and Swedish strains of NDV (Fig. 1A), as expected from the sequence identity. It has been demonstrated that genotypes VII and VI are the most common genotypes circulating in Asia [11]. In Pakistan, both of these genotypes have been characterized recently [6]. Genotype VII is further subdivided into subgenotypes [9], with the isolate under study clustered with NDV strains belong to subgenotype VIIb (data not shown). However, the topology of the tree indicated that Chicken/CP/Pakistan/2010 grouped apart from subgenotype VIIb and could be regarded as separate subgenotype (VIIf). The robustness of the branching for a new subgenotype was supported by high bootstrap values. In the tree based on the HN gene, the Chicken/CP/Pakistan/2010 sequences clustered with isolates from India, Sweden,

Russia and China, but still branched separately from all these isolates (Fig. 1B). Since this genotype is most prevalent in Asia, most of the isolates were reported from China, a country that neighbors Pakistan, and Chicken/CP/Pakistan/2010 showed high nucleotide identity (89%) to these isolates.

In conclusion, a virulent Newcastle disease virus was isolated from a commercial poultry flock. Complete genome sequence analysis showed that this isolate clustered with NDV isolates of genotype VII and, more specifically, in subgenotype VIIb (VIIf), which is reported to be the most common genotype in Asia. This report is the first record of a complete genome sequence of an NDV isolate from Pakistan, and these findings provide insights into the genetic nature of the NDV isolates circulating in Pakistan. Moreover, our results will also be useful for laboratory diagnosis and vaccine development for NDV.

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Conflict of interest The authors declare that they have no conflict of interest.

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