Acta Tropica 172 (2017) 64–69

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Short Communication

Emerging variant of genotype XIII Newcastle disease virus from Northeast India

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ARTICLE INFO

Keywords:
Newcastle disease virus
RNA
Paramyxovirus
Poultry
Phylogenetic

ABSTRACT

Northeast India with its rich and diverse avifauna acts as a hotbed for emerging virulent Newcastle disease virus (NDV) strains. The present work describes the molecular and pathogenic characterization of NDV strain isolated from Pandu, Assam, India. Clinicopathological and genetic analysis showed the virulent nature of NDV strain Pandu. On molecular phylogenetic and evolutionary distance analysis, the NDV strain Pandu formed a distinct clade within the genotype XIII of class II NDV, suggesting a new sub-genotype XIIIc. The accumulation of mutations in the NDV strain Pandu makes it divergent enough to be considered as a new sub-genotype. The proposed NDV sub-genotype XIIIc consists of strains recently reported from eastern and northeastern India.

Newcastle Disease (ND) is one of the most notable viral diseases of the wild and domestic avian species. ND outbreaks are a constant drain on the economy due to a high rate of mortality, trade restrictions and its control measures. In the developing countries, ND is the principal constraint to the increasing small-scale poultry production. ND is caused by Newcastle disease virus (NDV), a variant of avian paramyxovirus-1 (APMV-1). NDV is one of the best characterized *Avulavirus* under the family *Paramyxoviridae* (Alexander and Senne, 2008b; Lamb et al., 2005). NDV is an enveloped virus containing a non-segmented, negative-sense, single-stranded RNA genome, which falls into three different genome size categories of 15,186 nt, 15,192 nt and 15,198 nt in length (Czegledi et al., 2006; de Leeuw and Peeters, 1999; Ganar et al., 2014; Huang et al., 2004; Krishnamurthy and Samal, 1998; Romer-Oberdorfer et al., 1999). All NDV strains encode six essential proteins, namely, nucleoprotein [N], phosphoprotein [P], matrix [M], fusion [F], hemagglutinin-neuraminidase [HN] and RNA-dependent RNA polymerase [L] (Alexander and Senne, 2008b; Lamb et al., 2005; Samal, 2011). The F glycoprotein is an integral membrane protein and a major protective antigen (Samal, 2011). Based on disease signs and lesions produced, NDV isolates have been historically classified into three major clinicopathologic groups, namely, lentogenic (low virulent), mesogenic (moderately virulent) and velogenic (highly virulent) (Hanson and Brandly, 1955; Samal, 2011). The activation of NDV infectivity depends upon cleavage of the F protein and hence, the amino acid sequence at the F protein cleavage site is established as the molecular determinant of NDV virulence (Nagai et al., 1976; Toyoda et al., 1987). The mean death time (MDT) in embryonated chicken eggs and intracerebral pathogenicity index (ICPI) in day-old chicks are the internationally accepted tests to determine the pathotype of NDV isolates (Alexander, 2009; Samal, 2011). According to the world organization for animal health (former Office International des Epizooties [OIE]), the virus should have an ICPI of ≥ 0.7 or at least three arginine or lysine residues between residues 113 and 116 and phenylalanine at residue 117 to be considered as virulent (OIE, 2012). Phylogenetically, strains of NDV are divided into two classes (class I and class II), and further classified into eighteen genotypes (I–XVIII) based on genetic differences (Dimitrov et al., 2016). Out of the eighteen genotypes, genotype I, II, VI and VII are further divided into different sub-genotypes (a–h) (Dimitrov et al., 2016; Kim et al., 2007; Liu et al., 2003; Miller et al., 2009; Snoeck et al., 2013). The most ancestral strain of genotype XIII NDV was recovered from a cockatoo (family *Cacatuidae*) in India in 1982 (Benson et al., 2014). NDV genotype XIII strains are divided into sub-genotype XIIIa and a recent sub-genotype XIIIb (Dimitrov et al., 2016). All viruses of genotype XIII are recovered predominantly from chickens and are virulent in nature. But a potential spillover of sub-genotype XIIIa virus from poultry to wild birds was reported in Russia (Usachev et al., 2006). Viruses of sub-genotype XIIIa are distributed in Europe, Asia, Africa and the Middle East whereas, that of sub-genotype XIIIb are detected in India and Pakistan (Benson et al., 2014; Cattoli et al., 2009; Ebrahimi et al., 2012; Gogoi et al., 2015; Jakhesara et al., 2014; Khan et al., 2010; Linde et al., 2010; Munir et al., 2012; Shabbir et al., 2012). In addition, recurrent outbreaks and recovery of sub-genotype XIIIb viruses in vaccinated and unvaccinated flocks in commercial and backyard poultry were reported in Pakistan (Munir et al., 2012). In recent years, several occasions of NDV sub-genotype XIIIb and XIIIc outbreaks were reported

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http://dx.doi.org/10.1016/j.actatropica.2017.04.018
Received 29 March 2017; Received in revised form 18 April 2017; Accepted 21 April 2017
Available online 24 April 2017
0001-706X/

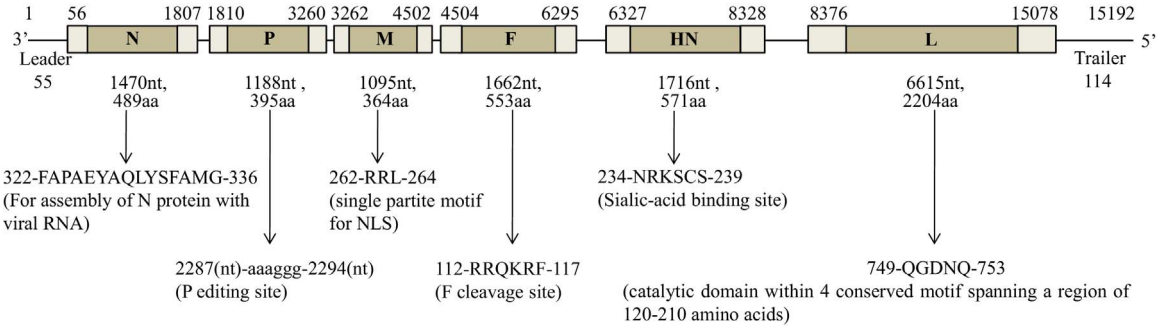

Fig. 1. Genetic characteristics, deduced protein characteristics and distinct features of NDV isolate Pandu.

from different geographically isolated parts of India (Das and Kumar, 2016; Gogoi et al., 2015; Kumar and Kumar, 2015; Morla et al., 2014; Morla et al., 2016; Nath et al., 2015).

NDV has a long history of evolution in Southeast Asia, which hosts the largest population of commercial and backyard poultry in the world (Roohani et al., 2015). Epidemiological data indicated origin of four major panzootics of ND in Southeast Asia (Alexander, 1988; Yu et al., 2001). The continuous accumulation of several point mutations leading to amino acid substitutions as well as selective immune pressure of several species of the host enhance the evolutionary process of NDV making Southeast Asia a hub of emerging virulent NDV strains which are divergent enough to be regarded as new sub-genotypes (Munir et al., 2012). In the Indian subcontinent, the Northeast region is a unique biodiversity hotspot with one of the richest and diverse avifauna. The region shares extensive and porous international boundaries with Bhutan, China, Myanmar and Bangladesh. Numerous short and long distance migratory birds criss-cross the region annually and semi-annually, which might be potential reservoirs of virulent NDV. Despite improved controls like adequate sanitary measures, good diagnostics and widespread vaccination, a remarkable percentage of poorly vaccinated commercial poultry and unvaccinated domestic poultry act as reservoirs of virulent NDV (Miller et al., 2015). Phylogenetically, genotype II, IV, VII and XIII strains are circulating in Southeast Asia (Esmaelizad et al., 2016; Jakhesara et al., 2014). In recent times, genotype XIII strain outbreaks are reported from eastern and northeastern India as well as from the neighboring countries (Das and Kumar, 2016; Miller et al., 2014; Nooruzzaman et al., 2013). Outbreaks of genotype XIII viruses are also reported from vaccinated flocks, suggesting its considerable divergence from the vaccine strains used, due to high rate of evolution (Khorajiya et al., 2016).

Chickens were reported to have died during January-February, 2015 at a poultry farm in Pandu, Guwahati, Northeast India. The Pandu port, located on the shores of the Brahmaputra river, is a major hub of river transport facilities. Even though the birds were vaccinated against NDV LaSota, respiratory distress, tracheal hemorrhage, caecal hemorrhage and lung congestion were the several clinical signs observed at death. Serum samples were collected from the ailing birds and were tested for the presence of NDV specific antibody by hemagglutination inhibition (HI) assay and commercial enzyme-linked immunosorbent assay (ELISA) [IDEXX, USA]. The virulence of the NDV strain Pandu was determined by the clinicopathological assays: MDT and ICPI following standard protocol (OIE, 2012). Infected tissue samples viz. brain, lungs, bursa, liver, kidney, intestine and spleen collected from the ailing and/or dead birds were homogenated and the supernatants were inoculated in the allantoic cavities of 9-days-old specific pathogen free embryonated chicken eggs. Allantoic fluids were collected 48 h post inoculation and the presence of the virus was confirmed by hemagglutination assay using 1% chicken erythrocytes. The isolated virus strain was plaque purified using chicken embryo fibroblasts (CEF) following standard protocol (Alexander and Senne, 2008a). A confluent monolayer of CEF cells were infected with tenfold serial dilutions of the virus (allantoic fluid) at a multiplicity of infection (MOI) of 0.01 and incubated at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) with 2% fetal calf serum (Gibco, USA) and antibiotics/antimycotics (Sigma, USA). The MOI of the NDV strain was established using the standard protocol (Kumar and Kumar, 2015). Viral genomic RNA was isolated from infected cell culture supernatant using TRIzol Reagent (Invitrogen, USA) and reverse transcribed by SuperScript™ III RT enzyme (Invitrogen, USA) and NDV specific consensus primers (Gogoi et al., 2015; Kumar et al., 2008; Paldurai et al., 2010). Genome editing and final assembly of the nucleotide sequence was performed using the Lasergene sequence analysis software package (Lasergene, version 5.07; DNASTAR, Inc., Madison, WI). Phylogenetic and evolutionary analysis of NDV strain Pandu was performed using MEGA6 software (Tamura and Kumar, 2002; Tamura et al., 2013). The F gene sequence of NDV strain Pandu was used to construct phylogenetic tree to infer its evolutionary relationship with other representative strains belonging to genotype I–XVIII. In addition, the F gene sequence was used to determine the phylogenetic relationships of NDV strain Pandu with other representative genotype XIII strains. The evolutionary distances between NDV strain Pandu and representative strains belonging to genotype II, IV, VII, XIIIa and XIIIb was computed since these are the commonly circulating genotypes in Southeast Asia. To further test sub-genotypes or clades within the genotype, evolutionary distances were computed between sub-genotype XIIIa, XIIIb and representative genotype XIII strain in India NDV isolate Cockatoo/India/7847/1982. For the assignment of sub-genotypes and genotypes the recently proposed nomenclature was followed (Diel et al., 2012).

The post-mortem analysis of the birds showed extensive hemorrhages in the lung. The collected serum sample was found positive for NDV by HI and ELISA. The MDT and ICPI of NDV strain Pandu were 49 h and 1.8, respectively. The plaque-purified NDV gave a titer of 2^7 HA units when grown in embryonated chicken eggs. The complete genome of NDV strain Pandu was sequenced and characterized. The genome was found to be 15,192 nt long (GenBank accession number KY774445). The features of all the six genes along with their deduced proteins are summarized in Fig. 1. The genetic characterization of NDV strain Pandu showed virulent cleavage site ^{112}-RRQKRF-^{117} in its F protein. The nucleotide and deduced amino acid sequence of the F and HN protein of NDV strain Pandu showed low sequence identity with the vaccine strain LaSota. The F and HN gene of NDV strain Pandu showed 83.5% and 81.7% nucleotide sequence identity with LaSota, respectively. Similarly, the F and HN protein of NDV strain Pandu showed 88.1% and 87.8% amino acid sequence identity with LaSota, respectively. On molecular phylogenetic analysis, NDV strain Pandu clustered with the strains of the genotype XIII (Fig. 2a). The NDV strain Pandu appeared to branch out as a topologically distinct clade within genotype XIII, along with two other isolates with a confident bootstrap value of 100% (Fig. 2b). On evolutionary distance analysis, NDV strain Pandu showed minimum distance of 9.2% and 11.2% with sub-genotype XIIIa and XIIIb viruses, respectively (Table 1a). In the present study, the data from phylogenetic and evolutionary analyses of NDV strain Pandu suggests its independent evolution among genotype XIII viruses. The