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Short communication

Molecular characterization of Newcastle disease virus strains isolated from different outbreaks in Northeast India during 2014–15

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ABSTRACT

Newcastle disease virus (NDV) isolates recovered from different outbreaks in chicken flocks in Assam during 2014–15 were genotypically and pathotypically characterized. Nucleotide sequence analysis of fusion (F) and hemagglutinin protein genes showed a close similarity with genotype XIII strains of NDV. Amino acid sequence of F protein showed a virulent cleavage site ^{112}R-R-Q-K-R-F^{117}. Furthermore, pathogenicity test in one-day-old chicks and embryonated chicken eggs showed a virulent pathotype of the isolated NDV strains. The study will help us to understand the biology of circulating strains of NDV in Northeastern part of India.

Newcastle disease (ND) is a highly infectious viral disease of domestic and wild avian species. ND was first reported from Java, in 1926 [1] and subsequently from the different parts of the world [2–8]. ND is endemic in many developing countries, including India. The Indian poultry industry suffers severe economic losses due to recurring episodes of ND outbreak in both vaccinated and unvaccinated flocks [9–12].

ND is caused by Newcastle disease virus (NDV) which belongs to the genus *Avulavirus* under the family *Paramyxoviridae* [13]. The genome of NDV comprises of a non-segmented, negative-sense, single-stranded RNA. The genome size of NDV varies between 15,186 and 15,198 nucleotides [14–18]. The typical NDV genome consists of six essential genes encoding nucleocapsid (N), matrix protein (M), phosphoprotein (P), fusion protein (F), haemagglutinin-neuraminidase protein (HN), and large polymerase protein (L). The F protein is considered to play a vital role in the virulence of NDV strains [17,19]. The F protein helps in the entry of the virus into the host cell by mediating fusion of the viral envelope with the plasma membrane [13]. The virulence of NDV depends upon the amino acid sequence at the cleavage site of the F protein [20,21]. The consensus amino acid sequence of the F protein cleavage site of velogenic and mesogenic strains is ^{112}R/K-R-Q-R/K-R↓F^{117}; whereas, that of lentogenic strains is ^{112}G/E-K/R-Q-G/E-R↓L^{117}. HN protein helps in the attachment of the virion to sialic acid-containing cell surface receptors [22]. HN also has neuraminidase activity which cleaves sialic acid from sugar side chains and releases progeny virions from the surface of infected cells [23]. The HN protein determines the tropism as well as the virulence of NDV by interacting with the F protein for fusion promotion [24–26].

NDV isolates can cause a wide range of clinical disease in chickens, ranging from asymptomatic to highly fatal. NDV isolates have been classified into three major pathotypes based on their disease symptoms and lesions produced in chickens: lentogenic (low virulent), mesogenic (moderately virulent) and velogenic (highly virulent). Velogenic viruses are further divided into viscerotropic and neurotropic based on its predilection site in the intestine and central nervous system, respectively [27]. Both live and inactivated vaccines against ND are commercially available. Lentogenic NDV strains B1 and LaSota are efficiently used as live attenuated vaccines worldwide including India [27,28]. Mesogenic NDV strains R2B, Mukteswar, Roakin and Komarov are used in countries where ND is endemic and where the risk of its outbreak is high.

In developing countries, the conventional live vaccines against NDV are not effective due to inappropriate cold chain and inappropriate storage condition. The problem aggravates more for the poultry production in tropical countries [29]. Inactivated vaccines

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are not that effective and occasionally cause necrosis because of adjuvants present in the vaccine formulation [30]. Moreover, recent studies from different countries suggested that there has been a substantial antigenic shift in the NDV strains circulating in poultry population [31–35]. The emergence of newer strains and failure of the vaccination are the major reasons of its outbreak in developing countries [29].

Geographically, ND imposes a serious threat to the poultry industry and economy of the Northeast part of India because poultry farming is a major source of people's livelihood. In addition, poultry products are integral parts of the diet for local inhabitants. Moreover, the Northeast India adjoins the countries like China, Bhutan, Myanmar and Bangladesh, where newer strains of NDV are regularly reported [36–40]. In the present study, we report the first comprehensive NDV outbreak from the Northeast part of India (Fig. 1). The results of our study will be useful to understand the biology of circulating strains of NDV in India.

Three different NDV outbreaks have been reported during the period of June 2014 to February 2015 from Northeast India. The details of the outbreak have been summarized in Table 1. The collected serum samples from the ailing birds were tested for the presence of NDV specific antibody by hemagglutination inhibition (HI) assay and commercial enzyme-linked immunosorbent assay (ELISA) [IDEXX, USA]. Infected tissue samples such as brain, bursa, lungs, liver, kidney, spleen and intestine were collected from ailing and/or dead birds. Collected tissues were fixed in 10% neutral buffered formalin for approximately 48 h. Formalin-fixed tissue samples were routinely processed for hematoxylin and eosin staining after paraffin embedding and sectioning (3 μm) following standard procedure [41].

Tissue samples containing viruses were inoculated in the allantoic cavities of 9-days-old specific pathogen free embryonated chicken eggs and infected allantoic fluids were collected 48 h post inoculation. The presence of virus in collected allantoic fluids was confirmed by hemagglutination assay using 1% chicken RBC. Moreover, the isolated virus strains were plaque purified using chicken embryo fibroblasts (CEF) following standard protocol [42]. Briefly, CEF were infected with virus at a multiplicity of infection (MOI) of 0.01 and incubated at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) with 5% fetal calf serum. The MOI of the NDV strains were established using the standard protocol [12].

The viral genomic RNA was extracted from the homogenized tissue samples using TRIzol® Reagent (Invitrogen, Grand Island, NY, USA). Reverse transcription (RT) was performed following the manufacturer's protocol using a SuperScript™III RT enzyme (Invitrogen, Grand Island, NY, USA) by NDV gene specific forward primer (NDV 4163 forward: 5′ AGC CTG CTA TCC YAT AGC AAA TGC 3′). The cDNA synthesised by the RT reaction was further amplified by PCR using NDV F and HN gene specific primer pairs (F 4544 forward: 5′ GCT GCT AGC ATG GGC TCC AGA CCT TC 3′ and F 6205 reverse: 5′ CGT GGT ACC TCA CAT TTT TGT AGT GGC 3′ HN 6412 forward: 5′ CGG GGT ACC ATG GAC MGC GCM GTT AG 3′ and HN 8147 reverse: 5′ CGG GAT CCC TAR CCA GAC CTG GCT TCT C 3′ where M stands for A/C, R stands for A/G. The number in the primer indicates the position of the gene with respect to the complete genome sequence of NDV). Degenerate consensus primers were designed using available GenBank sequences of different strains of NDV (GenBank accession numbers: NC_002617, FJ986192, AY562988, JF950510). The amplified 1661 and 1735 base pairs (bp) of F and HN genes, respectively were purified and cloned into pGEM®-T vector (Promega, Madison, WI, USA). The cloned gene products were sequenced by the Sanger sequencing method and analyzed by DNA Star software. The evolutionary relationship of isolated NDV strains was analyzed with available GenBank sequences using MEGA 5.2.2 software.

The mean death time (MDT) and intracerebral pathogenicity index (ICPI) values of NDV/Chicken/Hajo/01/14, NDV/Chicken/Polashbari/01/14 and NDV/Chicken/Pandu/01/15 suggest the velogenic nature of NDV strains (Table 1). All three isolates were named according to the place and year of isolation. The clinical signs observed among infected birds are summarized in Table 1. Microscopically, the liver showed severe haemorrhages along with multiple areas of focal aggregation of mono-nuclear infiltrating cells along with focal areas of coagulative necrosis and heterophils

Fig. 1. Geographical location of Northeast India where outbreaks occurred during 2014–15.