The Pharma Innovation Journal 2021; SP-10(11): 2512-2515

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; SP-10(11): 2512-2515

www.thepharmajournal.com
Received: 04-09-2021
Accepted: 22-10-2021

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Isolation and molecular detection of virulent Newcastle disease virus from outbreak in broilers in Assam

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Abstract
Poultry sector is the sustainable means of livelihood in developing countries like India. The major obstacle for the poultry producers comes from infectious diseases. Newcastle Disease (ND) caused by a virulent strain of *Avian orthoavulavirus 1* is the most economically important viral disease of poultry worldwide due to the substantial morbidity and mortality associated with it. This report describes an outbreak of an acute, highly lethal, ND in 3 weeks old age, vaccinated broiler chickens in a private broiler farm in Kamrup district, Assam. The farmer presented the infected and dead birds for diagnostic investigation. Ante mortem evaluation revealed severe neurologic signs, including tremors, ataxia, torticollis, and paralysis of wings and legs. Gross lesions included haemorrhagic intestinal lesions and characteristic haemorrhages at the proventriculus and in the caecal tonsils. The study aimed to isolate and identify Newcastle disease virus (NDV) by haemagglutination inhibition (HI) assay using NDV-specific hyperimmune serum and reverse transcription-polymerase chain reaction (RT-PCR) targeting *Fusion* (*F*) gene of NDV. Virus was isolated from samples of intestinal tissues, lungs, trachea and spleen in embryonated specific-pathogen-free (SPF) chicken eggs by allaontoic cavity route. The pathogenic evaluation of the isolated samples was determined on the basis of mean death time (MDT) in eggs and intracerebral pathogenicity test (ICPI) in day-old chicks. The suspected samples isolated in SPF chicken eggs were NDV positive in the haemagglutination assay. The virus was identified as NDV by the HI assay and RT-PCR of the isolate resulted in the amplification of 363 bp partial length *F* gene. Pathogenicity indices classified the NDV isolate as mesogenic strain. This study indicates that traditional NDV vaccines may not be sufficient to protect the birds against newly emerging virulent viruses, implying the requirement for a more efficient vaccine against virulent NDV infection.

Keywords: Newcastle disease virus, pathogenicity, reverse transcription-polymerase chain reaction, velogenic

Introduction
Newcastle disease (ND) also known as Ranikhet disease is a highly contagious viral disease of avian species causing serious economic losses in domestic poultry whose listed status with OIE marks its importance to both commercial poultry producers and poultry trading countries [1, 2]. ND is caused by *Avian orthoavulavirus1* (AOAV-1) formerly designated as *Avian avulavirus 1* (AAvV-1) or *Avian paramyxovirus 1* (APMV-1) classified under the genus *Orthoavulavirus* within subfamily *Avulavirinae* of the family *Paramyxoviridae*. The ND virus (NDV) genome is an enveloped, non-segmented, negative-sense, single stranded RNA of 15,186 to 15,198 nucleotide long flanking six genes. These genes encode a nucleocapsid protein (N), a phosphoprotein (P), a matrix protein (M), a fusion protein (F), a hemagglutinin-neuraminidase protein (HN) and a large polymerase protein (L). Additionally, two non-structural proteins, known as V and W. NDV strains have been broadly classified into two classes- class I and class II. The class I comprise a single genotype whereas the Class II viruses are genetically more diverse exhibiting wider range of virulence [3] as compared to the genetically less diverse Class I [4], and the complete analyses identified 21 distinct genotypes (I to XXI) [5]. The virus can infect more than 240 avian species [6]. The outbreak of ND in vaccinated flocks suggests the emergence of virulent strains resulting in high mortality and morbidity rate among affected birds. The pathotypes of NDV are viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic or respiratory and asymptomatic [7]. The assessment of virulence is conducted according to the mean death time (MDT) and intracerebral pathogenic test (ICPI) [8].
The present study aimed to isolate and identify NDV from specimens obtained from broilers during an outbreak in Kamrup district, Assam by haemagglutination inhibition (HI) assay

using NDV-specific hyperimmune serum and reverse transcription-polymerase chain reaction (RT-PCR) targeting *Fusion* (*F*) gene of NDV, and determine its pathogenicity.

Material and Methods

Ethical Approval

The Institutional Animal Ethics Committee, Assam Agricultural University, Khanapara, Guwahati-781022, Assam reviewed and approved this study (Approval No. 770/GO/Re/S/03/CPCSEA/FVSc/AAU/IAEC/19-20/736 dated 23.12.2019).

Case history

A total of 45 broilers 3 week old (30 infected and 15 dead) with suspected Newcastle disease virus (NDV) infection based on the characteristic clinical findings of greenish diarrhoea, depression, severe neurologic signs, including tremors, ataxia, torticollis, and paralysis of wings and legs were presented for diagnostic investigation. The broiler farm was well managed and ventilated, equipped with all necessary feeders, and water and light sources. The flock were routinely vaccinated against ND and Infectious bursal disease (IBD).

Sample collection and processing

Post-mortem examinations were conducted and gross evaluation of the organs were done. The lesions observed were recorded systematically and infected tissues i.e. intestinal tissues, lungs, trachea and spleen were collected under aseptic conditions from 25 diseased and/ or freshly-dead chickens. After collection, the samples were transported on ice packs and stored at -20^{0}C for further processing.

Virus isolation

Aseptically, small pieces of tissues, 2g each were ground in a sterile pestle and mortar, then transferred to sterile tubes containing antibiotic solution. The suspension of homogenized tissue samples were clarified by centrifugation, and the supernatant (100 µl) was used for inoculation in 9-days-old specific-pathogen-free (SPF) embryonated chicken eggs (ECE) (Venky's India Ltd., SPF Eggs Div., Pune) through the allantoic cavity route. Control embryonated eggs were inoculated with sterile phosphate buffered saline (PBS). Briefly, the inoculated eggs were incubated at 37^0 C for 3 days and checked daily for embryo viability. The embryos that died within 24 hours post-inoculation were discarded and considered death due to non-specific causes. All embryos that died after 24 hours or survived until the end of incubation were chilled at 4^0 C overnight and the allantoic fluid was harvested. The haemagglutination (HA) activity of the harvested allantoic fluid was checked by HA assay using 1% (v/v) chicken red blood cells (RBC).

Serological identification of NDV

The hyperimmune serum was generated in in-house chickens immunized with NDV LaSota vaccine strain (Venkateshwara Hatcheries Pvt Ltd, Maharashtra, India). The identity of the HA positive allantoic fluid were confirmed by haemagglutination inhinition (HI) assay following standard procedure [8]. Briefly, 4 HA units (HAU) of antigen were reacted against antisera against LaSota and serially diluted wo-fold in PBS for 30 minutes at room temperature. Thereafter, an equal volume of 1% chicken RBC was added. After an additional incubation of 30 minutes, the HI endpoint was determined as the reciprocal of the highest dilution of serum causing complete inhibition of 4HAU antigen.

Biological pathogenicity assessment of NDV

The pathogenic evaluation of the NDV isolate was detremined by MDT and ICPI.

MDT

The MDT was determined by inoculating in 9-days-old SPF ECE as described [8, 9] with ten-fold serial dilutions of NDV isolate and incubating at 37^0 C until the embryos died. The velogenic and lentogenic strains of NDV caused embryo death within 60 hours and >90 hours post-inoculation respectively, and those that died >60 hours but <90 hours were considered mesogenic.

ICPI

ICPI was determined in 1-day-old SPF chicks as described [8]. Briefly, 50 µl of a 1:10 dilution of fresh infected allantoic fluid with a HA titre 2^5 was inoculated intracerebrally into 10 chicks and examined every 24 hours for 8 days. The ICPI was obtained by calculating the mean score per bird per observation over the 8-day period and scored as (0) if normal, (1) if sick, ad (2) if dead.

RNA extraction and RT-PCR amplification of NDV F-gene

The presence of NDV in the allantoic fluid was further reconfirmed by RT-PCR. Allantoic fluid was used for RNA extraction using Qiagen RNeasy Extraction Kit (Hilden Germany) following the protocol provided by the manufacturer. RNA extracted was subjected to two step RT-PCR using Revert Aid (M-Mulv) (Thermo Fisher). RT-PCR was performed as described by [10] with slight modification. The amplification cycle started with reverse transcription at 50^0 C for 30 minutes, initial denaturation at 94^0 C for 5 minutes, followed by 35 cycles with denaturation at 94^0 C for 30 seconds, annealing at 56^0 C for 1 minute, extension at 72^0 C for 2 minutes, and final extension at 72^0 C for 5 minutes. The PCR products were subjected to 1.5% agarose gel electrophoresis containing ethidium bromide (0.5µg mL^{-1}) and visualized in a ultra-violet (UV) transilluminator.

Table 1: Specific primers used for RT-PCR

Sl. No.	Gene	Primer	Primer Sequence	PCR product	Reference
i.	F	NDV-F	5´-TTGATGGCAGGCCTCTTGC-3´	363 bp	[10]
ii.	F	NDV-R	5´-GGAGGATGTTGGCAGCATT-3´		

Results

Clinical manifestations

The clinical signs of the affected broiler chickens were greenish diarrhoea, depression, severe neurologic signs, including tremors, ataxia, torticollis (Fig 1), and paralysis of wings and legs.

Fig 1: Broiler chick showing torticollis (twisting of the neck)

Gross lesions

By observing the clinical signs and post-mortem findings, the case was tentatively diagnosed as ND. At necropsy, gross lesions included haemorrhagic intestinal lesions and characteristic haemorrhages at the proventriculus (Fig 2) and in the caecal tonsils.

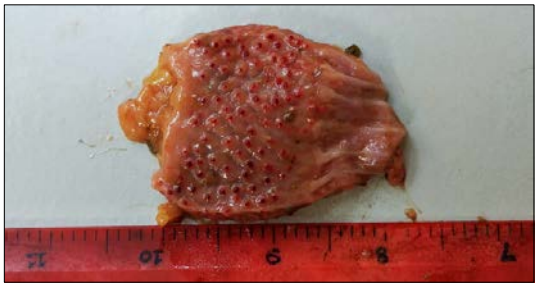

Fig 2: Haemorrhages at the tip of proventriculus

Isolation and identification of NDV

The isolated NDV were positive in the HA assay and the HA of these sample was inhibited by NDV-specific hyperimmune serum in HI assay, which is confirmatory for NDV [11]. All NDV-infected embryos showed haemorrhage in all parts of the body, whereas the control showed healthy embryos and no haemorrhage (Fig 3).

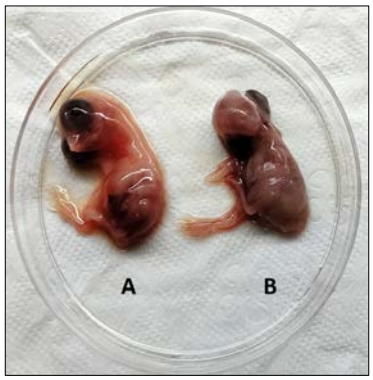

Fig 3: Gross pathology of the embryo post inoculation with the NDV isolate (A) Infected embryo showing haemorrhage (B) Control healthy embryo

Viral pathogenicity

In the present study, the biological pathogenicity assessment showed that the NDV isolate was mesogenic with MDT and ICPI 77.8±2.91 and 1.05±0.02 respectively.

Confirmation of NDV by RT-PCR

RT-PCR using NDV specific primers successfully amplified a 363 bp fragment covering a part of F gene of NDV (Fig 4).

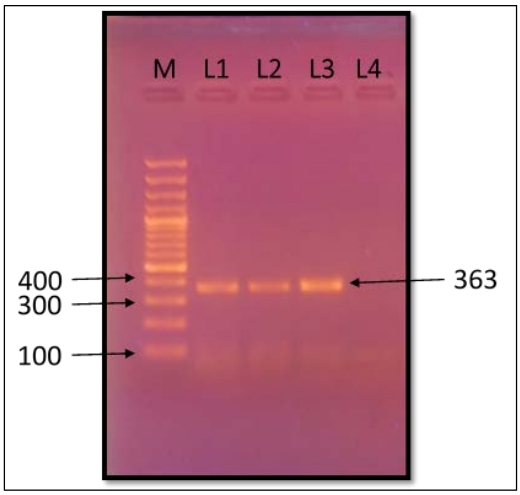

Fig 4: Confirmation of the NDV isolates through PCR. PCR carried out with *F* gene specific primers. Lane 1 and Lane 2 shows 363 bp *F* gene amplification product of NDV isolate; Lane 3, positive control (ND LaSota vaccine strain); Lane 4, negative control and Marker, a 100bp DNA ladder (Thermo Scientific)

Discussion

Biosecurity and vaccination are indispensable in controlling economically devastating diseases, such as ND. Despite immunization regimens, ND continued to occur in vaccinated flocks [12]. These vaccines when given to health birds prevent death and disease, but may not prevent shedding and transmission of challenge viruses [13]. Virulent Newcastle disease virus (vNDV), formerly known as Exotic Newcastle disease (END) is an OIE notifiable avian disease due to the socioeconomic impact on trading restrictions and its embargoes in endemic areas of its outbreak [8]. The virulence of NDV varies greatly with the host and chickens are highly susceptible [14]. The disease can vary from mild to severe and clinical signs in NDV infected birds also vary widely. On the basis of clinical and pathologic manifestations in infected chickens, NDV has been categorized into five pathotypes, namely, viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic or respiratory and asymptomatic [7]. Nevertheless, if the disease is endemic, clinical signs and lesions may be considered highly suggestive. Typical signs of ND are a state of prostration, depression in the infected birds, greenish white diarrhoea, neurological signs, including tremors, ataxia, torticollis, and paralysis of wings and legs. On necropsy, typical lesions are haemorrhage in the intestine, particularly in the proventriculus [15, 16].

Both isolation of virus and laboratory characterization are necessary for definitive diagnosis of ND. The present study isolated and identified NDV from samples collected from naturally infected broiler chickens. Formerly, pathogenicity assay like MDT and intravenous pathogenicity test (IVPI) have been used [17], but the OIE defines the assessment of virus virulence based on the ICPI. The pathogenicity indices indicated that the isolated NDV was mesogenic with MDT and ICPI ranging from >60 hours to <90 hours and 0.7 to 1.3 respectively [8]. Similar studies, reported the isolation of virulent NDV from different outbrelas in chicken flocks in Assam [18, 19]. Molecular assays like the RT-PCR has the potential for rapid and sensitive detection of NDV in different

tissue samples [20, 21] and therefore, can be introduced for routine diagnostic tool.

Conclusion

In the present study we isolated and identified a mesogenic strain of NDV that circulated among poultry farm in Kamrup district, Assam. This study indicates that traditional NDV vaccines may not be sufficient to protect the birds against newly emerging virulent viruses, implying the requirement for a more efficient vaccine against virulent NDV infection.

Acknowledgements

We acknowledge the DBT Twinning Project "An integrated omics approach to characterize circulating Newcastle disease virus and intervention strategies to control Newcastle disease in Northeast India" BT/ PR24911/NER/95/903/2017 Dated 26/06/2017, Department of Veterinary Microbiology, College of Veterinary Science, AAU, Khanapara, Guwahati-22, Assam for providing research facility to accomplish this study.

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