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Isolation and Characterization of the Newcastle Disease Virus (NDV) of Haryana Region Based on *F*-gene Sequence

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ABSTRACT

Newcastle disease virus (NDV) continues to cause serious economic losses in global chicken production. The objective of the present study was to use a rapid and efficient method for determining pathotype of NDV of poultry from field specimens by using reverse transcription polymerase chain reaction (RT-PCR) and *in silico* restriction endonuclease enzymes (REA) method. The field samples were investigated for identification, differentiation and pathotyping of NDV by using amplification of RT-PCR followed by *in silico* REA of F protein encoding gene. The RT-PCR amplification for *F* gene NDV generated DNA fragments in size of 356 bp. To differentiate virulent and avirulent NDV, the RT-PCR product of *F* gene in size of 356 base pairs (bp) was analyzed by *Hha*I and *Bgl*I restriction enzymes. Out of one hundred twenty field samples examined, nineteen were found positive for NDV and among these nineteen positive samples, sixteen samples were lentogenic, two samples were mesogenic and one sample was velogenic NDV. RT-PCR combined with *in silico* REA method by using *Hha*I and *Bgl*I enzymes can be used to determine pathotype of NDV from field specimens.

Keywords: Newcastle Disease Virus, Fusion protein, Restriction Endonuclease Analysis

Newcastle disease (ND) is a highly contagious viral disease of poultry and other birds causing significant economic loss to poultry industry worldwide. The disease is highly devastating, characterized by gastrointestinal and respiratory signs, often associated with nervous disorders with mortality up to 100%. ND is an OIE listed disease and is widespread in most of the countries worldwide except Oceania countries (Dimitrov *et al*., 2016). The causative agent, Avian Paramyxovirus Serotype-1 (APMV-1) synonymous with Newcastle disease virus (NDV) belongs to genus *Avulavirus*, of subfamily *Paramyxovirinae* within family *Paramyxoviridae* of order *Mononegavirales* (ICTV, 2017). NDV is an envelope, single stranded RNA virus with genome size of 15 kb with negative polarity which encodes for six major structural and non-structural proteins.

The pathotyping of NDV was done based on different criteria as defined by the Office International des Epizooties (2016). Conventionally, pathotyping is done on the basis of isolation of the virus in embryonated chicken eggs, followed by *in-vivo* tests, such as the intra-cerebral pathogenicity index (ICPI), Intravenous pathogenicity index (IVPI) and the Mean death time (MDT) in specific pathogen free (SPF) birds/embryos. These tests are labour intensive, time consuming, expensive and inhumane (Seal *et al*., 1995). The reverse transcription polymerase chain reaction (RT-PCR) targeting partial *F*-gene that encodes the cleavage site of the Fusion protein and restriction enzyme digestion using different RE enzymes is one of the easy way to detect the virus and to categorize the virus in different pathotypes (Seal *et al.,* 1995; Marin *et al.,* 1996; Kant *et al.,* 1997).We describe here the differentiation of Indian pathotypes (Haryana region) of NDV using RT-PCR amplified product sequence of *F*-gene encoding the Fusion protein cleavage site followed by *in-silico* restriction enzyme analysis.

MATERIALS AND METHODS

Sample collection

One hundred twenty (120) NDV suspected field samples were collected aseptically in 50% buffered glycerin from different flock, located in various parts of Haryana state *viz*. Jind, Safidon, Kaithal, Sirsa and Hisar. Investigated flocks were suffering from respiratory distress (rales, sneezing and wet eyes) and/or nervous manifestations (head deviation and torticollis). Dead and moribund birds were subjected to necropsy and tissue samples were collected including lung, proventriculus, liver, duodenum, spleen, and caecal tonsils. The tissues were pooled and kept at -20 °C till further processing.

Processing of samples

Tissue samples (around 50-100mg) from each flock were pooled and triturated in sterile phosphate buffer saline (PBS) pH 7.0–7.4 to make 10% suspension containing gentamycin (50 µg/ml) and mycostatin (1000 units/ml) in a 1:5 (w/v) dilution. After centrifugation, the supernatant was collected and stored at -20 °C till being used in virus detection and isolation.

Haemagglutination assay

Spot haemagglutination assay was performed by mixing equal volume of 10% chicken RBC's suspension prepared in PBS (pH 7.0-7.4) with equal volume of sample tissue homogenate supernatant on a glass petri plate and observed for the formation of agglutinates within five minutes (OIE, 2012). The agglutination of test sample was compared with the positive and negative controls. The suspension of commercial vaccine used in India was used as positive control and PBS was used as negative control.

Hyperimmune serum preparation

Live attenuated lentogenic NDV vaccine was mixed with the sterile water. Rabbit was inoculated subcutaneously (0.5 ml) with live attenuated vaccine containing approximately 128 HA unit of the virus. Rabbit received a booster immunization 2 weeks later and sera were collected 2 weeks after the booster dose. Sera were heat inactivated at 56 °C for 30 min (Allan and Gough, 1974). The hemagglutination inhibition (HI) test was conducted using 4 HA units of two-fold diluted serum and titers were determined using 1% chicken red blood cells (OIE, 2012).

RT-PCR amplification of NDV *F*-gene

Extraction of total RNA

Total RNA from the suspected samples homogenates was extracted using TRIzol reagent (Life Technologies, USA) as per the manufacturer's protocol. To 700 µl of tissue homogenate supernatant (containing 50-100 mg tissue), 1.0 ml of TRIzol was added in a tube, vortexed and incubated for five minutes at room temperature. After that, 200 µl of chloroform was added per one ml of TRIzol reagent, vortexed vigorously for 20 s and incubated at room temperature for 2–5 min. The suspension was centrifuged ≥ 12,000xg for 15 min at 4°C for phase separation. The upper aqueous phase was transferred carefully into fresh tube without disturbing the interphase. The RNA was precipitated from the aqueous phase by mixing of 500 µl of isopropyl alcohol per one ml of TRIzol reagent. Samples were incubated at 15–30°C for 10 min and centrifuged at 12,000xg for 10 min. The RNA pellet was then washed with 75% ethanol, by adding at least 1 ml of 75% ethanol per one ml of TRIzol reagent. The samples were mixed gently and centrifuged at 7,500xg for 5 min. The supernatant was discarded carefully and the RNA pellet was air dried and resuspended in 30 µl RNase free water and stored at -20 °C till further use.

Spectrophotometric analysis

The eluted RNA was quantified by NanoDrop 2000 spectrophotometer. The purity of the RNA was judged using A260/280 ratio and was ≥1.8-2.0. The RNA was stored at -80ºC until used.

cDNA synthesis and PCR

Total RNA isolated from tissue was used for the preparation of cDNA employing Revertaid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA), following manufacturer's instructions. Briefly, 2 µl of total RNA and 1 µl of random hexamer primer were made up to make total volume of 12 µl with nuclease free water (NFW) and then incubated at 25°C for 5 min. After that following mixture

was added: Reaction buffer 5 × (4 µl), Ribolock™ RNase inhibitor (1 µl), 10 mM dNTP mix (2 µl) and MMuLV (Moloney murine leukemia virus) reverse transcriptase (1 µl). The tube was spinned for proper mixing and incubated at 42°C in thermocycler for 60 min for cDNA synthesis. The reaction was terminated by heating at 70°C for 5 min. The cDNA product was stored at -20 °C, until further use.

PCR was performed using 6 µl of cDNA from previous step as template DNA and 10 pmol of each forward and reverse primers, 10 µl of 5X Dream Taq Green PCR Master Mix (Invitrogen, USA), NFW was used to make a total volume of 50 µl reaction mixture. A pair of published primers was used to amplify 356 bp sequence of the *F* gene encoding the fusion protein cleavage site (Table 1).

Table 1: Primers used to amplify *F gene* of ND Virus

Target Gene	Primer Sequence (5'-3')	Location	RT-PCR product
F-gene NDV	F-GCAGCTGCAGGGA TTGTGGT	158-177	356 bp
	R-TCTTTGAGCAGGA GGATGTTG	513-493	

The incubation temperature and duration of each cycle of the PCR were 94°C for 3 min for initial denaturation followed by 35 cycles of 94°C for 45s of cyclic denaturation, 58°C for 45s for annealing and 72 °C for 45s for extension. A final extension step was performed at 72°C for 5 min. The amplified PCR products was analyzed by agarose gel electrophoresis using 1.5% agarose gel with ethidium bromide along with the 100 bp DNA marker (Invitrogen, USA).

Gel extraction and sequencing

Gel purification of amplified PCR product was done using Gel extraction kit (Invitrogen, USA), according to manufacturer's guidelines. Purified PCR products of 19 NDV positive samples were submitted for nucleotide sequencing to the DNA sequencing facility in the Department of Animal Biotechnology, LUVAS, Hisar (Haryana), India using gene specific primers. Purified sequences were analyzed for identity using NCBI-BLAST. All the sequences were submitted to NCBI-GenBank.

Virus isolation

Nineteen processed positive samples were inoculated into the allantoic sac of 9-day-old specific pathogen free embryonated chicken eggs (SPF-ECE) (OIE, 2012). Inoculated eggs were incubated at 37 °C for 5 days and candled daily for embryo viability. All inoculated SPF ECE were kept at 4 °C for overnight. Allantoic fluids were harvested from chilled inoculated SPF ECEs (both dead and surviving embryos) and tested for hemagglutination using 1% washed chicken red blood cells (OIE, 2012). Initial virus identification was conducted using hemagglutination inhibition test with hyper immune sera raised against NDV raised in rabbit in Department of Veterinary Microbiology, LUVAS, Hisar.

Restriction enzyme analysis for pathotyping of NDV

In-silico RE analysis was done using software NEB cutter V2.0 (Biolab) with *Alu*I, *Bgl*I, *Hae*III, *Hinf*I, *Hha*I, *Rsa*I and *Sty*I enzymes as described previously for pathotyping of the NDV (Ballagi-Pordany *et al.,* 1996; Kant *et al*., 1997). Sequence having site for *Bgl*I was categorized as lentogenic, which had site for *Hha*I but devoid of *Bgl*I was categorized as mesogenic while samples devoid of site for *Hha*I and *Bgl*I was categorized as velogenic (Nathankumar *et al.,* 2000).

RESULTS AND DISCUSSION

Pathotyping of NDV was conducted by RT-PCR followed by *in silico* REA. In this study the specific sequence corresponding to the primers for partial *F*-gene was amplified by RT-PCR, which was shown as band of size of 356 bp in agarose gel electrophoresis. The specificity of the RT-PCR amplification was shown in negative control by the absence of 356 bp DNA fragment as well as the presence of 356 bp DNA fragment in positive control. Electrophoresis results of RT-PCR products were presented in Fig. 1. The nineteen samples were found spot HA test positive, the result of spot HA was shown in Fig. 2.

The BLAST analysis of the sequences of the PCR positive samples showed close homology with NDV sequences confirming the identity of the viruses as NDV. The *in-silico* RE analysis of positive sample amplicon sequence to determine the virus pathotype revealed the presence of all the three pathotypes of the NDV among the samples

under study including one velogenic, two mesogenic and sixteen lentogenic type (Table 2).

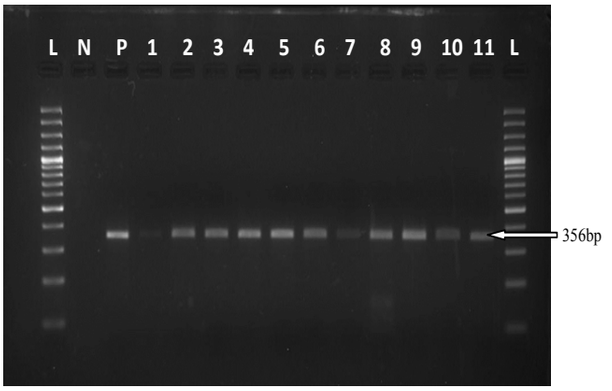

Fig. 1: Amplification of partial F-gene of NDV by RT-PCR

Lane L: 100 bp plus DNA ladder as molecular size marker; **Lane N**: Negative control; **Lane P**: Positive control; **Lane 1 to Lane 11**: Field samples positive for NDV *F*-gene amplification

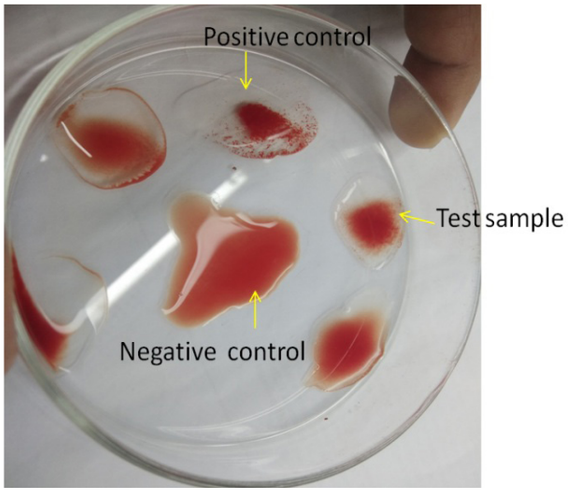

Fig. 2: Representative sample showing positive Spot HA

The technique of RT-PCR followed by restriction enzyme digestion has been exploited by many workers to differentiate the pathotypes of NDV (Ballagi-Pordany *et al.,* 1996; Kant *et al.,* 1997). In the present study, a product of NDV specific 356 bp of *F*-gene could be generated by RT-PCR of suspected field samples for NDV in positive cases. The authenticity of these primary amplicons was confirmed by agarose gel electrophoresis and RT-PCR.

Table 2: Pathotyping of samples on the basis of sequencing and *In-silico* RE analysis

Sample ID	Accession number	Haemagglutination Assay (SPOT)	RE site and Type	Pathotype
LUVAS/ NDV001	MH460386	Yes	*Bgl*I-188nt	lentogenic
LUVAS/ NDV002	MH460387	Yes	*Bgl*I-193nt	lentogenic
LUVAS/ NDV003	MH460388	Yes	*Bgl*I &*Hha*I-ab	velogenic
LUVAS/ NDV004	MH460389	Yes	*Bgl*I-175nt	lentogenic
LUVAS/ NDV005	MH460390	Yes	*Bgl*I-172nt	lentogenic
LUVAS/ NDV006	MH460391	Yes	*Hha*I-55nt	mesogenic
LUVAS/ NDV007	MH460392	Yes	*Bgl*I-188nt	lentogenic
LUVAS/ NDV008	MH460393	Yes	*Bgl*I-183nt	lentogenic
LUVAS/ NDV009	MH460394	Yes	*Bgl*I-188nt	lentogenic
LUVAS/ NDV0010	MH460395	Yes	*Bgl*I-168nt	lentogenic
LUVAS/ NDV0011	MH460396	Yes	*Bgl*I-167nt	lentogenic
LUVAS/ NDV0012	MH460397	Yes	*Bgl*I-190nt	lentogenic
LUVAS/ NDV0013	MH460398	Yes	*Bgl*I-183nt	lentogenic
LUVAS/ NDV0014	MH460399	Yes	*Hha*I-52 nt	mesogenic
LUVAS/ NDV0015	MH460400	Yes	*Bgl*I-188nt	lentogenic
LUVAS/ NDV0016	MH460401	Yes	*Bgl*I-167nt	lentogenic
LUVAS/ NDV0017	MH460402	Yes	*Bgl*I-175nt	lentogenic
LUVAS/ NDV0018	MH460403	Yes	*Bgl*I-188nt	lentogenic
LUVAS/ NDV0019	MH460404	Yes	*Bgl*I-188nt	Lentogenic

According to Nathankumar *et al*. (2000) restriction enzyme digestion with *Bgl*I could differentiate the lentogenic strains (F and LaSota) from the mesogenic (R2B) and velogenic strains (NP1-93, UP1-93 and WB1-94). Restriction enzyme *Hha*I and *Bgl*I could differentiate mesogenic and lentogenic strains from velogenic isolates. Therefore, based on restriction enzyme analysis with these two enzymes, it is possible to differentiate all three pathotypes of NDV, without any exceptions. We classified the pathotypes *in silico* using these two REs with the help of NEB cutter V2.0 (Biolab). The presence of *Bgl*I cutting site in the *F* gene, is classified as Lasota or lentogenic pathotype, Absence of *Bgl*I RE site refers to other than lentogenic pathotype. The sequences showing absences of *Bgl*1 RE was checked for the *Hha*I RE. Presence of this RE site (*Hha*I) were classified as mesogenic pathotypes while velogenic is classified as absence of both REs sites (*Hha*I *& Bgl*I). Thus with the help of software NEB cutter V2.0 (Biolab) the different RE site can be located. Based on the presence or position of specific RE site we can differentiate the virus in different pathotypes universally. Use of software reduces our laboratory work load and also the expenditure on chemicals. Thus, this method is helpful in hassle free pathotyping of virus.

CONCLUSION

Amplification of *F* gene NDV by RT-PCR generated DNA fragments in size of 356 bp. RT-PCR and *in silico* REA method by using *Hha*I and *Bgl*I restriction enzymes can be used to determine pathotype of NDV from field specimens. Out of hundred twenty field samples, nineteen samples were positive by RT-PCR, spot HA and HI using hyperimmune serum against NDV. Pathotyping on the basis of *in silico* REA revealed one sample was velogenic, two samples were mesogenic and sixteen samples were lentogenic NDV. RT-PCR and *in silico* REA methods is appropriate with DNA sequencing method to determine the pathotype NDV.

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