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## Detection of Newcastle Disease Virus Genome from the Field Outbreaks in Poultry by Reverse Transcription – Polymerase Chain Reaction

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**Abstract:** A total of 30 field samples (27 tissue samples comprising of either trachea, lungs or brain; and 3 allantoic fluid samples) were collected from broiler chickens for detection of Newcastle disease virus (NDV) by RT-PCR. In addition, vaccine strains (F, LaSota, R<sub>2</sub>B) were taken as positive control and trachea from unvaccinated healthy bird as negative control. Newcastle disease virus (NDV) was detected in five field samples as well as in all the vaccine strains by RT-PCR. All these samples as well as vaccine strains yielded a band of 356 bp on amplification of F region of NDV. Three more field samples yielded a band of 216 bp with nested PCR, thus making total eight field samples positive for NDV. The results of the present study indicated that RT-PCR followed by nested PCR can be used to detect NDV directly from tissue samples in poultry.

**Key words:** Newcastle disease virus, RT-PCR, Nested PCR

### Introduction

Newcastle disease (ND) is a highly contagious viral disease that affects over 250 species of birds of all age groups (Alexander *et al.*, 1997). It is caused by a single stranded, enveloped, non-segmented RNA virus i.e. Avian Paramyxovirus serotype-1 (APMV-1) belonging to genus *Avulavirus* of family Paramyxoviridae (Mayo, 2002). The genome of Newcastle disease virus (NDV) is of 15,186 bases (de Leeuw and Peeters, 1999) and encodes for six structural proteins in the order 3'-NP-P-M-F-HN-L-5' (Wilde *et al.*, 1986). Now it is believed that fusion (F) protein is a major determinant for virulence (Peeters *et al.*, 1999).

Newcastle disease virus is categorized into lentogenic, mesogenic and velogenic strains on the basis of pathogenesis and virulence. The disease, on the basis of virulence and clinical signs, is characterized into different pathotypes viz: viscerotropic velogenic (Doyle's form), neurotropic velogenic (Beach's form), mesogenic (Beudette's form), lentogenic (Hitchner's form) and asymptomatic enteric (Beard and Hanson, 1984). The morbidity and mortality in a flock varies according to the strain involved, thereby causing considerable economic losses to the poultry industry throughout the world.

In the past, the diagnosis of ND has been based on serological tests like complement fixation test, haemagglutination inhibition test, virus neutralization test, single radial immunodiffusion test, immunoperoxidase assay, enzyme-linked immunosorbent assay, plaque neutralization, agar gel precipitation test, virus isolation etc. These methods are

less sensitive as well as time consuming. In the recent past, molecular techniques like polymerase chain reaction (PCR) have been frequently used all over the world to detect NDV in the field samples using F protein (Kant *et al.*, 1997, Gohm *et al.*, 2000, Creelan *et al.*, 2002, Meulemans *et al.*, 2002 and Mathivanan *et al.*, 2004). The present work aims at detection of NDV from poultry in Haryana state by RT-PCR.

### Materials and Methods

**Collection of samples:** A total of 30 samples from broiler chickens were collected from Hisar, Bhiwani, Ambala, Jind, Sonapat, Karnal, Yamunanagar, Sirsa and Fatehabad districts of Haryana state. Of these, 27 were tissue samples (brain, trachea or lungs) and 3 samples were in the form of allantoic fluid suspected to be positive for NDV. In addition, six vaccine strains (LaSota, R<sub>2</sub>B and F- strain) of different firms, currently used in this region were also included in the study as positive controls. Tissue (trachea) from a healthy non-vaccinated bird was also taken as negative control. The tissue samples after collection in 50% buffered glycerine, were kept at -20° C till further use.

**Total RNA extraction:** Total RNA from the tissue samples (brain, trachea, lungs) was extracted with the TRIzol reagent (Life Technologies, USA) as per the manufacturer's protocol. In brief, about 100 mg of the suspected tissue was homogenized in pestle and mortar with 1.0 ml TNE buffer. It was taken in a microfuge tube and was then centrifuged at 12,000x g for

5min. The supernatant (600µl) was taken out and 1.0 ml of TRIzol was added to it. The lysate was then incubated at room temperature for 5min. to permit complete dissociation of nucleoprotein complexes. To it, 200µl of chloroform was added and was then shaken vigorously for 15sec. After shaking, it was again centrifuged at 12,000x g for 15min. The upper aqueous phase was carefully taken in another microfuge tube. To it, 0.5 ml of isopropanol was added so as to precipitate the total RNA from the supernatant. It was kept at room temperature for 10min. and then centrifuged at 12,000x g for 10min. for pelleting the RNA. After washing with 75% ethanol, the dried RNA pellet was dissolved in 30µl RNase free water and then stored at -20°C till use.

RNA from trachea of an unvaccinated, healthy bird was also extracted in the same manner. The same protocol was followed for RNA extraction from the vaccine strains as well as the allantoic fluid. The extraction was initiated with 500 µl of the vaccine strain and the allantoic fluid.

#### Reverse Transcription- Polymerase Chain Reaction

**(RT-PCR):** RT was carried out using 8µl of total RNA extracted following the protocol described by Nanthakumar *et al.* (2000). cDNA was synthesized using 50µg random hexamer primers, 100µg heat denatured viral RNA, 40 units RNase inhibitor, 1µl 10mM dNTPs, 2µl 0.1 M DTT, 4µl 5X RT buffer and 300 units MMLV-RT (MBI Fermentas) in 20µl reaction mixture. After allowing the random primers to anneal further at 25°C for 10 min., reverse transcription was carried out at 37°C for 1 hr. The enzyme reverse transcriptase was heat inactivated at 95°C for 3 min. The oligonucleotide primers forward- 5'- GCAGCTGCAGGGATTGTGGT-3' (nucleotide position 158-177) and reverse - 5'- TCTTTGAGCAGGAGGAT GTTG-3' (nucleotide position 493-513) as described by Nanthakumar *et al.* (2000) were used for the amplification of 356 bp amplicons corresponding the cleavage activation site of F gene of NDV. For PCR, 6µl of cDNA was incubated in a total volume of 50µl reaction mix containing 5µl 10X PCR buffer (without Mg<sup>++</sup>), 15 pmol each of the forward and reverse primers, 1µl 10mM dNTPs mix, 3 units Taq DNA polymerase (Bangalore Genei, India) and 0.9 mM Mg<sup>++</sup>. The incubation temperature and duration of each cycle of the PCR were 45 sec. at 94°C for denaturation, 45 sec. at 52°C for annealing and 45 sec. at 72°C for extension. The amplification was carried out for 35 cycles with final extension at 72°C for 5 min.

**Confirmation of RT-PCR:** The PCR products (6µl aliquots) were separated on 1.5% agarose gel stained with ethidium bromide. For determining the DNA segment size, the 100 bp DNA marker (Bangalore Genei, India) was used. For further confirmation, the nested PCR of the primary amplicons (356 bp) was

carried out using forward primer-5'-CCCCGTTGGAGGC ATAC-3' (nucleotide position 282-298) and reverse primer -5'-TGTTGGCAGCATTGTTGATTG-3' (nucleotide position 478-497) for amplifying 216 bp internal sequence of the cleavage activation site of F gene of NDV as described by Nanthakumar *et al.* (2000). Primary amplicon (1µl) of 356 bp diluted 1:10 in nuclease free water was taken for nested PCR. Other reagents and cycling conditions were same as for primary PCR except annealing temperature 56°C instead of 58°C.

#### Results and Discussion

Newcastle disease virus was detected in 8 of the 30 field samples as well as in all the six vaccine strains by RT-PCR. Of the positive samples, five samples had a single band of 356 bp by RT-PCR. However, a band of 216 bp was also noticed in three more samples on nested PCR, thus making total eight field samples positive by this technique. All the six ND vaccines yielded bands of 356 bp and 216 bp by RT-PCR and nested PCR, respectively. There was no amplification in the negative control with RT-PCR as well as with nested PCR (Fig. 1 and 2).

The total RNA was extracted from tissue homogenates using TRIzol reagent. It yielded sufficient pure RNA which could be used directly for reverse transcription-polymerase chain reaction (RT-PCR). The methodology of RT-PCR as described by Nanthakumar *et al.* (2000) was found suitable for amplification of 356 bp of F region of NDV. Nested PCR with primer pair no. 2 improved the sensitivity of PCR as three more samples had clear band of 216 bp. It has been reported that nested PCR is about 100 times more sensitive as compared to a non-nested PCR (Kho *et al.*, 2000). The specificity of RT-PCR was confirmed by absence of amplification in unvaccinated apparently healthy tissue sample taken as negative control.

The technique of RT-PCR has been successfully used for the detection of NDV by various workers (Kant *et al.*, 1997; Nanthakumar *et al.*, 2000; Creelan *et al.*, 2002). Recently, Mathivanan *et al.* (2004) used this technique to detect the NDV from an apparently normal guinea fowl in India. The authenticity of PCR products by size of the amplicons has been verified by other workers too (Collins *et al.*, 1993; King and Seal, 1997; Aldous *et al.*, 2003).

This technique using F glycoprotein therefore, can easily be used for the detection of NDV in clinical samples, without necessitating virus isolation and propagation in cell cultures or embryonated eggs, even when the virus is present in very minute quantity and has lost its infectivity. In the present study, the samples were collected from two types of syndromes in broiler chickens viz: a syndrome characterized by nervous signs particularly stretching of neck forward and legs

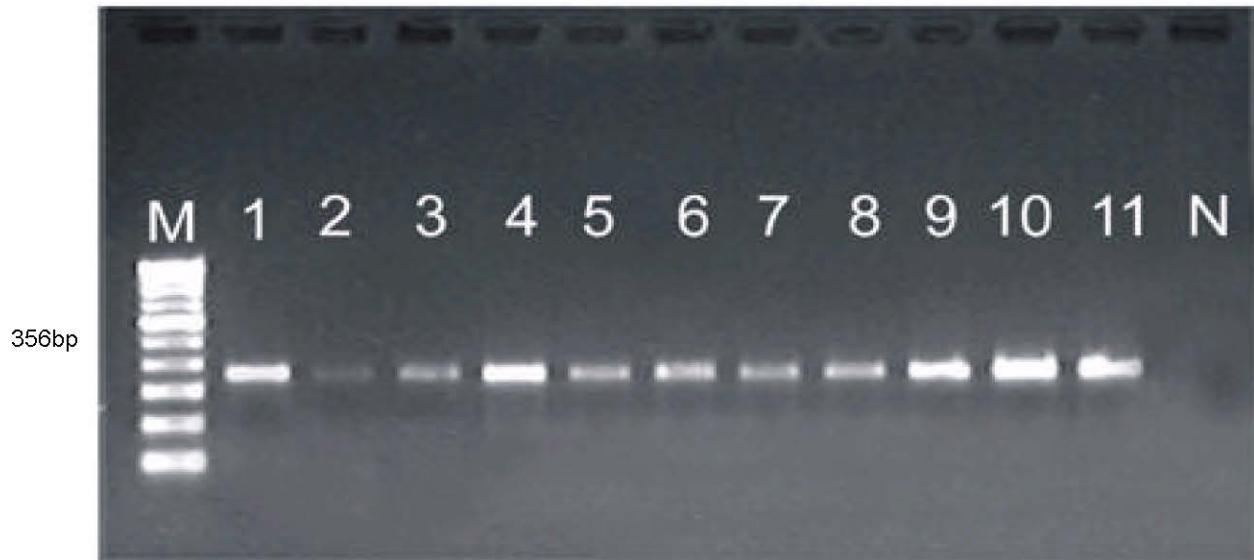


Fig. 1: Agarose gel electrophoresis of 356 bp PCR products of NDV field isolates and reference strains using primer pair no. 1

Lane M: 100 bp DNA Ladder as molecular size marker;  
Lane 1: HRR-01; Lane 2: HRR-02; Lane 3: HRR-03;  
Lane 4: HRR-04; Lane 5: HRR-05; Lane 6: Reference LaSota-I;  
Lane 7: Reference V1; Lane 8: Reference LaSota-II; Lane 9: Reference F;  
Lane 10: Reference LaSota-III; Lane 11: Reference Mesogenic R<sub>2</sub>B;  
Lane N: Negative control.



Fig. 2: Agarose gel electrophoresis of 216 bp nested PCR products generated from field isolates and reference strains using primer pair no. 2

Lane M: 100 bp DNA Ladder as molecular size marker;  
Lane 1: HRR-01; Lane 2: HRR-02; Lane 3: HRR-03;  
Lane 4: HRR-04; Lane 5: HRR-05; Lane 6: Reference LaSota-I;  
Lane 7: Reference V1; Lane 8: Reference LaSota-II; Lane 9: Reference F;  
Lane 10: Reference LaSota-III; Lane 11: Reference Mesogenic R<sub>2</sub>B;  
Lane 12: HRR-06; Lane 13: HRR-07; Lane 14: HRR-08;  
Lane N: Negative control.

backward; and secondly a syndrome characterized by respiratory distress. Only brain samples were collected from the flocks suffering from nervous disorder; whereas, trachea or lungs were collected from the flocks suffering from respiratory disorder. Only one sample was collected from an affected flock for detection of NDV. Of the five positive samples by RT-PCR, three were tracheal tissues, and one each was brain tissue and allantoic fluid. Of the three additional positive samples by nested PCR, one each was of brain tissue, allantoic fluid and trachea. It indicated that NDV could be detected more in tracheal tissue. The primary objective of the present study was to detect NDV in tissue samples from the field cases. There can be the involvement of other agents in such syndromes, however, no attempts were made to isolate or detect such etiological agents. It could have been possible that examination of more than one tissue samples from affected birds in a flock might have increased the number of positive samples for NDV. Using the technique of RT-PCR along with further classification of NDV field isolates of varying pathogenicity into velogenic, mesogenic and lentogenic strains by using restriction enzyme analysis and sequencing will be useful to know the type of strains circulating in a particular geographical location. It will also help to generate epidemiological data regarding the changes in antigenicity/ pathogenicity of the field isolates as well as in formulation of a vaccination strategy for effective control of the disease under field conditions.

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