

Isolation and Differentiation of Virulent and Non-Virulent Strains of Newcastle Disease Virus by Polymerase Chain Reaction from Commercial Broiler Chicken Flocks in Shiraz-Iran

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Abstract: Newcastle Disease (ND) is highly contagious infection of poultry that causes nervous signs and mortality in poultry. This study has been one during two years from 2010-2011 in Shiraz, Iran. The poultry industry in Fars province faced an almost heavy loss that was characterized by mild and in some flocks high mortality and respiratory distress. The aim of this study was designed to clarify the roles of the Newcastle Disease Virus (NDV) by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) assay and Mean Death Time (MDT) test in recent outbreak in Fars province in Iran. In virology tests, Newcastle Disease Virus (NDV) was isolated from tracheal and cloacal swabs samples. Diagnosis implies the differentiation to virulent and non-virulent Newcastle disease virus. During the period of this study a total 30 commercial broiler flocks with high mortality in Fars province were visited. Samples were collected from chickens with respiratory distress. Two oligonucleotide primers, representing the sequence at the cleavage site of the F protein of both virulent and non-virulent NDV strains, respectively, were used to differentiate NDV. Using the RT-PCR was able to differentiation 6 NDV reference strains 6 of which were virulent.

Keywords: Broiler flocks, Iran, MDT, newcastle disease, RT-PCR, virulent strain

INTRODUCTION

Newcastle disease has been considered as one of the major contagious disease of poultry worldwide (Aldous and Alexander, 2001; Glickman *et al.*, 1988). Newcastle disease is caused by Avian Paramyxoviruses type-1 (APMV-1) which is classified with the other avian paramyxoviruses in the genus Avularius, subfamily paramyxovirinae, family paramyxoviridae, order Mononegavirales (Lamb *et al.*, 2005; Ghiamirad *et al.*, 2010). NDV strains have been divided into three groups: virulent (Velogenic), moderately virulent (Mesogenic) and non-virulent (Lentogenic), which differ in the number of basic amino acids at cleavage site of the fusion protein (Kant *et al.*, 1997; Aldous and Alexander, 2001). The viral genome codes for six proteins, including RNA-directed RNA polymerase (L-gene), Haemagglutinin-Neuraminidase (HN gene), Fusion (F gene), Matrix (M gene), Phosphoprotein (P gene) and Nucleocapsid (NP) protein, in that order from 5 terminus to the 3 terminus (Alexander, 1997). The fusion protein encoded by the F gene is cleaved into F2-F1 protein by post-translational cleavage by host proteinases.

The virulent type of NDV has multiple basic amino acid sequence at the C-Terminus of F2 protein. These multiple basic amino acid sequences can be recognized

by ubiquitous host proteinases. Pathotype prediction and diagnosis of the virulence of NDV can be performed using the fusion protein cleavage site sequence analysis (Collins *et al.*, 1993; Seal *et al.*, 1995; Marin *et al.*, 1996; Gould *et al.*, 2001). The virulence of NDV strains is related to the cleavability of the F protein, whereas the F protein of non-virulent strains is cleaved only in cells containing trypsin-like enzymes (Toyota *et al.*, 1987; Glickman *et al.*, 1988; Kant *et al.*, 1997). There are several methods for pathotyping and characterization of NDV, such as Intra Cerebral Pathogenicity Index (ICPI), Intra Venous Pathogenesis Index (IVPI) and (MDT) in Specific Pathogen Free (SPF) embryonated eggs (Fenner *et al.*, 1987). Recently, the molecular methods such as RT-PCR and amino acid sequencing are used for diagnosis of NDV and amino acid sequence of the cleavage site of F protein of NDV strains which is considered as a virulence criterion (Panda *et al.*, 2004; Alexander and Sene, 2008). Application of one-step RT-PCR (Reverse transcription-polymerase chain reaction) to various NDV samples, including wild-type virulent isolates and a virulent vaccine strains, demonstrated the potential for rapid identification of NDV isolates as well as the differentiation of virulent from non-virulent strains (Wang *et al.*, 2001; Creelan *et al.*, 2002). In this study we have used an RT-PCR that enable us to

differentiation virulent and non-virulent NDV strains, also used MDT for determining types of NDV.

MATERIALS AND METHODS

Sample collection: The samples of present research collected from dead and morbid birds showing signs of respiratory distress for laboratory investigations from poultry industry Fars province in Iran in 2010-2011. A total of 30 commercial broiler flocks, were collected that included cloacae swabs and tracheal. Virus isolation followed standard procedures for NDV as described. All of the samples were processed for virus isolation in embryonated chicken eggs (SPF) and were tested by RT-PCR.

Virus isolation and characterization: Virus isolation and characterization of the samples collected were performed at the Razi Vaccine and Serum Research Institute, Shiraz, Iran. For checking bacterial and fungal contamination, swabs and tissue samples were placed into tubes containing 1 mL PBS solution (PH 7.2) and antibiotics (10,000 IU/mL penicillin, 1 mg/mL streptomycin sulphate, 1 mg/mL gentamicin sulphate (Noroozian *et al.*, 2007). Frozen tissues were thawed; grinded and squashed in a sterile pestle and a 10% suspension with transport medium were made (Numan *et al.*, 2008). Homogenate was centrifuged at 3000 rpm for 15 min and supernatant was collected. Frozen cloacal swabs were thawed, vortexed and pooled from each flock and centrifuged at 3000 rpm for 15 min (Numan *et al.*, 2008; Noroozian *et al.*, 2007). Supernatants were collected and filtered through 0.22 µm syringe filter (Gelman, USA). A part of filtered material was stored at -70°C for PCR testing until used (Numan *et al.*, 2008). Two hundred µL of original specimens were included into the allantoic cavity of 9 to 11 day old SPF chicken eggs according to the method described by Swayne *et al.* (1998). The amino allantoic fluids were harvested and analyzed for HA (Swayne *et al.*, 1998) and then Hemagglutination Inhibition (HI) assay was used on HA positive allantoic for virus isolates subtyping.

Pathogenicity test: Avian paramyxovirus-1 isolates were tested for pathogenicity by Mean Death Time (MDT) in embryonated SPF eggs according to the guidelines of the standard procedures provided the world Organization for animal health (OIE).

Mean Death Time (MDT) in eggs: Fresh and sterile allantoic fluids were diluted in sterile saline to give a tenfold dilution series between 10⁶ and 10⁹ for each dilution 0.1 mL was included into the allantoic cavity of each of five 10-day-old embryonated SPF fowl eggs, which were then incubated at 37°C. Each egg was examined twice daily for 7 days and the times of any

Table 1: Sense, sequence and location of use primers (Kant *et al.*, 1997)

Code	Sense	Sequence	Location
A	+	5'-TTGATGGCAGGCCTCTTGC-3'	141-159
B	-	5'-GGAGGATGTTGGCAGCATT-3'	503-485
C	-	5'-AGCGT(C/T)TCTGTCTCT-3'	395-380

embryo deaths were recorded. The MDT is the mean time in hours for the minimum lethal dose to kill all the inoculated embryos and has been used to classify NDV strains into the following groups: velogenic (taking under 60 h to kill) mesogenic (taking between 60 and 90 h to kill) and lentogenic (taking more than 90 h to kill).

RNA extraction: RNA was extracted from all aminoallantoic fluid samples using viral Gene-spin TM viral DNA/RNA extraction kit (Vetek, Korea) following manufacturer's instructions.

Reverse transcription-polymerase chain reaction: To confirm biological results RT-PCR was performed using three pair's oligonucleotide primers designed by Kant *et al.* (1997). The reaction mixture was used to perform RT-PCR using three different primer pairs A+B, A+C (Table 1). The RT-PCR using primer pair A+B should result in a 362 bp fragment with all NDV strains, using primer A+C in a 254 bp fragment with virulent NDV (Kant *et al.*, 1997). Amplification was carried out in a thermal cycler with an initial denaturation at 94°C for 5 min followed by PCR consist of 35 cycle (denaturation 94°C for 1 min annealing 58°C for 1 min, extension 72°C for 1 min) and final extension hold at 72°C for 10 min. The PCR products were detected and analyzed by electrophoresis on 1% agarose gel containing ethidium bromide.

RESULTS AND DISCUSSION

In this study the RT-PCR and MDT methods were optimized for isolation of virulent strains of NDV in referred samples. Virulent NDV strains in the allantoic fluid samples were detected and differentiated successfully. NDVs were isolated from 6 samples and identified as AMPV-1 by results obtained the HI test with NDV-specific antibodies and RT-PCR method. All 6 NDV isolates of this study were pathotyped by MDT that put them within the range of velogenic strains 46.2 to 60 h (<60 h). HI results for 6 out of 30 samples were shown positive for NDV and 24 samples were shown negative. On the other hand all NDV isolated broiler chickens were vaccinated against Newcastle disease by live attenuated LaSota vaccine strain. RT-PCR with primer pair A+B confirmed isolation for all NDV from allantoic fluid samples an expected size PCR product, 362 bp were detected (Fig. 1). Reaction with primer pair A+C specific for virulent NDV strains, an expected size PCR product 254 bp was detected (Fig. 2). The result of this study showed that all 6 isolates from

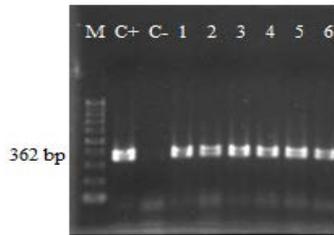


Fig. 1: A+B: result of RT-PCR using primer pair A+B specific for all NDV
An expected size PCR product, 362 bp were detected; M: Marker 100 bp DNA ladder; C+: Positive control; C-: Negative control; Lane 1 to 6: Field positive samples

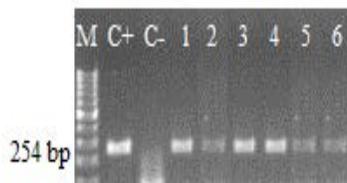


Fig. 2: A+C: result of RT-PCR using primer pair A+C specific for virulent NDV strains
An expected size PCR product 254 bp was detected; M: Marker 100 bp DNA ladder; C+: Positive control of virulent strain; C-: Negative control; lane 1 to 6: Field positive

6 flocks infected with NDV of velogenic type, on the other hand 24 flocks were negative for velogenic Newcastle disease.

Many reports have been published on NDV in chicken and other poultry. In the laboratory, the pathogenicity of NDV strains can be estimated by different means of which the most widely used is the Mean Death Time (MDT) in embryonated eggs (Alexander and Allan, 1974; Alexander, 2003; Momayez *et al.*, 2007). The MDTs are under 60 h for velogenic, between 60-90 h for mesogenic and more than 90 h for lentogenic strains (Alexander, 2003). The speed of the diagnosis can be considerably increased by using methods based on molecular biology e.g., RT-PCR (Aldous and Alexander, 2001; Creela *et al.*, 2002; Gohm *et al.*, 2000; Jestin and Jestin, 1991; Kho *et al.*, 2000; Śmietanka *et al.*, 2006). Although the egg passage test is more sensitive than HA and ELISA. It will take several days to obtain results, therefore, a rapid and sensitive method will be helpful to detect virus in varied samples. RT-PCR has been developed to detect and pathotype ND in clinical samples (De Franchis *et al.*, 1988; Farkas *et al.*, 2007). However, RT-PCR has limited sensitivity in detecting complicated samples, such as feces, tissue samples and contaminated water. Thus, an effective and simple virus concentration and purification method will be great helpful to enhance the detection sensitivity (Ghom *et al.*, 2000; Jestin and Jestin, 1991). RT-PCR for the

detection of NDV was first described by Jestin and Jestin (1991) and to date it has been successfully developed in different modifications (Aldous and Alexander, 2001; Śmietanka *et al.*, 2006) e.g., using universal primers to detect all NDVs (Creelan *et al.*, 2002; Gohn *et al.*, 2000). 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; Mathivanan *et al.*, 2004; Singh *et al.*, 2005). Kant has developed an RT-PCR which detects and differentiates virulent and non-virulent NDV strains directly in tissue homogenate, the technique is easy to perform and can be completed within 1 day (Kant *et al.*, 1997). Two oligonucleotide primers, specific for the sequence at the cleavage site of the F protein of either virulent or non-virulent NDV strains respectively, were used for differentiation (Kant *et al.*, 1997). The nucleotide variation around the cleavage site of fusion gene has been exploited for the pathotype characterization of NDV isolates using molecular methods (Wang *et al.*, 2001; Creelan *et al.*, 2002; Nanthakumar *et al.*, 2000; Pham *et al.*, 2005; Baratchi *et al.*, 2006). The results of RT-PCR using primer pair A+B specific for all NDV and in the virulent strains, primer pair and A+C could amplify specific 254 bp fragments. The test has proved to be reliable in the detection and differentiation of virulent and non-virulent NDV and could be used as a sure test in laboratories.

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