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Short Communication

Differentiation of virulent and non-virulent Newcastle disease virus isolates using RT-PCR

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Abstract
Newcastle disease is one of the main concerns of poultry farmers. Detection of virulent strains of Newcastle disease virus (NDV) has a great impact on control measures against the disease. In this study RT-PCR was optimized in high sensitivity in order to differentiate the virulent from non-virulent NDV isolates directly in tissue homogenates. The vaccinal NDV strain and known field isolates were tested by this technique. RT-PCR was performed using two sets of primers chosen from a section of the F gene. The PCR product was cloned in to a pTZ57R/T vector and sequenced. The sequence data confirmed the specificity of the test. Detection of viral virulence was determined based on the amplification of PCR products. The above optimized RT-PCR produce can be used to confirm the diagnosis of Newcastle disease within 24 hrs using RNA isolated directly from tissue homogenate or passaged in SPF (Specific Pathogen Free) embryonated eggs.
Keywords: *Newcastle disease virus; Virulent strains; Non-virulent strains; Differential detection; RT-PCR*

Newcastle disease has been considered as one of the major contagious diseases of poultry worldwide ever since the advent of high-density confinement husbandry systems. The F glycoprotein of the virus is responsible for fusion between the cellular membrane and the virus and subsequent virus genome penetration (Aldous and Alexander, 2001; Glickman *et al.,* 1988). Using the clinical signs observed in experimental the NDV infection of chickens, 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). There are several methods for pathotyping and characterization of NDV; such as intra cerebral pathogenicity index (ICPI), intra venous pathogenesis index (IVPI) and mean death time (MDT) in SPF embryonated eggs (Fenner *et al*., 1987). These methods are cumbersome, time consuming, inhumane and sometimes not decisive. Different molecular diagnostic techniques have been employed for detection and differentiation of NDV strains. 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). Recently, in order to rapidly detect and differentiate NDV isolates, a method based on real-time PCR SYBR Green I melting-curve analysis of the fusion (F) protein gene was also developed and used for this purpose (Pham *et al*., 2005). In this study RT-PCR method using degenerate primers from the F gene for differentiation of virulent and non-virulent strains of Iranian NDV isolates was optimized, and clinical tissue samples from poultry farms were tested. Seven samples of allantoic fluid containing virulent NDV isolates (Velogenic) and also live attenuated LaSota vaccine strain were received from the Razi Vaccine and Serum Research Institute (Karaj, Iran). Four clinical tissue samples from suspected birds were also received from veterinary clinics. All samples were kept at -20°C until use. Total RNA was extracted from 300 µl of clarified allantoic fluids or tissue homogenates using the RNAfast kit (Gene Fanavaran, Iran), as described previously (Beladi *et al*., 2005). Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was conducted

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using 2 virulent (Velogenic) NDV samples (allantoic fluids) and a vaccine strain (LaSota) as the non-virulent strain. Related negative control samples were also included in each experiment. Clinical tissue samples and other allantoic fluids were tested by the optimized RT-PCR procedure and results were recorded. Oligonucleotide primers (primers A+B and A+C) A-Reverse-5´-TTGATGGCAGGCCTCTTGC-3´, B-Forward-5´-AGCGTCT-CTGTCTCCT-3´ and C-Forward-5´-G(A/G)CG(A/T)-CCCTGT(C/T)TCCC-3´ used in RT-PCR were selected from published data (Kant *et al*., 1997) and used as described previously (Tiwari *et al*., 2004). The RT-PCR conditions were performed as reported before (Beladi *et al*., 2005). For determination of sensitivity of the test, viral RNA was extracted from a virulent isolate and its quality and quantity was measured by spectrophotometrically. Ten-fold serial dilutions (10^{-1} to 10^{-8}) of extracted RNA were made in DEPC-dH_2O and RT-PCR was carried out on each dilution. The highest RNA dilution with a positive result in RT-PCR was recorded as sensitivity of the assay. To sequence the PCR products, the desired PCR fragment was ligated into the pTZ57R/T vector (Fermentas, Lithuania). Plasmids containing PCR product were then sequenced using a direct sequencing method and data were compared to those in the GenBank database. The RT-PCR of non-virulent isolates and LaSota vaccine strains resulted in a single fragment of 255 bp only, when primer pairs A+C were used. However DNA fragments were not amplified with the A+ B primers. On the other hand, for virulent isolates of NDV from tissue homogenate and allantoic fluid, both primer pairs of A+B and A+C resulted in 255 bp fragments (Fig. 1). RT-PCR of the seven allantoic fluid samples that were received from the Razi Institute, resulted in the generation of 255 bp fragments with both A+B and A+C primer pairs whereas only three out of four clinical tissue samples were found to be virulent. The remaining tissue sample did not produce any DNA band and remained negative. However, allantoic fluid from the first passage of this sample produced a 255 bp DNA fragment only with A+C primer pairs which indicates that the sample contained non-virulent NDV. The nucleotide sequence of PCR amplicons were determined by sequencing. The obtained sequence could be accessed in the GenBank with the Accession Number DQ334274. Comparison of sequence data with those in the GeneBank database revealed that the sequence belongs to the F gene of NDV and therefore the specificity of the test was confirmed. Positive results involving generation of 255 bp fragments were observed with primers A+B and A+C using 10 fold NDV RNA dilutions (720×10^{-1}-720×10^{-5} ng). In further diluted RNA samples (720×10^{-6}-720×10^{-8} ng), DNA amplification was not observed (Fig. 2). In this study the RT-PCR method was optimized for differentiation of virulent and non-virulent strains of NDV in referred samples. Both virulent and non-virulent NDV strains in the allantoic fluid samples were detected and differentiated successfully. The virulent NDV strain was also directly detected in clinical tissue samples. However, one non-virulent NDV strain which was not detected in a tissue sample could only be identified after the first passage of the sample in

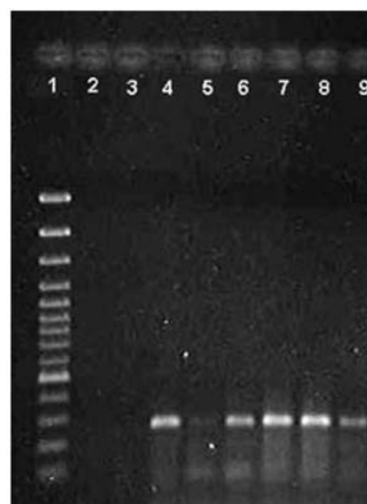

Figure 1. RT-PCR of NDV samples using A+B and A+C primers for each sample in 1% agarose gel. Lane 1: DNA molecular weight marker, lane 2: Negative control (A+B), lane 3: Negative control (A+C), lane 4: LaSota strain (A+C), lane 5: LaSota strain (A+B), lane 6: Field isolate, sample1, allantoic fluid (A+C), lane 7: Field isolate, sample 1, Allantoic fluid (A+B), lane 8: Field isolate, sample 2, Tissue homogenate (A+ C), lane 9: Field isolate, sample 2, Tissue homogenate (A+B).

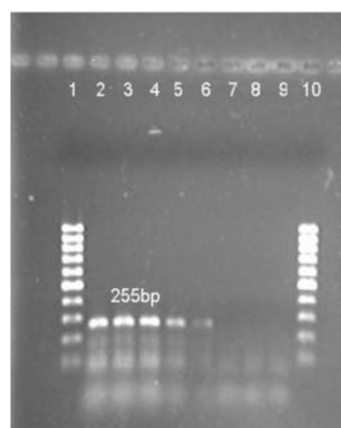

Figure 2. Detection of NDA RNA by RT-PCR in allantoic fluid. Lane 1 and 10: DNA molecular weight marker (100bp DNA ladder); Lane 2-9: diluted RNA corresponding to 10^{-1} - 10^{-8} ng of RNA, respectively.

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SPF embryonated eggs. This failure might be due to the low titer of non-virulent virus in the tissue sample. To detect the virus in a clinical tissue sample, at least 720×10^{-5} ng of viral RNA should be available. The concentration of wild virulent virus in the tissue of diseased birds is high enough to be detected by this test; whereas, the titer of the residual vaccine strain of NDV present in related tissues of vaccinated birds was too low to be detected. Two pairs of oligonocleotide specified for the cleavage site of the F protein could distinguish between the virulent and non-virulent strains of NDV. In the virulent strains, both primer pairs A+B and A+C could amplify specific 255 bp fragments; however, in non-virulent strains only the primer pair A+C could amplify specific 255 bp fragments. Similar results have been reported by Tiwari *et al.* (2004) who used the same primer pairs but in different PCR conditions. However, different findings were reported by Kant *et al*. (1997), who used similar primers to amplify the F gene sequence specifically from virulent and non-virulent strains of NDV. These different results could be due to different PCR conditions or PCR sensitivities. However, imperfect matching of primers with the template due to the low degeneracy of primer pairs could also lead to unexpected results. Therefore, in order to verify the results, more NDV isolates with known pathogenicity must be tested. The primary determinant for virulence among NDV isolates is the presence or absence of dibasic amino acids in the fusion protein's cleavage activation site (Berinstein *et al*., 2001). The nucleotide variation around the cleavage site of the fusion gene has been exploited for the pathotypic characterization of NDV isolates using molecular methods (Wang *et al*., 2001; Creelan *et al*., 2002; Nanthakumar *et al*., 2000; Pham *et al*., 2005). Wang *et al*. (2001) designed three sets of oligonucleotides, each specific for amplifying the NDV fusion protein gene-specific RNA from virulent, non-virulent or all NDV isolates respectively. The sensitivity of this test was reported to be 10^{-5} HA (Hemagglutination) units. However the sensitivity of the present study was found to be 270×10^{-5} ng of viral RNA. In comparison to the previous pathotyping methods of distinction between virulent and non-virulent strains such as ICPI, IVPI and MDT in SPF embryonated eggs, the technique that is used in this study is efficient and less time-consuming. Since the sensitivity and specificity of NDV detection tests are important factors in controlling the disease, RT-PCR is a suitable candidate for the quick diagnosis of virus in clinical samples as well as differentiation between virulent and non-virulent isolates. The test has proved to be reliable in the detection and differentiation of virulent and non-virulent NDV and could be used as a robust test in veterinary diagnostic laboratories.

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