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Original Article

Two in-House One-Step rRT-PCR Assays, Developed for Accurate and Rapid Molecular Identification of Newcastle Disease Virus, on the basis of SYBR Green and Specific TaqMan Probe

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Abstract

Background and Aims: *Newcastle disease* virus (NDV) is an avian paramyxovirus (A-PMV 1) and one of the major pathogens in poultries. Vaccination is intended to control the disease, nevertheless this virus is a growing threat to the poultry industry. So, early detection of the virus can prevent the spread of illness and avoid huge economic losses. Towards this goal, in this research, we developed two novel in-house one-step rRT-PCR assays; based on SYBR Green and specific TaqMan probe for accurate and rapid molecular identification of Newcastle disease virus.

Materials and Methods: In this experimental study, after preparation of viral sample, respective RNA was extracted from virus by using viral RNA extraction kit. The specific probe and primers were designed, based on the conserved region of matrix (M) protein encoding gene of NDV, and used to produce c-DNA and subsequently to amplify and detect this region by two novel one-step real-time reverse-transcription polymerase chain reaction (rRT-PCR) assays. These diagnostic appraisements were carried out using SYBR Green and matrix (M) gene specific TaqMan probe.

Results: The presence of M gene in RNAs, extracted from NDV samples, was confirmed by both of these one-step rRT-PCR assays and the results of these diagnostic tests were positive.

Conclusion: This study showed that these two developed one-step rRT-PCR assays are the proper molecular methods for rapid and accurate diagnosis of NDV by detection of matrix (M) protein encoding gene.

Keywords: Matrix (M) gene; *Newcastle Disease* Virus (NDV); One-step rRT-PCR; Rapid diagnosis; SYBR Green; TaqMan probe

Introduction

Newcastle disease virus (NDV) is one of the major pathogens in poultry, affecting most of the avian species and characterized by impairment of respiratory, gastro-intestinal and central nervous systems (1). This agent can cause significant damage to the poultry industry (1). Transmission occurs by exposure to fecal and other excretions from infected birds, or through contact with contaminated feed, water, equipment and plumage (1). NDV belongs to the *Rubulavirus* genus within the *Paramyxoviridae* family of

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negative-stranded RNA viruses. The single stranded and non-segmented viral RNA genome (15 kb) encodes for six major structural and non-structural proteins from the 5' terminus to the 3' terminus: RNA-directed RNA polymerase (L gene), hemagglutinin-neuraminidase (HN gene), fusion (F gene), matrix (M gene), phosphoprotein (P gene), and nucleocapsid (NP gene) protein (2). Both HN and F glycoproteins on the surface of NDV are important for virus infectivity and pathogenicity (3-9). The F protein is responsible for membrane fusion required in the first stage of infection (10). Furthermore, if inserted into the host cell it can mediate fusion between the plasma membranes of the two cells. The molecular basis of NDV pathogenicity depends on the F protein cleavage site amino acid sequence (11). The F protein is synthesized as an inactive precursor F_0 (M_r 68,000) which is proteolytically cleaved into two disulfide-linked subunits F_1 and F_2 that is necessary for the infectivity of paramyxoviruses (Le *et al.*, 1988). NDV causes a disease that varies in clinical severity and transmissibility depending on the pathotype involved. NDV strains can be categorised into three groups based on their virulence, as lentogenic (nonvirulent), mesogenic (intermediately virulent) and velogenic (highly virulent). Lentogenic strains, especially in adult chickens may cause minimal or no clinical sign. However, the disease produced by mesogenic strains may cause mortality that can reach 25% and that by velogenic strains may be reached up to100% (12). Vaccination is intended to control the disease as an effective solution, nevertheless this virus is a growing threat to the poultry industry. So, early detection of the virus can prevent the spread of illness and avoid huge economic losses (1). Towards this goal, in this research, we developed two novel in-house one-step rRT-PCR assays; based on SYBR Green and specific TaqMan probe for accurate and rapid molecular identification of Newcastle disease virus.

Methods

Sample preparation and viral RNA extraction

In this research, after preparation of the sample (that was NDV vaccine) from Razi Institute (Tehran, Iran), Viral RNA was extracted from 200 µl of the virus-containing sample using a High Pure Viral RNA Extraction Kit (Roche-Germany) within 30 min, according to the manufacturer's instructions. Sample residues were stored at 4°C.

Design and synthesis of specific probe and primers

Specific probe and primers (forward and reverse) targeting NDV M gene, were designed by primer designing softwares (such as Oligo) and afterwards synthesized by Bioneer Company (Korea). The sequences of specific probe and primers used for amplification and detection of NDV M gene, has reported in table 1. None of the primer sequences showed genomic cross-reactivity with other viruses, human genome and other probable interfering genomes in a BLAST search analysis and only detected a 138 bp fragment of the NDV M-region (results not presented).

SYBR Green based one-step rRT-PCR assay

The novel in-house SYBR Green based one-step rRT-PCR assays were developed using the QuantiFast SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) on Corbet (Rotor-Gene) 6000 (Qiagen, Germany) real-time PCR instruments. The amplifications were accomplished within 100 min in 20 µl reaction mixtures containing 5 µl of the target viral RNA and 2 µl (20 pmol) of each primer (10µM). Cycling conditions were as follows: a single cycle of 10 min at 50°C, 5 min at 95°C; followed by 45 cycles of 10 sec at 95°C; and a final cycle of 30 sec at 60°C (analysis of fluorescence performed at the end of each 60°C step). So, the rRT-PCR products were detected via an increase in fluorescence from cycle to cycle. Also negative control or no template control (NTC) was used as the quality control of the process. Both cycling and melting curves in the real-time analysis were evaluated with respect to negative control and

the test assay. The rRT-PCR amplicons were then confirmed by electrophoresis analysis.

TaqMan probe based one-step rRT-PCR assay

The novel in-house TaqMan based one-step rRT-PCR assay was developed using the QuantiFast RT-PCR Kit (Qiagen, Hilden, Germany) on Corbet (Rotor-Gene) 6000 (Qiagen, Germany) real-time PCR instruments. The amplifications were accomplished within within about 3 h in 20 µl reaction mixtures containing 5 µl of viral RNA and 2 µl (20 pmol) of each primer (10µM). Cycling conditions were as follows: a single cycle of 30 min at 50°C, 15 min at 95°C; followed by 45 cycles of 20 sec at 95°C; 40 sec at 50°C and a final cycle of 30 sec at 72°C (analysis of fluorescence performed at the end of each 50°C step). So, rRT-PCR products were detected via an increase in fluorescence from cycle to cycle. Also negative control or no template control (NTC) was used as a quality control of the process. It should be mentioned that common one-step real-time RT-PCR kits, formulated for application with probes, should all provide satisfactory results with default reaction mix compositions as suggested by manufacturers.

Repeatability of the assay

All evaluations were performed with assay repeating by at least three different users and three times by each one of them in different days and the repeatability of the assays were confirmed.

Evaluation of analytical specificity by in silico prediction

As previously mentioned, the highly conserved M-region sequence of NDV genome was adapted from genbank after alignment of the nucleotide sequences of available NDV strains (obtained from NCBI database) and the specific probe and primers targeting this region were designed. None of the probe and primer sequences showed genomic cross-reactivity with other viruses, human genome and other probable interfering genomes in a BLAST search analysis and only detected a 138 bp fragment of the NDV M-region (results not presented).

Results

Gel-based analysis of amplified fragment using the corresponding specific primers

After amplification of NDV M-region sequence by the specific primers, expected 138 bp amplicon, was confirmed by analysis of 2% electrophoresis gel (Figure 1).

SYBR Green based one-step rRT-PCR assay

Melting curve analysis: In this novel in-house SYBR Green based one-step rRT-PCR assay, the products were identified based on T_m curve analysis and the PCR products from each primer-pair were generated based on individual T_m value. After 45 amplification cycles, the melting curve analysis revealed the melting temperature (Tm) of 86.5 ± 0.5°C for various viral samples, quite different from that of primer-dimers and the positive samples showed only a small variation in parameters.

So, only T_m peaks between 86.5 ± 0.5°C can be acceptable as positive (observed in all tested

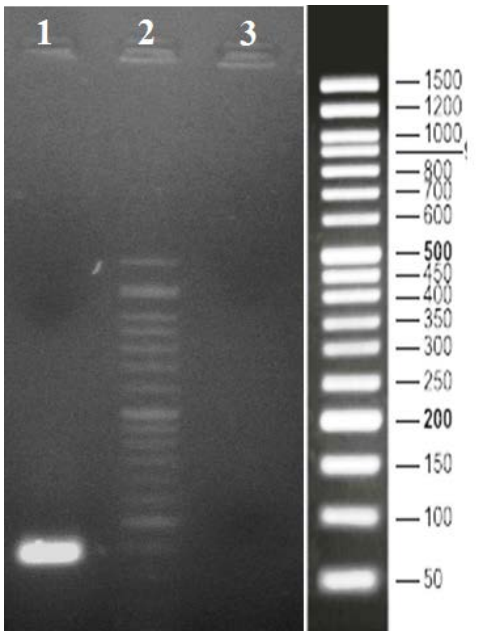

Fig. 1. Electrophoresis analysis of NDV M-region, amplified by gel based one-step RT-PCR assay. Lane 1: PCR product with a length of ~ 150 bp fragment showing the positive result of assay, Lane 2: 50 bp DNA Ladder (SinaClon), Lane 3: Negative control or NTC (No template control).

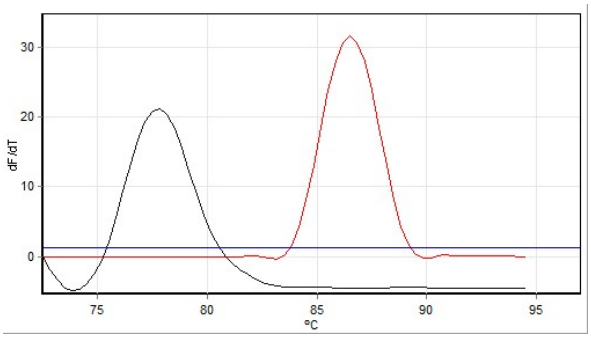

Fig. 2. Melting curve analysis of one sample showed that melting temperature (Tm) of specific amplicon was 86.5°C (red curve) and quite different from primer-dimers (black curve).

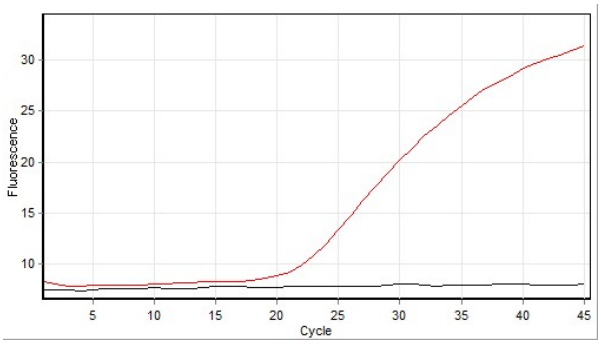

Fig. 3. Representative amplification plot of developed NDV rRT-PCR assay. Red curve indicates the positive result of diagnostic assay conducted in one of NDV samples and black curve is the negative control or NTC (No template control).

samples) as shown in the melting curve in Figures 2 for one of these samples.

TaqMan probe based one-step rRT-PCR assay

Diagnostic test of rRT-PCR using the corresponding specific TaqMan probe and primers: The results of this novel in-house TaqMan based one-step rRT-PCR assay, accomplished on viral samples were positive, showing the presence of M-gene in RNAs, extracted from NDV samples. Figure 3 shows one result of these diagnostic tests, carried out on NDV RNA samples, using matrix gene specific TaqMan probe.

Discussion

NDV has a variety of strains that differ widely in virulence, from causing an asymptomatic infection to lethal disease. Although vaccination programs have provided significant protection against NDV outbreaks, infections by ND viruses have been reported frequently around the world in recent years. Correct and fast diagnosis of NDV will surely help in effective controlling the disease.

Unfortunately, rapid detection of NDV at the onset of the disease has always been hampered, because of the lack of a sensitive and fast detection method. On the other hand, current diagnosis of NDV infection by conventional virus isolation and serological tests such as haemagglutination inhibition (HI) and serum neutralization, are either time-consuming or lacking the required sensitivity (1). Feces from NDV infected birds play an important role within the spread of NDV and the bacterial contamination and toxic substances in feces limit NDV detection by virus isolation in embryonated eggs, however until now, time-consuming isolation of ND virus in embryonated chicken eggs has been used for NDV detection, as above mentioned (1).

For rapid and accurate diagnosis, reverse-transcription polymerase chain reaction (RT-PCR) on extracted RNAs is a valuable alternative (1). Traditional (gel-based) RT-PCR methods require amplification in a thermo cycler (after cDNA preparation via RT process) and product analysis by

Table 1: The sequences of specific probe and primers used for amplification and detection of NDV M gene.

Name	Sequence
Newcastle F Primer	5'-TCCTCAGGTGGCCAAGATAC-3'
Newcastle R Primer	5'-TGCCCCTTCTCCAGCTTAGT-3'
Probe	DYXL-5'-TTTTAACGCTCCGCAGGCAC-3'-BBQ

electrophoresis gel, both of which are time-consuming and laborious (1). By eliminating the need for post-amplification product processing, real-time RT-PCR method enables shortened turnaround times for reporting results, which is critical for deciding on suspected case patients isolation and contact tracing. Real-time RT-PCR assays are sensitive and rapid and they can be automated. They have also greater reproducibility and since the need for post-PCR processing is eliminated, they can prevent carry-over contamination (1). TaqMan probe-based real-time RT-PCR assays, not only provide confidence in identification of target genes due to specific probing, but also reduce the risk of facing with laboratory product contamination, that is because the amplification reaction and detection of PCR products are performed in a single tube (1).

Despite all advantages of rRT-PCR assay, this technique is two-step. Step 1 is reverse transcription (RT) of RNA extracted from the virus, for cDNA preparation and step 2 is the real-time PCR. In this study, we developed two novel one-step rRT-PCR assays, based on SYBR Green and specific TaqMan probe, instead of traditional two-step rRT-PCR assays, as the both of steps were outandout in single step in these assays that it can increase sensitivity of the assays and is affordable economically, in addition of other advantages, mentioned above.

Conclusion

This study showed that the developed one-step rRT-PCR assays based on SYBR Green and specific TaqMan probe, are the proper molecular methods for rapid and accurate diagnosis of NDV by detection of M gene.

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