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Detection of Newcastle disease virus of poultry by real time reverse transcription-polymerase chain reaction

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Abstract

A real-time reverse transcription - polymerase chain reaction (rRT-PCR) was used for the detection of Newcastle disease virus (NDV) of poultry. A panel of seven known isolates of NDV in the form of allantoic fluid, obtained from a laboratory repository, was used for the development of the test. RNA was extracted from the allantoic fluid with a magnetic processor based automated RNA extraction system. The identity of the reference virus was first reconfirmed by a conventional RT-PCR specific for the Fusion (F) protein gene. Using these RNA, the rRT-PCR protocol was optimized with regard to the reaction mix and thermal profile using published primers and probes specific for M gene. The sensitivity of standardized rRT-PCR was compared to that of the conventional RT-PCR using serial 10-fold dilutions of the RNA of a selected sample. The thermal profile was modified from the published one; the annealing and extension steps were combined to a single step performed at 60°C. The adopted rRT-PCR successfully amplified M gene from all the seven reference samples with a C_T value ranging from 15.28 to 32.68. The rRT-PCR for M gene was 100-fold more sensitive than the conventional RT-PCR for F gene. This is the first report of the use of rRT-PCR for the detection of NDV in Bangladesh. This test will be useful for virological surveillance, particularly for screening NDV in respiratory infections. (*Bangl. vet*. 2016. Vol. 33, No. 1, 16 – 22)

Introduction

Newcastle disease (ND) or Ranikhet disease is an economically important disease of poultry in Bangladesh. Newcastle disease virus (NDV), also known as virulent forms of Avian Paramyxovirus serotype 1 (APMV-1), is a single-stranded, negative-sense RNA virus within the genus of *Avulavirus* in the family *Paramyxoviridae* (Lamb *et al.,* 2005). Isolation of NDV in embryonated chicken eggs and their identification by haemagglutination (HA) and haemagglutination inhibition (HI) test with a NDV-monospecific antiserum is the gold standard for the detection of NDV (OIE, 2012), but the procedure is time-consuming. Reverse transcription-polymerase chain reaction (RT-PCR) has been established to identify NDV (Jestin and Jestin, 1991; Kho *et al.,* 2000, Zhang *et al.*, 2010). Real time RT-PCR (rRT-PCR) is more sensitive and quicker, and has recently been used for the identification and typing of NDV (Wise *et al.*, 2004; Tan *et al.*, 2004; Pham *et al.*, 2005; Farkas *et al.*, 2009; Khan *et al.*, 2010; Qiu *et al.*, 2014).

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Previous research on NDV in Bangladesh has focused on isolation, characterization and pathogenicity testing (Chowdhury *et al.*, 1981; Saha *et al.*, 1997; Noor *et al.*, 2005; Barman *et al.*, 2010; Mazumder *et al.*, 2012) and use of conventional RT-PCR for the detection of the virus (Islam *et al.*, 2010; Khan *et al.*, 2014; Nooruzzaman *et al.*, 2015). The present study aimed at the adoption of an rRT-PCR technique for the detection of Newcastle disease virus.

Materials and Methods

Virus isolates

A total of seven known isolates of NDV, previously detected by RT-PCR and propagated in embryonated chicken eggs, were obtained in allantoic fluid from a previous study (Barman, 2016).

Primers and probes

The details of the primers and probes used in the conventional RT-PCR and rRT-PCR are given in Table 1. The primers and probe were synthesized from a commercial source (Integreted DNA Technologies, Inc., USA).

Table 1. Primers and probes used in conventional RT-PCR and rRT-PCR for the detection of NDV

RT-PCR format	Primer name (Reference)	Primer/probe sequence (5′ – 3′)	Target gene	Product size
Conventional RT-PCR	P 1 (Wang *et al.*, 2006)	ATG GGC YCC AGA YCT TCT AC	F gene	535 bp
	P 2 (Wang *et al.*, 2006)	CTG CCA CTG CTA GTT GTG ATA ATC C		
Real time RT-PCR	M+ 4100 F (Wise *et al.*, 2004)	AGT GAT GTG CTC GGA CCT TC	M gene	121 bp
	M- 4220 R (Wise *et al.*, 2004)	CCT GAG GAG AGG CAT TTG CTA		
	NDVM + 4169 Probe (Khan *et al.*, 2010) modified after (Wise *et al.*, 2004)	[FAM] TTY TCT AGC AGY GGG ACA GCY TGC [TAMRA]		

Extraction of RNA, RT-PCR and rRT-PCR protocols

Viral RNA was extracted from infected allantoic fluid with King Fisher ML automated RNA extraction machine using Ambion MagMAX™-96 Viral RNA Isolation Kit (Life Technologies, USA) following manufacturer's instructions. As the kit was designed for 96-well format, the volumes of reagents were adjusted for tube format of the RNA extraction machine.

For amplification of the F gene fragment by conventional RT-PCR, SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA polymerase (Life Technologies, USA) was used as recommended by the manufacturer. The thermal profile consisted of RT at 50°C for 30 min followed by initial denaturation at 94°C for 2 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 1 min. The amplified cDNA was analyzed by electrophoresis on 1.5% agarose gel.

The rRT-PCR was performed in AB 7500 Fast real time PCR machine (Applied Biosystems, USA) using AgPath–ID™ One Step RT-PCR Kit (Life Technologies, USA) as recommended by the manufacturer. The reaction was performed in 25 µL volumes in 0.1 mL MicroAmp Fast PCR 8-tube strips; 5 µL RNA templates were added to 20 µL reaction mix. The thermal profile used by Wise *et al.* (2004) was modified as shown in Table 2 considering the requirements of the present kit and the real-time PCR machine.

Table 2. The original and modified thermal profile of rRT-PCR for the detection of Matrix (M) gene of NDV

	Original thermal profile (Wise *et al.*, 2004)	Modified thermal profile
Reverse transcription	50°C for 30 min	45°C for 10 min
Initial denaturation	94°C for 15 min	95°C for 10 min
40 cycles of PCR		
Denaturation	94°C for 10 sec	94°C for 15 sec
Annealing	52°C for 30 sec	60°C for 30 sec*
Extension	72°C for 10 sec*	

* Signals were collected at this cycle

Comparison of the sensitivity of real time and conventional RT-PCR

To compare the sensitivity of conventional and real-time RT-PCR, RNA extracted from a selected sample was serially diluted 10-fold from undiluted to 10^{-9}. All the dilutions were tested in conventional and real time RT-PCR. The highest dilution at which the test gave positive result was considered as the detection limit.

Results and Discussion

All the seven reference isolates of NDV retested by conventional RT-PCR were positive for NDV F gene. A 535 bp product was amplified (Fig. 1a).

The initial trials of rRT-PCR using the thermal profile described by Wise *et al.* (2004) were not successful. However, after some modification of the thermal profile all the seven isolates were successfully amplified (Fig. 1b). In the modified thermal profile

the annealing and extension steps were combined to a single step at 60ºC for 30 sec. This was needed as a different real-time PCR machine was used in the present study. The temperature and time for reverse transcription and initial denaturation were also modified as suggested by the manufacturer of the RT-PCR kit used in the present study. The C_T values for the seven reference isolates were 15.98, 32.34, 32.68, 25.89, 32.60, 17.92 and 22.72. Typical amplification curve was observed with four out of seven samples. Two samples having very good C_T values (15.98 and 17.92) did not show good exponential rise in the curve. Another sample also having good C_T value (25.89) did not show typical curve. The reason for this atypical curve needs to be investigated further. Inadequate mixing of reagents could result in such atypical curves.

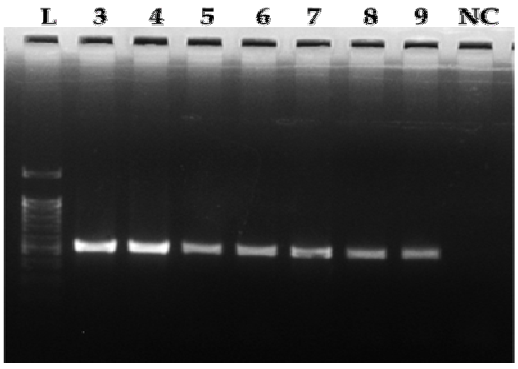

Fig. 1a. Amplification of 535 bp fragment of F gene from NDV isolates by conventional RT-PCR (L = DNA ladder, 3-9 = Samples with ID # 3-9)

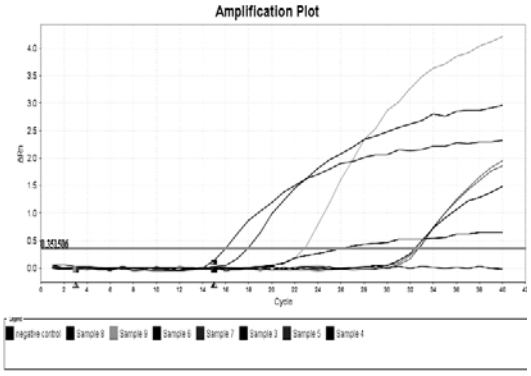

Fig. 1b. Amplification plot (linear view) of rRT-PCR for M gene for seven NDV samples

Wise *et al.* (2004) developed three protocols for NDV rRT-PCR, two for M gene and one for F gene. The present protocol is one of these three protocols, which was based on the conserved region of M gene and was able to detect NDV strains of lentogenic, mesogenic and velogenic pathotypes. This protocol is considered as USDA-validated protocol. However, it has been recently demonstrated that within class II APMV-1 viruses, the matrix gene is not highly conserved and false negatives occurred in outbreak investigations or routine surveillance in poultry using the USDA-validated real-time RT-PCR assay targeting this gene (Cattoli *et al.*, 2009; Khan *et al.*, 2010). To overcome this problem, Khan *et al.* (2010) modified the probe of Wise *et al.* (2004) by including three degenerate bases in the probe. In the present study the primers of Wise *et al.* (2004) and the modified probe of Khan *et al.* (2010) were used.

To compare the sensitivity of conventional RT-PCR for F gene fragment and rRT-PCR for M gene fragment, 10-fold serial dilutions of RNA of one selected isolate (isolate #9) were tested by both these methods. On conventional RT-PCR a clear and distinct band of RT-PCR product of 535bp was observed in undiluted, 10^{-1} and 10^{-2} dilutions of RNA (Fig. 2a). cDNA band was hardly visible in the next two dilutions. However, in case of rRT-PCR the undiluted sample as well as the serial dilutions from 10^{-1} to 10^{-4}

were positive with C_T value ranging from 23.07 to 36.59 (Fig. 2b). Hence, the rRT-PCR appears to be 100-fold more sensitive than the conventional RT-PCR. Very high sensitivity of rRT-PCR has been reported (Wise *et al.*, 2004).

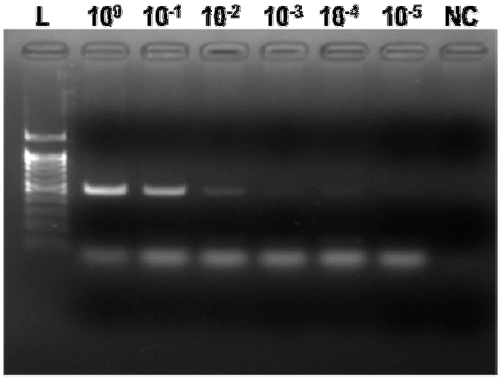

Fig. 2a. Amplification of 535 bp fragment of F gene by conventional RT-PCR from the undiluted and 10-fold serial dilutions of RNA of NDV Isolate # 9

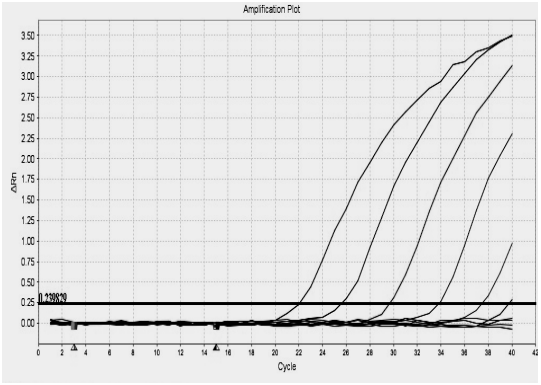

Fig. 2b. Amplification of matrix gene of NDV by rRT-PCR from the undiluted and 10-fold serial dilutions of the Isolate # 9

Newcastle disease is endemic in Bangladesh. With the emergence of highly pathogenic and low pathogenic avian influenza, differential diagnosis between NDV and avian influenza is often required because of the clinical and pathological similarity. This is the first report of the application of rRT-PCR for the detection of NDV in Bangladesh. Availability of this modern, sensitive and quick diagnostic tool will be very useful in confirmation or ruling out NDV from suspected avian influenza outbreaks. This test will be useful for initial screening of samples in NDV surveillance.

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