RESEARCH PAPER

Detection of newcastle disease virus from wild quail (*Coturnix coturnix*) by reverse transcription polymerase chain reaction in Isfahan and Loretan Provinces of Iran

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Abstract

The present research work was carried out to isolate and identify Newcastle disease virus (NDV) by using haemagglutination inhibition (HI) test and reverse transcription-polymerase chain reaction (RT-PCR) assay. A total of 500 clinical and post-mortem (brain, lung, colon and spleen) samples were collected from of two populations of *Coturnix Coturnix* of Newcastle disease (ND) in 2013, one in Lorestan province and other one in Isfahan province . All the samples were inoculated onto 10-day-old embryonated through allantoic sac route and in the chicken embryo fibroblasts (CEFs) cell culture. The allantoic fluid (AF) of the dead embryos and the infected culture fluid (ICF) of the CEF were harvested at 48 and 96 hours of post-infection,respectively. The HI and RT-PCR were employed to detect NDV in tissue homogenates of all the clinical and post-mortem samples as well as laboratory samples (AF and ICF). In CEF cell culture system, the rate of virus isolation from all the aforesaid samples was found 100% with the exception of serum samples. The isolation rate of NDV was higher in CEF culture system (93.8%) compared to that of avian embryos (80%). The anti-NDV hyperimmune serum revealed complete inhibition of the 4 haemagglutination unit of each isolate of viruses isolated from broiler and layer chickens present in the laboratory samples (AF and ICF). The NDV specific primers used in the direct RT-PCR for genome detection of NDV showed equal sensitivity and specificity with the RNA extracted from the clinical, post-mortem and laboratory samples (AF and ICF) as with the genomic RNA of reference NDV. Higher rate of detection of NDV was recorded with RT-PCR assay than HI test. Therefore, the molecular method (RT-PCR) can be introduced for rapid and confirmatory detection of NDV from different forms of Isfahan and Lorestan province but there is no report on Newcastle disease *Coturnix Coturnix* in Iran.

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Introduction

Coturnix coturnix) is a small bird in the pheasant family Phasianidae. It is widespread and is found in parts of Iran; it should not be confused with the domesticated common Quail, *Coturnix Coturnix*, native to Asia which although visually similar have very distinct calls.Newcastle disease virus (NDV) which is also called avian paramyxovirus serotype 1 (APMV-1) is a singlestranded, enveloped, negative sense RNA virus belonging to the genus *Rubulavirus* of subfamily *Paramyxovirinae* and family *Paramyxoviridiae* (Barbezange and Jestin, 2005; Mayo, 2002). Based on pathogenic and virulence properties, NDV is categorized into three major pathotypic strains *i.e.* lentogenic, mesogenic and velogenic strains (Beard and Hanson, 1984). NDV causes Newcastle disease (ND), which is an acute highly contagious viral disease of domestic poultry as well as other species of birds regardless of age and sex variation (Alexander, 2003). According to the strain variation of NDV, the rate of morbidity and mortality of poultry in a flock due to ND varies from 90-100%, thereby poultry industry all over the world facing serious economic losses every year (Gohm *et al.*, 2000). ND is also considered as one of the major threats to the poultry raisers of I too, because of its high morbidity, mortality and reduced productivity of poultry resulting remarkable economic losses every year. According to Chowdhury *et al* (1982), ND alone is responsible for at least 40-60% of total mortality of poultry population in the asia Therefore, when there is any outbreak of ND, rapid and confirmatory detection of the viruses are crucial for the adaptation of an effective control of the disease. But, due to lack of availability of rapid and confirmatory diagnostic methods on wild quail in Isfahan and Lorestan province confuse ND with other infectious diseases of poultry. Traditionally, the methods of diagnosis of ND are being practiced at field level of Iran are very much limited within the recording of the clinical signs and post-mortem findings manifested by ND affected birds and some serology-based diagnostic methods like haemagglutination inhibition (HI), virus neutralization (VN) and agar gel immunodiffusion (AGID) tests etc. Although virus isolation followed by identification using most of the serological tests such as enzyme linked immunosorbent assay, fluorescent antibody technique, serum neutralization test, plaque reduction neutralization test and AGIDT are considered as the standard methods for the confirmation of the disease (OIE, 1996) but these are always considered as time consuming and expensive compared to that of genome detection of the virus as a single method. Therefore, genome detection of NDV both from the clinically sick and dead birds is very important for early and rapid confirmatory diagnosis of ND in poultry during outbreak of the disease compared to any other single or combined conventional methods of diagnosis (Jestin and Jestin, 1991; Pedersen *et al.*, 2000). The reverse transcription-polymerase chain reaction (RT-PCR) has established its position top over other confirmatory test methods in the field of diagnosis of ND (Singh *et al.*, 2005; Krzysztof *et al.,* 2006). This paper describes the isolation and molecular detection (RT-PCR) of NDV from the clinical and post-mortem samples of naturally infected *Coturnix Coturnix*

Materials and methods

Collection of samples

A total of 500 clinical (blood, tracheal and cloacal swabs) and post-mortem (lung, brain, colon, spleen)samples, All the samples were transported to thelaboratory maintaining 4°C on ice and were either processed immediately or stored at -86°C.

Virus isolation

Virus isolation from clinical and post-mortem samples was performed in 9-11-day-old embryonated chicken eggs and in chick embryo fibroblast (CEF) cells (Uruakpa, 1997). Collection and storage of allantoic fluid (AF) and infected culture fluid (ICF) were done properly.

Haemagglutination (HA) and haemagglutination inhibition (HI) tests

Tissue homogenates of post-mortem samples, clinical samples, AF and ICF were subjected for slide as well as micro-plate HA test to determine the presence of haemagglutinating virus using 1.5% and 0.5% freshly

prepared cRBC suspension (Steph en *et al.,* 1975). The HI test using anti-NDV hyperimmune sera raised in chickens was employed with the HA positive samples for identification of NDV.

Extraction of viral RNA

The genomic viral RNA was extracted from reference NDV, clinical samples, post-mortem samples, ICF and AF by using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacture's protocol and was stored at −86°C until use.

Oligonucleotide primers

A set of oligonucleotide primers, forward (NDVF) - 5´-GCAGCTGCAGGGATTGTGGT-3´ (nucleotide position 158-177) and reverse (NDVR) - 5´-TCTTTGAGCAGGAGGATGTTG-3´ (nucleotide position 493-513) as designed and used by Nanthakumar *et al.* (2000) were used for the amplification of 356 bp amplicons corresponding the cleavage activation site of F gene of NDV.

Reverse transcription (RT)

A volume of 4 µl of eluted RNA of NDV and 8.3µl DEPC were taken into individual PCR tube and mixed properly with the help of the minispin. The tubes were then placed in a forty eight wells thermocycler (MJ Mini thermocycler, BIORAD®, USA) and heated at 94oC for 5 minutes for linearization of coiled RNA followed by snap cooling on ice for 2 minutes. Meanwhile, for the synthesis of cDNA from RNA of NDV, reaction mixture containing 4.0 µl 5X RT buffer, 2.0 µl 10 mM dNTP, 1.0 µl prime RNase inhibitor, 0.2 µl AMV-RT, and 0.5 µl primer (RH 100 pmol) was prepared and kept on ice. After adding this reaction mixture onto the PCR tube containing linearized RNA placed into the thermocycler and followed the thermal profile as 42oC for 40 minutes followed by 85oC for 5 minutes.

Polymerase chain reaction (PCR)

PCR was carried out in a total volume of 50 µl containing 5.0 µl 10X LA buffer, 2.0 µl 25 mM MgCl2,

2.0 µl 10 mM dNTP, 0.2 µl LA-Taq, 0.8 µl NDVF primer (100 pmol), 0.8 µl NDVR primer (100 pmol), 1.5 µl cDNA, and 37.7 µl DEPC was added to each tube and mixed with the micropipette and minispin. The tubes were immediately returned to the thermocycler and the cycling programme was resumed at 94oC for 2 minutes (initial denaturation) followed by 30 cycles of 94oC for 30 seconds (denaturation), 45oC for 45 seconds (annealing),60oC for 1 minute (elongation) and final elongation of 60oC for 5 minutes.

Agarose gel electrophoresis

The PCR products were separated in 1.5% agarose gel in TAE buffer stained with ethidium bromide and compared with molecular mass marker (100 bp DNA marker) and visualized by ultraviolet (UV) transillumination.

Results and discussion

Isolation of NDV

Results of virus isolation of the present study revealed that among 160 samples including clinical and postmortem samples of both the naturally infected broiler and layer chickens, Newcastle disease virus was isolated from 128 (80%) samples using the avian embryos and confirmed by HA and HI tests (Table 1, 2).

Among the clinical samples of naturally infected broiler and layer chickens, virus isolation rate was found higher from tracheal swab (90%) compared to those of cloacal swab (85%) and serum (65%) (Table 1, 2).

Higher rate of NDV isolation from tracheal swab in this study might be due to presence of maximum load of the virus in the trachea during the course of infection. As NDV normally spreads through aerosol and ingestion, therefore the virus may have a chance to shed rapidly through trachea. The tracheal swab may be considered as the best source of NDV during sample collection both from the clinical signs and experimental cases. On the other hand, among the four different types of post-mortem samples, virus

isolation rate was found higher in spleen (100%) compared to those of lungs (80%), colon (60%), and brain (80%) samples. As spleen is a lymphoid organ and it filters the blood, during viremic condition there is a chance of infiltration of NDV from the blood to the spleen. For that virus concentration and rate of infection may be higher in spleen and it may be the best source of virus compared to other organs collected during post-mortem sampling of both the natural and experimental cases. The findings of NDV

isolation of the present study support the findings of Majid and Peter (2006), Krzysztof *et al.* (2006) and Singh *et al.* (2005). Among them, Majid and Peter (2006) isolated NDV from blood at 4 days after infection and other scientists isolated NDV at almost similar rate as of the present findings from tracheal swab, cloacal swab, spleen, lung, and colon and brain from the natural cases of ND.

Table 1. Newcastle disease virus isolated from the clinical and post-mortem samples of naturally infected quail from Isfahan province

Type of samples		No. of samples inoculated	Avian embryos	CEF
Clinical samples	Tracheal swab	20	18 (90)	20 (100)
	Cloacal swab	20	17 (85)	20 (100)
	Serum	15a/ 20b	13a (86.7) /13b (65)	15a (100)/15b (75)
Post-mortem samples	Spleen	5	5 (100)	5 (100)
	Lung	5	4 (80)	5 (100)
	Brain	5	4 (80)	5 (100)
	Colon	5	3 (60)	5 (100)

aSerum of day 1 and day 2, bPool serum of day 1, day 2 and day 3, CEF = Chicken embryo fibroblasts of fibroblasts

Table 2. Newcastle disease virus isolated from the clinical and post-mortem samples of naturally infected quail from Lorestan province

Type of samples		No. of samples inoculated	Avian embryos	CEF
Clinical samples	Tracheal swab	20	18 (90)	20 (100)
	Cloacal swab	20	17 (85)	20 (100)
	Serum	15a/ 20b	13a (86.7) /13b (65)	15a (100)/15b (75)
Post-mortem samples	Spleen	5	5 (100)	5 (100)
	Lung	5	4 (80)	5 (100)
	Brain	5	4 (80)	5 (100)
	Colon	5	3 (60)	5 (100)

aSerum of day 1 and day 2, bPool serum of day 1, day 2 and day 3, CEF = Chicken embryo fibroblasts of fibroblasts

NDVs were isolated from 150 (93.8%) out of a total 160 collected samples propagating in CEF cell culture

system and confirmed by HA and HI tests in this study (Table 1, 2). The rate of virus isolation in CEF

cell culture system from all the aforesaid of the clinical and post-mortem samples was found 100% with the exception of serum samples. As serum samples were collected at day 1, day 2 and day 3 of post-infection and virus isolation was possible from the blood samples collected at day 1 and day 2 only. None of the serum samples collected at day 3 post-infection was found positive for virus isolation both in avian embryo and CEF cell culture system. The failure of virus isolation from the blood samples at day 3 post-infection might be due to the lodgment of the virus in different tissues of the infected birds at day 3 of post infection. The findings of NDV isolation

using CEF cell culture system has close agreement with the findings of Mohan *et al.*(2005) In their study, the isolation rate of NDV was higher in CEF culture system compared to that of avian embryo. It was found that virus isolation rate both from the clinical and post-mortem samples except serum of day 3 post-infection was 100% in cell culture compared to that of the avian embryo as the isolation rate varies (Table 1, 2). The reduced rate of virus isolation in avian embryo may be due to the presence of maternal antibody.

Table 3. Detection of Newcastle disease virus from the clinical and post-mortem samples of naturally infected quails from Isfahan and Lorestan provinces.

Type of samples		Days post-infection and Direct tissue suspension	AF	ICF	RT-PCR
Clinical samples	Tracheal swab	Day 1	-	+	+	+
		Day 2	-	+	+	+
		Day 3	-	+	+	+
	Cloacal swab	Day 1	-	+	+	+
		Day 2	+	+	+	+
		Day 3	-	+	+	+
	Serum	Day 1	-	+	+	+
		Day 2	-	+	+	+
		Day 3	-	-	-	-
Post-mortem samples	Spleen	All the PM samples were collected immediately after death of the birds within 3-4 days of post-infection	-	+	+	+
	Lung		-	+	+	+
	Colon		+	+	+	+
	Brain		-	+	+	+

There is no report on NDV in *Coturnix Coturnix* in Iran. Among the clinical and post-mortem samples, inoculum of only cloacal swab and colon showed HA and HI activities (Table 3). This might be due to the presence of high concentration of free NDV in both types of samples during early and advanced stage of the disease. The anti-NDV hyperimmune serum revealed complete inhibition of the 4 haemagglutination unit of each isolate of viruses isolated from quail chickens present in the laboratory

samples (AF and ICF). Therefore results of HI test of the present study similar with the findings of Manin *et al.* (2002), Peroulis and O'Riley (2004) and Seal *et al.* (2005) who confirmed NDV by HI test. The genomes of NDV were detected by direct RT-PCR using NDV specific primers isolated from all the clinical and post-mortem samples of the infected quail chickens. The NDV specific primers used in the direct RT-PCR for genome detection of NDV showed equal sensitivity and specificity with the RNA

extracted from the clinical, post-mortem and laboratory samples (AF and ICF) as with the genomic RNA of standard NDV (Fig. 1 and Table 3). Results of the molecular detection of the present study highly agree with the findings of Jestin and Jestin (1991), Creelan *et al.* (2002) and Wambura *et al.* (2006).
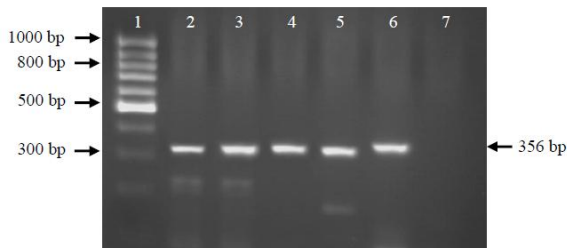
Fig. 1. RT-PCR products (356 bp) amplified from field samples and a reference strain of NDV visualized following electrophoresis on ethidium bromide stained 1.5% agarose gel. Lane 1 = DNA marker (100bp), Lane 2= Positive control (Komarov strain of NDV), Lane 3-4 = Clinical samples (tracheal and cloacal swabs), Lane 5 =Post-mortem sample (spleen), Lane 6 = Laboratory sample (allantoic fluid), Lane 7 = Negative control (IBDV).

This is a terrestrial species, feeding on seeds and insects on the ground. It is notoriously difficult to see, keeping hidden in crops, and reluctant to fly, preferring to creep away instead. Even when flushed, it keeps low and soon drops back into cover. Often the only indication of its presence is the distinctive "wet-my-lips" repetitive song of the male. The call is uttered mostly in the mornings, evenings and sometimes at night. It is a strongly migratory bird, unlike most game birds. Upon attaining an age of 6–8 weeks, this quail breeds on open arable farmland and grassland across most of Iran and Asia, laying 6-12 eggs in a ground nest. As Newcastle disease is one of the most important infectious as well as viral disease of poultry and responsible for remarkable economic losses in poultry sector every year, rapid detection and identification of the viruses are crucial for the adaptation of an effective control of the disease. Therefore, the molecular method (RT-PCR) can be introduced for rapid and confirmatory detection of NDV from any form of outbreak of ND in the context of Iran

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