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RESEARCH ARTICLE

Detection and Differentiation of Newcastle Disease Virus Strains Affecting Commercial Poultry in Northwest of IRAN using RT-PCR

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ABSTRACT

Newcastle disease (ND) is a highly contagious infection of poultry worldwide, caused by virulent strains of avian *paramyxovirus* type1. In order to rapidly detect and differentiate clinical isolates of ND virus (NDV) strains in poultry farms in Northwest of Iran, a RT-PCR assay was developed. RNA was extracted from ten NDV isolates and a one-step RT-PCR was performed. Three sets of primers were used to detect and differentiate virulent and non-virulent NDV strains by targeting their *F* gene. In the first step, a pair of general primers was employed to produce a product in all samples independent of the strain. Then, two sets of primers, specific for the F protein cleavage site sequence of virulent and non-virulent NDV strains, were used to differentiate NDV strains. Using RT-PCR, we detected the virulent strains of NDV in the poultry farms in Northwest of Iran. The results of the present study revealed that virulent strains of NDV affect the poultry in the region and the clinical diagnosis for the conformation of this disease can be made by RT-PCR.
To our knowledge, this is the first report of NDV detection and pathotyping in Northwest of Iran using molecular tools.

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INTRODUCTION

Newcastle disease is a highly contagious and fatal viral disease of poultry, categorized in list A diseases by the *Office* International *des Epizooties* (OIE) (Alexander 2000; Aldous *et al*. 2001). The causative agent, Newcastle disease virus (NDV), also known as avian *paramyxovirus* type1 (APMV-1), belongs to the *Avulavirus* genus within the *Paramyxoviridae* family (Alexander 2000; Kattenbelt *et al*. 2006). The enveloped virus has a non-segmented single-stranded negative-sense RNA. The genome of ~ 15.2 kb contains 6 genes in the order: 3´-NP, P, M, F, HN, L-5´ and codes for at least six structural and non structural proteins. The coded proteins include nucleo (N) protein, phospho (P) protein, matrix (M) protein, fusion (F) protein, haemagglutinin-neuraminidase (HN) protein and RNA polymerase (L) (Yusoff *et al*. 2001; Czegledi *et al*. 2006). The two interactive surface glycoproteins, F and HN, are involved in cell surface attachment and cell membrane fusion. These two proteins serve as the targets for the host's immune response (De Leeuw *et al*. 1999; Panda *et al*. 2004).

Infection with NDV can lead to a broad range of clinical signs, such as asymptomatic enteric to systemic infections with up to 100% mortality (Nanthakumar *et al*. 2000). Based on the severity of the disease in chickens, NDVs are categorized into asymptomatic enteric, lentogenic (low virulence), mesogenic (intermediate virulence) and velogenic (high virulence) pathotypes (Alexander 2000; Aldous *et al*. 2008). Further, the velogenic NDV subdivision pathotypes are classified as viscerotropic and neurotropic based on the clinical manifestation (Alexander *et al*. 2008b). All velogenic and some mesogenic strains of NDV are considered virulent for poultry (Aldous *et al*. 2008).

The virulence of NDV strains correlates to the cleavability of the F precursor protein, F0, which is cleaved into two disulfide-linked polypeptide subunits, F1 and F2 (De Leeuw *et al*. 2003). In the virulent strains post-translational cleavage of F0 is done by ubiquitous

host proteases that are found in most tissues. In contrast the F0 protein of non-virulent strains is cleaved only in cells containing trypsin-like enzymes which are limited to respiratory and alimentary tracts (Panda *et al*. 2004). So the spread of virulent strains is faster within the host (Lamb *et al*. 2001). The molecular basis for different levels of NDV pathogenicity is determined by the amino acid sequence of the F0 protein cleavage site (FPCS) (Aldous *et al*. 2001). In the virulent strains two pairs of basic amino acids, either lysine (K) or argienine (R) between residues 113 and 116 at the C-terminus of F2, as well as phenylalanine (F) at residue 117 in the N-terminus of F1 exist. In comparison, low virulence strains have a monobasic cleavage motif and leucine (L) at residue 117 (Collins *et al.* 1993; Peeters *et al*. 2000). Therefore pathotype prediction and diagnosis of NDV virulence can be determined by FPCS sequence analysis (Alexander *et al*. 2008a).

During recent years severe outbreaks of the disease with high mortality, suspicious to ND, in poultry of Northwest of Iran have increased markedly. The consequence has a great economic impact on the poultry industry in this region. To our knowledge, no study has been done to detect and pathotype of NDV strains in industrial poultry of the region. Hence, the objective of the present work was to isolate and differentiate NDV strains obtained from clinical cases in Northwest of Iran using RT-PCR as a sensitive and rapid diagnostic method.

MATERIALS AND METHODS

Collection of samples

A total number of 52 clinically suspicious birds believed to be infected with ND, 4 from each farm, were chosen from 13 different commercial poultry farms in various areas of Northwest of Iran. The birds were transferred to the virology laboratory, Faculty of Veterinary Medicine, Urmia University. The infected birds were euthanatized and the tissue samples from brains, lungs, spleens, tracheas and caecal tonsils of each farm (4 birds as a single sample) were removed and transferred into sterile tubes separately (Westover *et al*. 2001; OIE 2012). The samples were stored at -20°C for further use.

Isolation and confirmation of virus

As most of the flocks predominantly showed nervous signs of ND, brain samples of birds from each farm were separately triturated and homogenized using a sterilized pestle and mortared. The samples were homogenized in 5 ml tryptose phosphate broth solution containing penicillin (2000 IU/ml), gentamicin (50 µl/ml) and mycostatin (1000 units/ml). The suspension was incubated at 25°C for 30 minutes and centrifuged at 1000 *g* for 10 minutes. Afterwards, five, 10-days-old specific pathogen free (SPF) embryonated chicken eggs (ECE) (*Lohmann*, Australia) were inoculated with 0.2 ml of supernatant through allantoic cavity route. The inoculated eggs were incubated at 37°C for 48 hours and chandelled every 12 hours. The eggs with live embryos were incubated 4 additional days and candelled every 12 hours continuously. At the end of the incubation period, the eggs were chilled at 4°C and the allantoic fluids were collected (Collins *et al*. 1998; OIE 2012).

The presence of NDV in the allantoic fluid was determined using spot haemagglutination and micro-agglutination tests. Positive samples were confirmed as NDV through haemagglutination inhibition (HI) test with NDV specific antiserum (Razi Serum & Vaccine Research Institute, Karaj, Iran) according to the OIE standard procedures (OIE 2012). The infected allantoic fluids were stored at -70ºC as viral batches.

Viral RNA extraction

RNA was extracted from the infected allantoic fluid (as a viral source) using High Pure Viral Nucleic Acid Kit (Roche, Germany) according to the manufacturer's instructions. The RNA pellet was re-suspended in 20 µl nuclease-free water (Roche, Germany) for immediate use or stored at -70°C.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using Titan one tub RT-PCR system (Roche, Germany) and 3 sets of primers targeting the *F* gene (Table 1). RT-PCR was carried out in 3 separate reactions with primer pair A+B for detection of all NDVs, primer pair A+C for detection of virulent NDVs and primer pair A+D for detection of non-virulent NDVs (Kant *et al*. 1997).

The RT-PCR reaction mixture for each sample consisted of 2 µl of dNTPs mix (10 mM), 1 µl of forward primer (10 pM) A, 1 µl of reverse primer (10 pM) B or C or D, 7 µl of the purified template RNA, 1.25 µl of DTT solution, 0.25 µl of protector RNase inhibitor, 5 µl of 5x RT-PCR buffer and 0.5 µl of M-MuLV reverse transcriptase enzyme mix (Vivantis, Malaysia) in a final volume of 25 µl. Herts/33 strain as a velogenic strain, Komarov as a mesogenic strain and B1 as a lentogenic strain were used as positive control samples in this research. A master mix without viral RNA was used as a negative control.

One-step RT-PCR was performed using an Eppendorf gradient thermo cycler (Model 22331, Germany) according to the following protocol: 45 min at 45°C for cDNA generation and 2 min at 94°C for initial denaturation followed by 35 cycles of three steps: denaturation at 94°C for 1 min, annealing at 58°C (for primers A+B and A+D) or 53°C (for primers A+C) for 1 min and elongation at 68°C for 1 min, with a final extension step at 70°C for 7 min. Amplified products were separated by 1.5% agarose gel electrophoresis at 100 V for 1 h in 1x TAE buffer stained with ethidium bromide and visualized using a UV transilluminator (Model DOC-008, EEC). A 100 bp DNA ladder (Fermentas, Germany) was used as weight marker.

RESULTS

Among the 13 specimens collected from 13 different commercial poultry farms (4 birds from each farm combined into one sample), suspected of ND, in Northwest of Iran, NDVs were isolated from 10 out of 13 brain homogenates by inoculation of ECEs. The viruses were isolated after one or two passages in the allantoic

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

Code	Sense	Sequence	Location on *F* gene
A	+	5'-TTGATGGCAGGCCTCFFGC-3'	141-159
B	-	5'-GGAGGATGTTGGCAGCATT-3'	503-485
C	-	5'-AGCGT(C/T)TCTGTCTCCT-3'	395-380
D	-	5'-G(A/G)CG(A/G)CCCTGT(C/T)TCCC-3'	395-380

Table 2: NDV isolation from Northwest poultry farms of Iran

No. of flocks	Tissue sample	No. of samples positive for virus isolation	RT-PCR		
			Primers A+B	Primers A+C (virulent)	Primers A+D (non-virulent)
13	Brain	10	10	10	0

cavity. Identification of the isolates using NDV-specific polyclonal antiserum in HI test confirmed the viruses as NDV.

F gene targeting RT-PCR was performed on purified RNA using primers as described by Kant *et al*. (1997). The summarized results are shown in table 2. The RT-PCR assay using primer pair A+B would result in a 362 bp fragment with all NDVs regardless of strain or pathotype (Kant *et al*. 1997). The expected amplicon was obtained in all of the 10 field isolates (Fig. 1). Virulence of NDV isolates was evaluated based on the differences in *F* gene cleavage site motif in virulent and non-virulent strains. The NDV pathotypes were differentiated in two separate RT-PCR reactions using pathotype specific primers A+C and A+D representative of virulent and non-virulent strains, respectively. A 254 bp fragment generation with primer pair A+C in 10 isolated samples indicated that all of the viruses were virulent (Fig. 2). Moreover, in the third step, no isolate was detected as non-virulent using primer pair A+D, producing a 254 bp fragment only with the positive control (Fig. 3). From negative controls, no product was obtained.

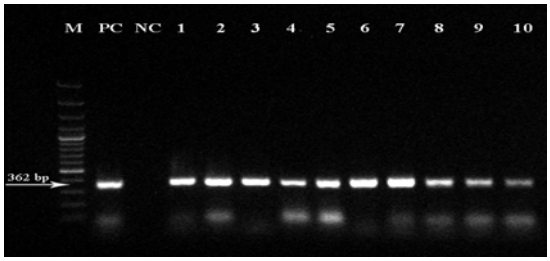

Fig. 1: RT-PCR products (362 bp) generated from virulent and non-virulent strains of NDV using primer pair A+B: M=100 bp DNA Marker (Fermentas, Germany), PC=positive control, NC=negative control, Lanes 1-10=field samples.

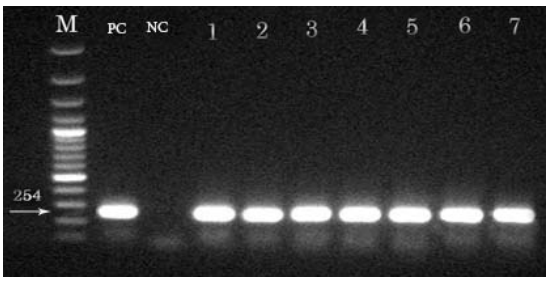

Fig. 2: RT-PCR products (254 bp) generated from virulent isolates of NDV using primer pair A+C: M=100 bp DNA Marker (Fermentas, Germany), PC=positive control, NC=negative control, Lanes 1-7=field samples.

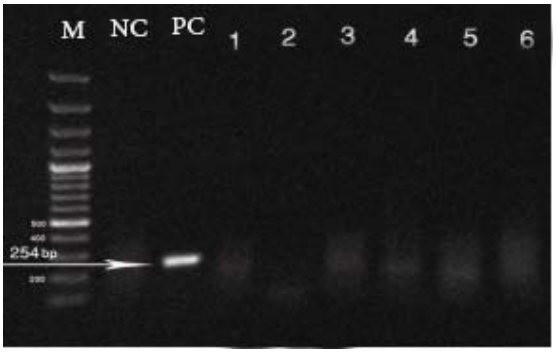

Fig. 3: RT-PCR using primer pair A+D did not result in any product related to non-virulent strains: M=100 bp DNA Marker (Fermentas, Germany), NC=negative control, PC=positive control, Lanes 1-6=field samples.

DISCUSSION

Despite routine vaccination programs, outbreaks of ND have frequently occurred in the Northwest of Iran. The disease remains a constant threat to commercial poultry and leads to huge economic losses. Furthermore, no epidemiological information is available on NDVs in the Northwest of Iran; thus, the present study was proposed to detect and pathotype the circulating viruses in the region by RT-PCR as a first step to achieve this aim. All 10 NDV isolates were examined by designing primers as described by Kant *et al*. (1997). Production of a 362 bp fragment in all 10 isolates using general primer pair A+B confirmed the virus isolation. Amplification of a 254 bp product with A+C pair of primers indicated the high virulence of these strains. As the RT-PCR using primer pair A+D did not result in any fragments, it indicates that non-virulent isolates were not responsible for NDV infections.

Since ND is highly contagious, rapid detection and identification of the virus is crucial for an effective control and/or eradication of the disease (Smietanka *et al*. 2006). Variation in NDV virulence and widespread use of live vaccines mean that identification of a virus as NDV does not confirm an accurate diagnosis of ND. This emphasizes the need for a reliable method of virulence assessment (Aldous *et al*. 2001). The *in vivo* pathogenicity tests developed for differentiation of high and low virulent NDV strains include the intracerebral pathogenicity index (ICPI), the intravenous pathogenicity index (IVPI) and the mean death time (MDT) (Alexander 2000; Khan *et al*. 2005; Alexander *et al*. 2008b). These methods are slow and laborious, requiring unacceptable use of animals.

Above all, they are highly expensive (Aldous *et al*. 2001). The knowledge of NDV virulence dependence on FPCS sequence aids in the development of molecular techniques such as reverse transcription-PCR. RT-PCR as a routine and reliable laboratory procedure, offers significant advantages over the conventional methods of NDV virulence assessment. The speed of diagnosis can considerably be increased to one day which allows rapid identification of many samples (Swayne *et al*. 2003). Identification of the virus can be performed directly from organ homogenates without the need for virus expansion. Another advantage of RT-PCR is the possibility of sequencing PCR products and consequently extensive evaluation of genomic changes and molecular epidemiology (Dortmans *et al*. 2010). However there are some main drawbacks for this procedure: a high potential for cross contamination of the samples because of post-amplification processing, and/or limited sensitivity in detecting complicated samples, such as faeces. Thus, virus concentration and purification method will be of great help to enhance the detection sensitivity (Cattoli *et al.* 2011).

The first RT-PCR for detection of NDV was described by Jestin *et al*. (1991) and to date it has been successfully developed with different modifications. Seal *et al* (1995) and Smientanka *et al.* (2006) could detect isloates of NDV using RT-PCR. The use of pathotype specific primers in our study enables to establish virulence of an isolate directly; versus the virus virulence in both these studies can be determined by the subsequent RT-PCR products' sequencing. In 1997 the method was used for differentiation of velogenic and lentogen NDV strains directly in tissue homogenates by Kant *et al*. Despite the efficacy of the method for directly viral RNA detection in tissue homogenates, we did not omit the prior virus isolation in ECE to have the viruses in our archive. Our results are in agreement with the results obtained by Kant *et al*. Detection of NDV in tissues and faecale samples using RT-PCR has been investigated by Gohm *et al*. (2000). Rapid pathotyping of NDV from allantoic fluid and organs was demonstrated by Li *et al.* (2004). In both these studies the screening samples were from experimentally infected chickens, in comparison with our samples which were collected from field infected cases. Wize *et al.* (2004) could detect NDVs in clinical samples by real time RT-PCR method. Real time RT-PCR (rRT-PCR) assay has the advantage over conventional RT-PCR assay in that fluorogenic hydrolysis probes or fluorescent dyes are used to monitor the present of target DNA after each PCR cycle, thus providing results in real time. The same as rRT-PCR, using a one-step assay in this study eliminated the post-amplification processing step. Singh *et al*. (2005) investigated NDV in samples colleceted from poultry farms. They applied nested PCR for further conformation of the primary amplicons as an advantage over our method, but the used set of primers in their study were not able to simultaneously pathotype the isolates.

Conclusions

In conclusion, the results of the present study prove that ND manifests as a viral disease in commercial poultry in Northwest of Iran. We conclude that RT-PCR can be used for identifying NDV as a fast and reliable method that enables clear differentiation of pathotypes of the virus strains and isolates. Our results aid in efficient control/ eradication of the disease and epidemiological analysis of NDVs in the region.

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