Virus isolation, identification and characterization of Newcastle disease virus strains isolated from two captivated falcon species (*Falco peregrinusperegrinus*) in Qatar

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ABSTRACT

A first trail of isolation, identification and characterization of Newcastle disease virus (NDV) field strains was reported in two captivated falcons(*Falcoperegrinusperegrinus*) in Qatar.Virus cultivation in specific pathogen free (SPF) embryonating chicken eggs (ECE),haemagglutination (HA), haemagglutination inhibition (HI) and mean death time (MDT)used in the investigation to reveal virus identity and pathogenicity. Both NDV isolates were found to be velogenic strains. Real time reverse transcriptase polymerase chain reaction (rRT-PCR) used as a confirmatory technique, successfully amplified and detected cDNA fragments corresponding to the NDV RNA extracted from amnioallantoic fluids (AAF). Sequence analysis and phylogensis of the NDVQF13 strain using 150bp primer sets revealed ^{112}R-R-Q-K-R-F^{117} amino acid motif confirming velogenicity of the strain and its relation to genotype group VII, respectively. NDVQF13 failed qualification for sequence and phylogentic analysis using the same primers.

Key Words: Newcastle disease, Newcastle disease virus, captivated falcons, virus isolation, pathotyping, real time reverse transcriptase polymerase chain reaction, sequence, phylogensis, genotyping.

Abbreviations: Newcastle Disease (ND), Newcastle Disease Virus (NDV), Real Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR), Haemagglutination (HA), Haemagglutination Inhibition (HI), Mean Death Time (MDT), Complementary DNA (cDNA)

INTRODUCTION

Newcastle disease (ND) is a known highly contagious disease of a significant worldwide impact causing velogenic, mesogenic and lentogenic infections to hundreds of avian species. The virus belongs to avian paramyxovirus type 1(APMV-1) serotype, genus *Avulavirus*that relates to the family *Paramyxoviridae*and *Mononegavirales* order(Alexander and Senne, 2008). The virus is approximately 15 kb RNA genome, negative-sense, single-stranded in an order of 5'-L-HN-F-M-P-N-3' encoding for largeprotein, haemagglutinin-neuraminidase protein, fusion protein, phosphoprotein, matrix protein(Alexander, 2008).

Since the first informative detection, isolation and pathotyping of Newcastle disease virus (NDV) in Qatar (Mohran*et al.,* 2011) continuous reporting of the disease primarily among chickens and other domesticated birds were observed (Anon, 2011, 2012, 2013). Although reporting ND among falcons was early (Hills *et al.,* 1953), and it is worldwide (Okoh, A.E.J., 1979; Werner*yet al.,* 1992; Elvira *et al.,* 2003) nothing is known about the disease in Qatar.This situation

signals public health hazards among traditional rearing feral avid species including falcons, and the conventional and industrial growing poultry businessin the country.

Aiming atinvestigating Newcastle disease (ND) in falconspecies in Qatar, virus isolation, identification and characterization attempts were conducted. Conventional virological methods, real time reverse transcription polymerase chain reaction (rRT-PCR) technique, sequence and phylogenic analysis were used to testsamples collected from apparently healthy and ND-suspected captivated birds.

MATERIALS AND METHODS

Samples: A total of 70tracheal and cloacal swabs from 35apparently healthy and ND-suspected captivated falcons were collected. All swabs were immediatelytransported in cool chamber condition and frozen at - 40^{o}Ctill used.Amnioallantoic fluids (AAF)of specific pathogen free (SPF) embryonatingchickeneggs(ECE) inoculated with the suspected samples were either used freshly or asfrozen stocks.

Virus isolation: Approximately 10% elution of each tracheal and cloacalswab collected from each falconwas prepared in sterile phosphate-buffered saline (SPBS). Then, the elutions werecentrifuged for 10 min at 2500 rpm at 20^{o}C. Each 2 mL of the final inoculum was supplemented with 100µL 200 IU mL^{-1} cell culture grade penicillin, 200 mg mL^{-1} streptomycin and 20 mg mL^{-1} gentamycin suspensions. Finally, 200µL of each elution was inoculated into the allantoic sac of five 10-day-old SPF ECE, Loghman breed (VALO, Germany). Controls received the same dose of SPBS. All eggs were incubated at 37^{o}C for 8 days observation. Two hundred microlitre of each neat AAF obtained from the first passage was inoculated for a successive second passage using the same inoculation conditions.

Haemagglutiantion (HA) and Haemagglutination Inhibition (HI) test: Ten per cent 4-week-old washed chicken RBC was used for rapid spot HA testing individual AAF of each dead embryo prior to harvesting and pooling. Pools of positive AAF passage level two were used for virus identification applying HA and HI assay following standard procedures (OIE Terrestrial Manual, 2012). Four HAU determined by HA assay of each isolatewas used for virus specificity testing using a reference anti-NDV serum (PA0155, VLA, UK).

Determination of the 50 Embryo Lethal Dose (ELD50) and Mean Death Time (MDT): A single-step determination of both ELD50 and MDT was conducted according to Allan and coworkers (1987). Two hundred microlitre of 10^{5}-10^{12} AAF dilutions prepared from the second passage was inoculated each into the allantoic sac of five 10-day-old SPF ECE. All eggs including uninfected controls were incubated at 37^{o}C and observed for 8 days at 12 h intervals. ELD50 was calculated according to Reed and Muenchmethod (1938).

Viral RNA extraction: Poolsof AAF from positiveNDV haemagglutination (HA) and haemagglutination inhibition (HI)isolates were used for isolation of viral RNA using QIAamp (QIAamp Viral

RNA Mini Handbook, 2005). Briefly, 140µL from each pool was added to 560µL buffer AVL RNA carrier, pulse-vortexedfor 15 sec and incubated at room temperature (15-25^{o}C) for 10 min. The mixture was briefly centrifuged. 560µL 96-100% ethanol was added and the mixture was pulse-centrifuged for another 15 sec. carefully, 630µLof this solution was applied to QIAamp Mini spin column contained in a 2 mL collection tube, centrifuged at 6000 X g (8000rpm), for 1 min. The column was placed in a new 2mL collection tube and additional 630µLof the solution was applied to column repeating the above mentioned process. Then, 500µL Buffer AWI was added to column, centrifuged at 6000 X g (8000rpm), for 1 min and the column was placed in a clean 2mL collection tube. 500µL Buffer AW2 was added and the content was centrifuged at 10500 X g (14000 rpm) for 3 min.The QIAamp spin column was transferred to a clean 1.5mL microcentrifuge tube, and 80µL AVE Buffer was added, incubated for 1 min at room temperature and centrifuged at 6000 X g (8000rpm) for 1 min. All viral RNA extracts were stored at -20^{o}C till amplification.

Real time reverse transcription polymerase chain reaction (rRT-PCR): QualitativerRt-PCR used as a confirmatory test was performed according to QIAampdirections (Qiagen, GMbH, Germany, 2005directions. For each 10.5 µL 1% dilution RNA template12.5 µL 1X RT-PCR buffer, 1 µL1X RT-PCR enzyme mix and1 µL 1X NDV primer probe mix was prepared and added to form a final volume of 25 µL. Positive and negative controls were treated the sameusing 10.5 µL of approximately 0.1% NDVpositive controlpreparationand10.5 µL NTC (Nuclease- Free Water), respectively.ABI 7300 Real Time Cycler was used for amplification and analysis of cDNA fragmentsfollowinga program of 48^{o}C for 10 min (RT step), 95^{o}C for10 min (RT inactivation/denaturation), 40 cycles of amplification of 95^{o}C for 15 sec and60^{o}C for 45 sec, respectively.

Reverse transcription polymerase chain reaction (RT-PCR): The forward (5'-GCA GCT GCA GGG ATT GTG GT-3', nucleotide position 158-177) and reverse (5'- TCT TTG AGC AGG AGG ATG TTG-3', nucleotide position 513-493) oligonucleotide primer sets were used for amplification of 356 bp amplicons corresponding the cleavage activation site of NDV-F gene (Nanthakumar*et al.*, 2000). RT-PCR amplification followed Access QuickRT-PCR Kit (Cat. no.A1700).25.5µL total reaction mix was prepared using 12.5µL 2X Access Quick Master Mix, 2.5µL 1 µM upstream F-primer and control R-primer each, 2.5µL Nuclease-free water, 0.5 µL 5 U/µ AMV RT enzyme. A three-step amplification method of 35-cycle-programmed PCR machine (9800 Fast Thermal Cycler) was used for cDNA amplification as follows: 45^{o}C for 45 min and 95^{o}C for 2 min (RT-step), 3-step cycling of 94 ^{o}C for 45 sec

(denaturation), 58 ^{o}C for 45 sec (annealing), 72^{o}C for 45 sec (extension) and 72^{o}C for 5 min (final extension).

Nested RT-PCR: One µL of each 10% diluted primary amplicon was amplified with the F-primer (5'-CCC CGT TGG AGG CAT AC-3', nucleotide position 282-298) and R-primer (5'-TGT TGG CAG CAT TTT GAT TG-3', nucleotide position 497-478) targeting the 216 bp internal sequence of the cleavage activation site of the NDV-F gene (Nanthakumar*et al.,* 2000). GeneAmp Gold PCR Reagent kit was used for amplification in a total volume of 50µL reaction mix containing 36.4µL ddH_2O, 5 µL 10X PCR buffer, 1µL 10mM each F and R primers, 5µL20mM each dNTP, and 0.6µL DNA polymerase. MQ H_2O was included as negative control in both primary and nested RT-PCR techniques. Amplification phases were as that in RT-PCR using BioRad –I Thermocycler, SN: 582BR011467, and cycles were adjusted following 95^{o}C for 5 min (initial activation), 3-step cycling 94^{o}C for 45 sec (denaturation), 56^{o}C for 45 sec (annealing), 72 ^{o}C for 45 sec (extension) for 35 cycles followed by 72^{o}C for 5 min (final extension). All amplicons were determined by electrophoresis, visualization and documentation following standard procedures.

Sequence analysis of the cDNA fragments: cDNA Nested RT-PCR products measured for concentration, run on 1.5% agarose gel according to standard procedures. cDNA fragments purification was done according to ExoSAP-IT instructions. Amplificationusing the 216bp nested RT-PCR primer sets was done according to ExoSAP-IT instructions (PCR: 78200/01/02/05/50-Affymetrix, USA) using 16.2µL ddH_2O, 0.8µL each F and R primers, 1µL purified cDNA product. Deduced amino acid sequences were constructed and aligned using MEGA (Version 5.05).

RESULTS

Epidemiological and clinical observations: Both falcons were non-vaccinated against ND. Sick falcons exhibited nervoussigns characterized by torticollis. No postmortem was performed.

Isolation of NDV from swabs: TwoNDVfield strains were successfully recovered intothe 10-day-old SPF ECE inoculated with the NDV-suspected swabelusions with successive maximum egg passage level two. The mean HA and HI titerswere determined. The isolated strains were designated NDV-QF10 and NDV-QF13 (Table 1).

ELD50 andMDT of the isolates: The ELD50 and MDT of the isolates are shown in Table (1). All AAF of the dead ECEwere positive on rapid spot HAassay indicative of specificity to NDVinfection.

Detection of NDV from AAF: NDV cDNAfragments were successfully detected in the AAF of the infected SPF ECE using rRT-PCR. The positive and negative controls were within the expected detection threshold.

Table (1): Properties of the NDV falcon strains studied at egg passage level two

NDV strain	SHA	HA (log 2)	HI (log 2)	ELD50 (log 10) (0.1mL)	MDT (hour)
NDVQF10	Positive	10	11	8.8	48
NDVQF13	Positive	8	11	8.7	48

Sequence analysis of NDV-QF10 and NDV-QF13: Sequence of the cDNA of NDV-QF13 isolate strain was shown in Fig. (1). The strain was proved to be velogenic showing amino acid sequence^{112}R-R-Q-K-R-F^{117}and classifying the virus under genotype VII. cDNA of the NDV-QF10 failed thrice to undergo amplification sequence analysis.

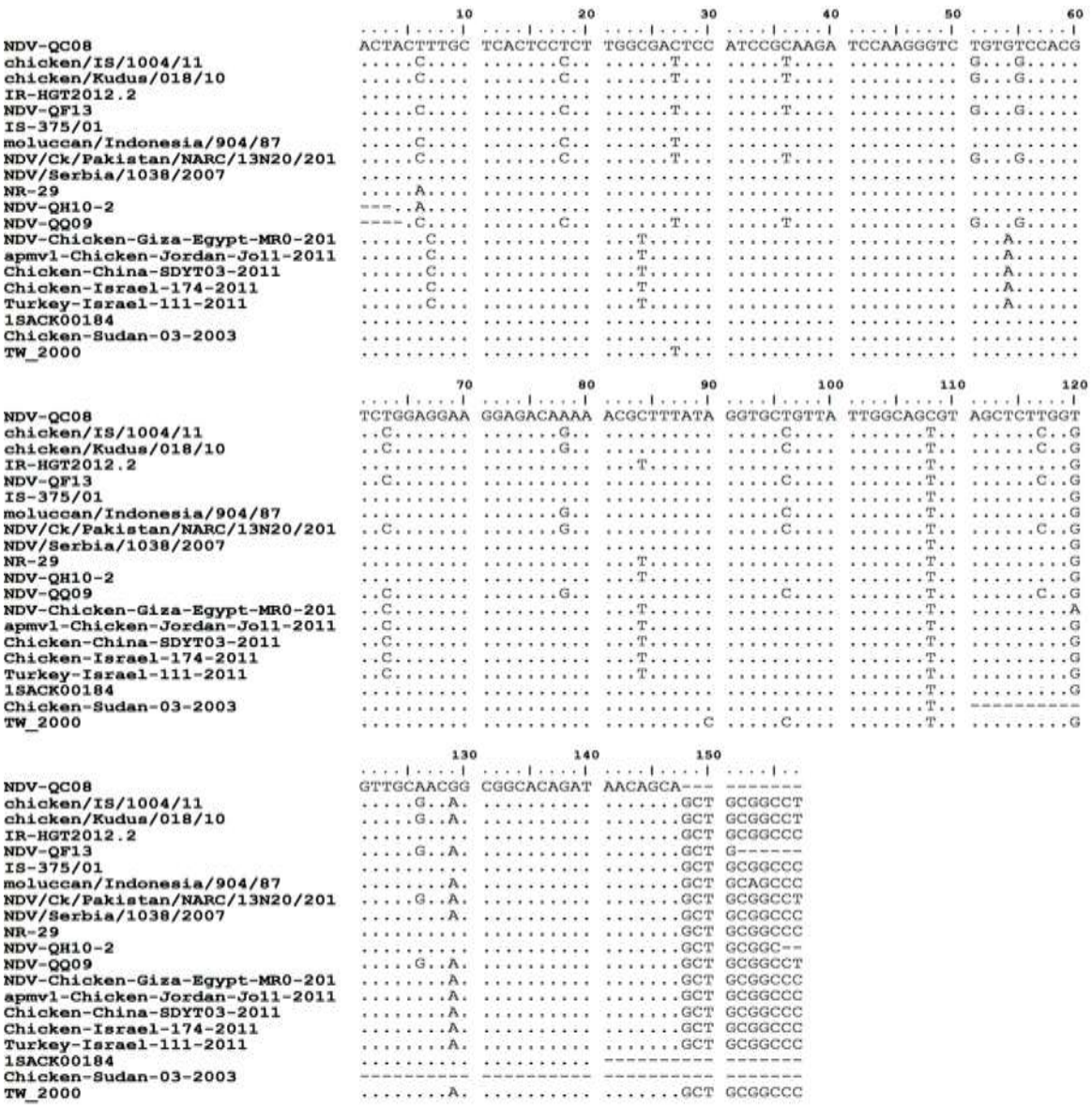

Fig. (1): Sequence analysis of the amplified F-gene of NDV-QF13 showing the gene cleavage site and its deduced amino acid sequences compared to NDV-QC-08 and other GenBank worldwide strains (MEGA, Tamura-Nei model 1993)

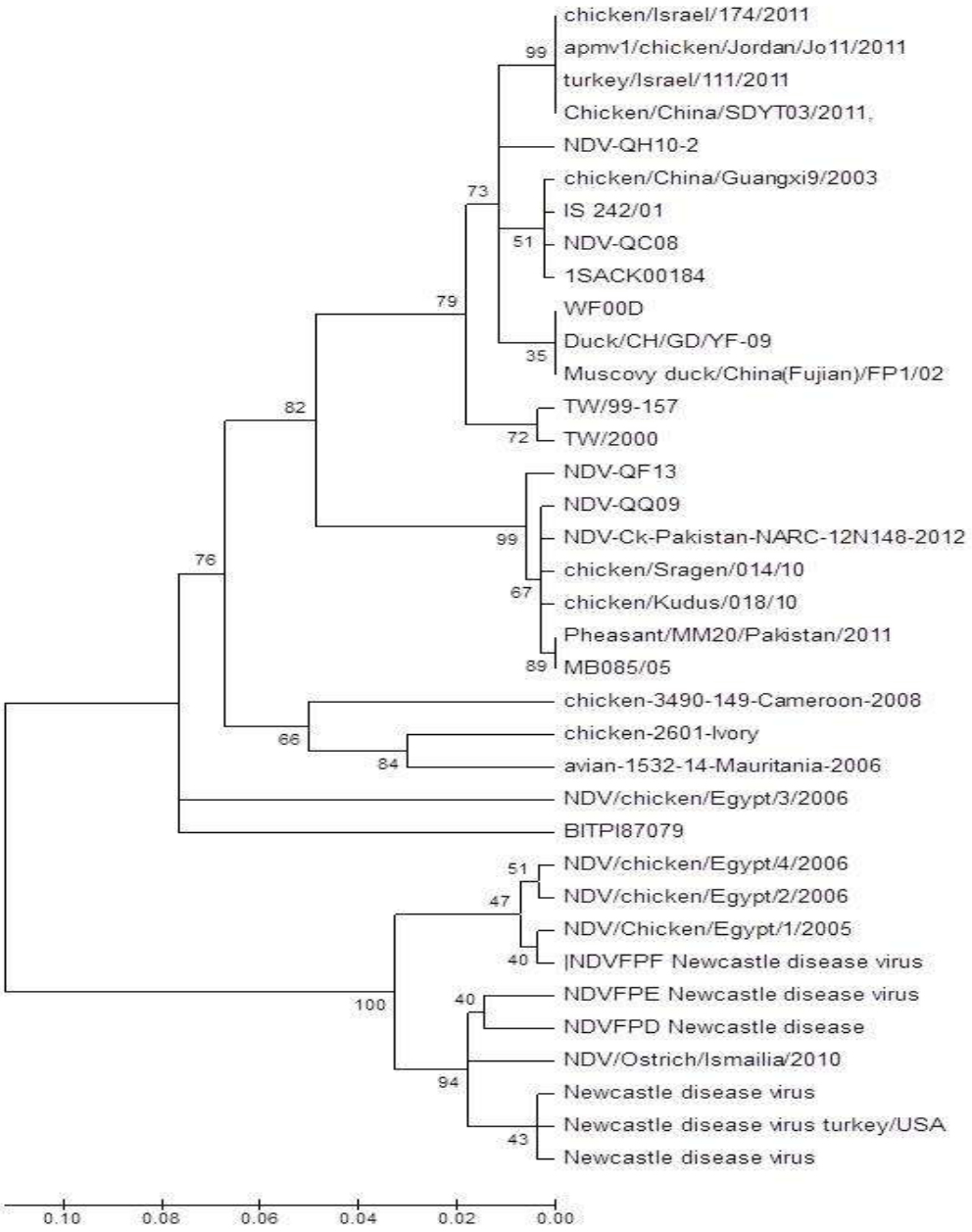

Fig. (2): The phylogenetic tree analysis (MEGA) of NDV-QF13 isolate (shaded triangle) compared to NDV-QC08, NDV isolate in Qatar, and to other NDV sequences retrieved from the GenBank (Modern Evolutionary Genetic Analysis "MEGA"), Tamura-Nei model 1993).

DISCUSSION

Two NDV field strains were isolated and characterized based on clinical epidemiology of the disease, virus isolation, MDT, sequence and phylogenic analysis. MDT of 48 hr classifies both NDV-QF10 and NDV-QF13 isolates among velogenic strains. Revealing amino acid motif ^{112}R-R-Q-K-R-F^{117} on sequencing the F-gene cleavage site of the NDV-QF13 strain, sustains the conventional categorization of the isolate velogenic. Although the investigation used a relatively short internal sequencing primer sets, alignment of the cleavage site results showed complementation with that of NDV-QC08, the first reported isolate in Qatar (Mohran*et al.* 2011). Despite lowering the annealing temperature of the 150bp internal primer sets to 42^{o}C, failure of the cDNA of NDV-QF10 strain to amplify might be attributed to the relatively short segment of the primers used in this investigation. However, not necessary genetically related, the insignificant difference in the ELD50 values of NDV-QF10 and NDV-QF13 strains ($10^{8.8}$ and $10^{8.7}$ $mL^{-0.1}$), respectively, and the similarity of the MDT (48h) might indicate the close relationship between the two NDV strains.

Beside its using primarily to rapidly detect virus genomes in various microbial susceptible samples, the use of rRT-PCR in this study to detect NDV replication in AAF of the infected SPF ECE could be a useful tool to rapidly confirm virus isolation in labs where standard HA and HI facilities are not available.

The fact that falcons play a significant role in the traditions of Gulf citizens, and that it is an important worldwide cross-boundary avian subspecies, beside its feeding habits on both ND-susceptible and reservoirs birds, warrants more strict measures to guard investment in.

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