Molecular detection and pathotyping of a Newcastle disease virus field strain from backyard chickens

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Abstract
A first trial of detection, isolation and pathotyping of a Newcastle disease virus (NDV) field strain was conducted in Qatar. NDV RNA was isolated, amplified and detected from oropharyngeal and cloacal swabs from clinically diseased backyard chickens and amnioallantoic fluids (AAF) of infected chicken embryos using primary RT-PCR, nested RT-PCR and gel electrophoresing techniques. The 356 bp and 216 bp RNA fragments were retrieved in primary and nested RT-PCR, respectively. NDV was isolated, identified and pathotyped in chicken embryos using haemagglutination (HA), haemagglutination inhibition (HI) tests, mean death time (MDT) and intracerebral pathogenicity index (ICPI). The causative agent was found to be a highly virulent strain.

Key words: Newcastle disease, Newcastle disease virus, chicken, RT-PCR, nested RT-PCR, virus isolation, pathotyping.

Introduction

Newcastle disease (ND) is a highly contagious viral malady of avid species known to cause significant worldwide afflictions to poultry industry. The virus belongs to avian paramyxovirus type 1 (APMV-1) serotype, genus *Avulavirus* that relates to the family *Paramyxoviridae* **(Mayo, 2002).** Up-to-date, over 250 species were estimated to be susceptible **(Alexander, 2008).** Although ND was diagnosed earlier in Saudi Arabia neighboring Qatar **(El-Zein, 1986)** and frequent virulent isolates had been reported in the Middle East **(Alexander *et al.,* 1987).** Only clinical reports of presence of the disease in Qatar were known. Information about the history, incidence and prevalence of the disease is limited. Because of lack of laboratory facilities at that time, no detection and isolation trials were conducted to substantiate the presumptive diagnosis describing the disease. The newly establishment of such means has paved the way to carry definitive laboratory diagnosis.

In order to investigate the presence of Newcastle disease virus (NDV) in samples from backyard chickens during ND-suspected outbreak, reverse transcription polymerase chain reaction (RT-PCR), nested RT-PCR, virus isolation and pathotyping techniques were used.

Materials and Methods

Samples: Oropharyngeal and cloacal swabs, spleens, kidneys, tracheae and livers were collected from apparently diseased native breed chickens reared on backyard. Swabs were preserved individually into 1 mL virus transport medium and were refrigerated for future processing. Necropsies were stored frozen at -40^{o}C till used. Amnioallantoic fluids (AAF) from dead organ tissue of the inoculated chicken embryos were either used freshly or as frozen stocks.

Viral RNA extraction: Separate pools of oropharyngeal and cloacal swab fluids and AAF from dead organ tissue of the inoculated chicken embryos were used for isolation of viral RNA using AM 1929 MagMax^{TM} AI/ND Viral RNA Isolation Kit (Ambion ABI, USA) following the manufacture's instructions. LaSota B_1 type, clone N79 and HN79 mass (Schering-Plough, USA) vaccine strains, used for vaccination in the country, were included as positive controls. Briefly, 400 µL from each pool was added to 802 µL viral lysis/binding solution, vortexed gently for 30 sec followed by

brief centrifugation. The lysate was mixed with 20 µL RNA magnetic bead resuspension, vortexed gently for 4 min and centrifuged briefly for 2 sec. Then, the mixture was captured to a magnetic stand for 3 min. The supernatant was removed and the trapped viral RNA was washed three times with two washing solutions. The beads were dried for 2 min before elution of the isolated RNAs in 100 µL elution buffer. All RNAs were collected after magnetically trapping the beads, and were stored at -20 ^{o}C for amplification.

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). Reverse transcription and PCR amplification followed QiaAmp one step RT-PCR procedure (Qiagen). A 50 µL total reaction mix was prepared using 10 µL 5X Qiagen RT-PCR buffer, 2 µL dNTPs, 10 µL 5X Q-solution, 6 µL 5 µM F and R primers each, 2 µL enzyme mix, 0.5 µL 20U/ µL Rnase inhibitor, 9 µL MQ H_2O and 100 µg viral RNA. A three-step amplification method of 35-cycle-programmed PCR machine was used for cDNA amplification as follows: 50 ^{o}C for 30 min (reverse transcription), 95 ^{o}C for 15 min (initial PCR activation), 3-step cycling 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: Nested RT-PCR was used for confirmation. About 1 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 50 µL reaction mix containing 5 µL 10X PCR buffer, 15 pmol each of the F and R primers, 200 µM each dNTP, 2.5 mM $MgCl_2$ and 3 units AmpliTaq Gold 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 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.

Virus isolation: Tissue homogenates were used for virus isolation. The 10 % pools were prepared in sterile Hank's-Balanced Salt Solution (SHBSS) (Euro C-lone), followed by centrifugation for 10 min at 1000 rpm at 20 ^{o}C and filtration was done through 0.45 µm Millipore membranes. Each of the final inocula was supplemented with 50 µL of 200 IU/ mL cell culture grade penicillin, 200 mg mL^{-1} streptomycin and 20 mg/ mL gentamicin. About 200 µL of each homogenate was inoculated into the allantoic sac of five 10-days-old commercial chicken embryos (Loghman breed). Controls received the same dose of SHBSS. All eggs were incubated at 37 ^{o}C for 8 days observation. About 100 µL of AAF obtained from the first passage was inoculated successively for another two passages using the same inoculation conditions.

Haemagglutiantion (HA) and Haemagglutination Inhibition (HI) test: HA and HI were used for virus identification following the standard procedure (OIE Terrestrial Manual, 2008). The 10 %, 4 weeks,old washed chicken RBC was used for spot HA testing individual AAF from the dead embryos prior to harvesting. Four HAU were used in the HI test using 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. About 100

µL of 10^5-10^{12} AAF dilutions prepared from the second passage was inoculated each into the allantoic sac of five 10-days-old commercial Loghman breed chicken embryos while controls were inoculated with SHBBS (Euro C -lone). All eggs were incubated at 37 ^oC and observed for 8 days at 12 h intervals. ELD50 was calculated according to Spearman-Kärber method **(Villegas and Purchase, 1980).**

Determination of the Intracerepral Pathogenicity Index (ICPI): The 50 µL of 10% ($10^{2.2}$ ELD50 mL^{-1}) third passage AAF was inoculated intracerbrally to each of the ten 30 h old Loghman breed commercial chicks. Twenty chicks were used as controls, 10 were inoculated with the same dose and technique using antibiotic-free sterile phosphate-buffered saline and the other 10 were kept untreated. Normal, sick and dead birds were recorded daily at the same time of inoculation for 8 successive days. The test followed standard procedures **(OIE Terrestrial Manual, 2008).** ICIP was determined according to **Allan *et al*. (1978).**

Results

Epidemiological and clinical observations: The disease was of a rapid pattern (1-3 days). All the infected chickens were of native breed, reared in backyard and non-vaccinated. Mortality rate exceeded 60%. Nervous signs were predominant. Necropsized birds showed ecchymotic haemorrhages in the mucosae of the proventriculus and intestines.

Molecular detection of viral RNA in oropharyngeal and cloacal swabs and aminioallatoic fluids (AAF): Newcastle disease virus was detected in the swabs, AAF of infected chicken embryos and the vaccine strains. cDNA coinciding with the positive controls measuring 356 bp and 216 bp, were detected in the primary RT-PCR (Fig.1) and nested RT-PCR (Fig. 2), respectively. Of the positive samples, cDNA from AAF had highly distinct 356 bp band followed by the oropharyngeal swabs. No bands were detected from the cloacal swabs. Positive bands of 216 bp were noticed in all tested samples using nested RT- PCR. No bands were obtained from the negative controls in both techniques.

Recovery of NDV from tissues: NDV virulent field strain was successfully obtained from 10 days old commercial chicken embryos inoculated with the NDV-suspected tissue homogenates. Three successive viral passages were attained. The mean HA and HI titres are shown in Table 1.

ELD50, MDT and ICIP of the isolate: The ELD50 and MDT of the isolate are shown in Table 1. All the AAF of the dead chicken embryos showed positive HA on spot testing indicative of viral infection. The MDT value was found to be equivalent to the 10^8 dilution (10^8 ELD50 mL^{-1}). ICIP of the isolate was shown in Table 1.

Table (1). Properties of the isolated NDV field strain

HA (log2)	HI (log2)	ELD50 (log10)	MDT (hours)	ICPI
9	**7**	**8.2**	**60**	**1.58**

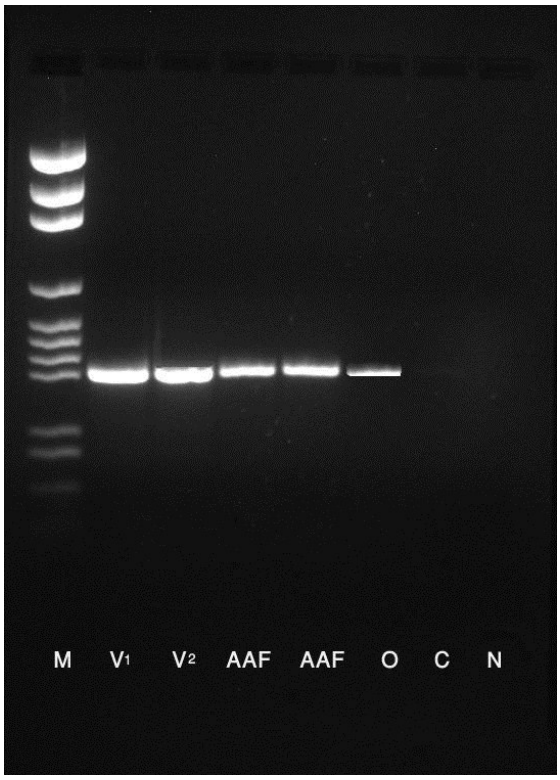

Fig.(1). Agarose gel electrophoresis of RT PCR products of NDV field isolate and reference vaccine strains using 356 primer sets. Lane M: pGem DNA molecular size marker.Lane V1: LaSota cl N79 vaccine strain. Lane V2: LaSota cl HN79.mass vaccine strain.Lane AAF: Amnioalantoic fluid.Lane O: Oropharyngeal swabs.Lane C: Cloacal swabs.Lane N: Negative control.

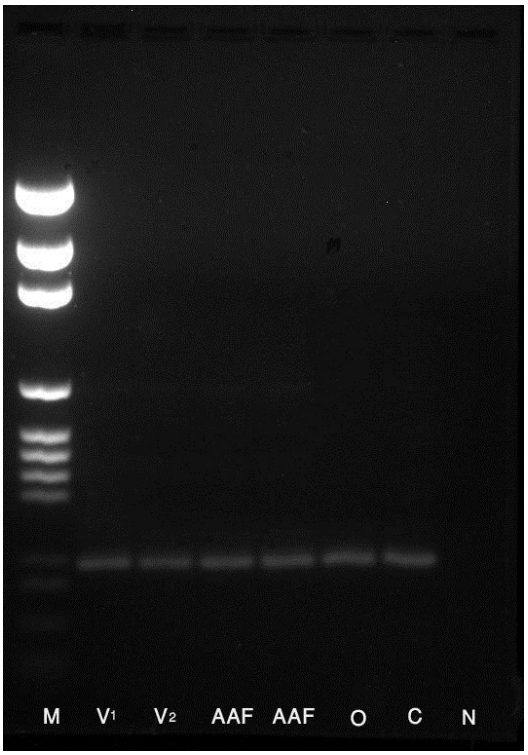

Fig.(2). Agarose gel electrophoresis of nested RT PCR products of NDV field isolate and reference vaccine strains using 216 bp primer sets. Lane M: pGem DNA molecular size marker. Lane V1: LaSota cl N79 vaccine strain. LaneV2: LaSota cl HN79.Mass vaccine strain. Lane AAF: Amnioalantoic fluid. Lane O: Oropharyngeal swabs. Lane C: Cloacal swabs. Lane N: Negative control.

Discussion

In this investigation, a high virulent NDV field strain was detected, isolated and pathotyped based on clinical picture of the disease, RT-PCR, nested RT-PCR virus isolation, MDT and ICPI results. The successful use of RT-PCR to rapidly diagnose ND indicates the efficacy of the technique as a primary diagnostic tool especially where standard virus isolation facilities are not available. The application of the nested RT-PCR for confirmation showing almost similar band intensity in all samples has further reflected the sensitivity and identity of the primary RT-PCR results **(Jestin *et al.*, 1993; Kho *et al.*, 2000** and **Yousof *et al.*, 2005).** In addition, it is proved in this study that the kit and the single step amplification technique is followed were suitable for ND diagnosis. The use of the highly conserved F genes for detection of the viral RNA amplicons could also further help for future studies aiming at probing the biology, ecology and molecular epidemiology of the isolate.

Although virus isolation is a gold standard measure for diagnosis yet it is time consuming and laborious. Owing to the less sensitivity of this tool compared to specific pathogen free eggs **(Wakamatsu *et al.*, 2006),** and because of diversity in tissue affinity among variant NDV virulent strains **(Brown, 1999; Hofstad, 2000** and **Alexander, 2008),** pooling of samples from different organ tissues was followed. This process was recommended to attain productive results **(Smietanka *et al.*, 2006)**.

Many people in Qatar have backyard chickens stocked with variety of birds including turkeys,

pigeons, geese, ducks and peafowls. Although the study did not include the sources of infection, it suggests incrimination of imported birds, an appreciable source of birds in the country. Live market birds, and to a lesser extent, the wild species, could be involved in spreading. This assumption is based on the absence of active surveillance programmes and lack of consistent vaccination schedules against ND in most rearing communities. Only individual immunization at the will of the owner is practiced.

In this study, it is concluded that NDV is a wide host range virus, and it could aggravates the sequence of other infective pathogens **(Bano *et al.,* 2003; Monne *et al.,* 2006** and **Aamir *et al.,* 2007)**, isolation of a high virulent strain from non-vaccinated flocks with such rearing system warrants attention to the growing poultry industry in the country.

Efficient public awareness and empowerment of bird trade legislations are needed. Further, epidemiological and molecular studies to give insights on the candidate vaccines and recommended vaccination policy against the disease are needed.

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الكشف الجزيئى وتحديد الأمراضية لمعزول حقلى لفيروس النيوكاسيل فى الدجاج

خالد مهران

قسم البيوتكنولوجى – معهد بحوث صحة الحيوان

الملخص العربي

في محاولة أولى تم الكشف عن وعزل وتحديد درجة الإمراضية لمعزول حقلي لفيروس النيوكاسل من الدجاج المنزلي بدولة قطر. تم عزل الحمض النووي الريبوزي واكثاره والكشف عنه من مسحات بلعومية ومذرقية من الدجاج المنزلي المصاب اكلينيكيا وكذلك السائل الأمنيوتي لأجنة البيض المحقون باستخدام تفاعل البلمرة المتسلسل العكسي والمتعشش. تم الكشف عن القطعة ذات 356 وحدة باستخدام التفاعل العكسي والقطعة ذات 216 وحدة باستخدام التفاعل المتعشش. تم عزل فيروس النيوكاسل وتمييزه وتحديد درجة الإمراضية له في أجنة بيض باستخدام تفاعل التجلط ومانع التجلط ومتوسط فترة الموت ومعامل الإمراضية بالحقن بالمخ, وكان الفيروس المسبب من النوع عالي الإمراضية.