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Detection of Newcastle Disease Virus in Clinical Samples from Experimentally Infected Chickens using Nested RT-PCR Assay

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Abstract: In this study, a nested Reverse Transcriptase (RT) polymerase chain reaction (RT-PCR)-based assay, for detection of Newcastle Disease Virus (NDV) Ribonucleic acid (RNA) in clinical samples from experimentally infected chicks, was evaluated. The clinical samples used included, blood, tracheal, cloacal, liver, spleen, heart, lung, kidney and brain. The nested RT-PCR was performed in two amplification steps. In the first step, a pair of primers (nd^1 and nd^2) was used to amplify a 356 bp specific region in the F gene of NDV. In the second step, a nested pair of primers (nd^3 and nd^4) was employed to produce 216 bp amplification products, internal to the annealing sites of primers nd^1 and nd^2. The 356 bp PCR products were amplified only from lung homogenate, cloacal and tracheal tissues, kidney, heart and brain. However, the 216 bp nested amplification was detected in all tissue samples collected from experimentally infected chicks. The nested amplification confirmed the identity of the first amplified product and increased the sensitivity of RT-PCR assay. RNA samples extracted from Infectious Bursal Disease Virus (IBDV) and Infectious Bronchitis virus (IBV) or total nucleic acid extracted from blood of non infected birds failed to demonstrate the primary or the nested PCR products. The described nested RT-PCR assay provide reliable, rapid, sensitive and specific diagnostic assay for detection of an outbreak of NDV infection among susceptible Birds.

Key words: Newcastle disease virus, RNA, RT-PCR, diagnosis

IINTRODUCTION

Newcastle disease is caused by avian paramyxovirus type-1 which is a single stranded RNA virus belonged to the family Mononegavirale[1]. Different pathotypes or clinical forms of the disease were reported in different countries including Sudan[2-7]. The wide range of variation in virulence and pathogenicity of the virus strains were responsible for the great losses encountered among commercial and rural flocks with great economic impact.

Control of the disease depends mainly upon accurate diagnosis. In this respect, the virological and serological diagnostic procedures are known to be expensive, time consuming and cumbersome. However, the advanced molecular biological approaches of diagnosis became reliable alternatives[8-11]. The Reverse Transcriptase (RT) polymerase chain reaction (RT-PCR) had been applied for the first time by Jestin and Jestin[12]. The virus could be detected directly from the clinical samples without previous isolation[10]. They performed a semi-nested RT-PCR assay for detection and subsequent differentiation of virus isolates. Nanthakumar *et al.*[13] detected the virus from suspected clinical samples by RT-PCR but they differentiated the virus strains by restriction enzyme analysis of the PCR product. Aradaib *et al* [14] calculated their method of PCR product detection to be 10 folds more sensitive than electrophoresis. In addition, they claimed the nested PCR to be 100 folds more sensitive than standard PCR or 1000 folds as described.

In this study a nested RT-PCR assay was evaluated for direct detection of NDV in clinical samples from chicks experimentally infected with local Sudanese isolate of a virulent strain of NDV.

MATERIALS AND METHODS

Experimental infection of birds: A local isolate of NDV designated KU-p2 was use to induce experimental infection in six weeks old white leghorn chicks. Twenty chicks were randomly selected and were divided into 2 Groups (A and B). Group A (10 chick) received two drops of undiluted infective allantoic fluid through nasal and ocular routes. Group B (10 chicks) were kept in another facility and left as controls. The experimental

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birds were fed a balanced ration with free access to water. The birds were then observed daily for onset of clinical signs.

Collection of samples: On day five post infection, blood samples were collected from the wing vein in sterile tubes containing EDTA. The blood was washed twice with sterile PBS and kept at 4°C till used for virus isolation and viral RNA extraction.

Tracheal and cloacal swabs were taken in sterile PBS containing antibiotics (5000 units mL^{-1} penicillin, 4 mg mL^{-1} streptomycin and 2000 units mL^{-1} mycostatin) and kept at -20oC. In the same day chicks were slaughtered and parts from internal organs were collected separately. Liver, heart, spleen, lung, kidney, bursa and brain tissues were removed in sterile petri dishes and homogenized by sterile pair of scissors. PBS containing antibiotics was used to make 20% w/v of the homogenized tissues. The homogenates were then kept at -2°C till used.

Virus isolation in Embryonated Chicken Eggs (ECE): Embryonated chicken eggs 9-10 days of incubation were kindly supplied by the viral vaccine production Department of the Central Veterinary Research Laboratory (CVRL) Khartoum, Sudan. Each sample was briefly centrifuged and the supernatant fluid was used to inoculate five ECEs via the allantoic cavity with 0.1 mL.

Extraction of viral nucleic acids: The QIAamp extraction kit (QIAamp, Hamburg, Germany) was used to extract viral nucleic acids. RNAs were extracted from supernatant of infected cell cultures and homogenate from infected tissues using QIAamp viral RNA kit (QIAmp, Humburg, Germany) as per manufacturer's instructions. Briefly, 140 μL of virus suspension were added to 560 μL AVL buffer containing carrier RNA into a 1.5 mL micro-centrifuge tube and mixed by pulse-vortexing for 15 sec. The mixture was incubation at room temperature for 10 min. 560 μL of absolute ethanol were added and mixed by pulse-vortexing for 15 seconds. 630 μL of the mixture were transferred to QIAamp spin column mounted on 2 mL collection tube and centrifuged at 6000 x (8000 rpm) for 1 minute. The column was then transferred to another collection tube and the remaining 630 μL of the mixture was again spin at the same speed. The column was then washed twice by 500 μL of washing buffers WB^1 and WB^2 respectively. Finally, dsRNAs were carefully eluted by 60 μL of buffer AVE equilibrated to room temperature. Total nucleic acid was quantified using a spectrophotometer at 260 nm wavelength. RNA Extracts were then kept at 4°C till used for PCR amplification.

Primers design: Two pairs of universal primers (nd^1, nd^2) and (nd3, nd4) were synthesized by Roth (Carl Roth GmbH+Co., Karlsrube, Germany) according to the published F gene sequence of the velogenic NDV Miyadera strain[15]. These pairs produced 356bp and 216bp PCR products, respectively[13]. The nd1 have the sequence of: 5'-GCAGCTCGAGGGATTGTGGT-3'nucleotide position 158-177, nd2 have the reverse sequence of: 5'-TCTTTGAGCAGGAGGATGT TG-3' nucleotide position 513-493, nd3 have the sequence 5'-CCCCGTTGG AGGCATAC-3' nucleotide position 282-298 and nd4 have the sequence: 5'TGT TGGCAGCATTTTGATTG-3'nucleotideposition497-478. All primers were synthesized in DNA synthesizer and received in a lihpolized tubes (Germany).

RT-PCR amplification: Titan one tube enzyme mix system was used to prime synthesis of first strand cDNA and to perform PCR in one step. The reaction mixture (5 μL of the sample RNA extract, 1ul from each primer of the first pair, 2 μL of dNTPs, 2.5 μL DDT, 0.5 μL Rnase inhibitor, 10 μL of 5x PCR buffer, 4 μL magnesium chloride, 1 μL titan enzyme mix and 23 μL of dd H^2O)were pipetted in 0.5 PCR tube. The tube was then incubated at 50°C for 30 min for reverse transcription, then cycled 40 times at 94°C for one min, 52°C for one min at 68°C for one min and finally, incubated at 68°C for 10 min.

Nested RT-PCR: For nested amplification, 2 μL of the first amplified 356 bp PCR product were transferred to a PCR tube containing amplification buffer. For each PCR amplification, the amplification buffer consisted of (10 μL of 10X PCR buffer; 10 μL Mg Cl_2 of 1.5 mM concentration; 2 μL of primers (BTV^3 and BTV^4) at a concentration of 20 picogram; 8 μL of dNTPs including ATP, TTP, GTP, CTP; 1 μL of Taq DNA polymerase (Perkin Elmer Corporation, Norwork, CT) at a concentration of 5.0 units μL^{-1}. Double distilled water was added to each PCR tube to obtain a total volume of 100 μL The PCR tubes were replace in the thermal cycler for another 40 cycles at the same temperature per cycles described above.

Following amplification, 20 microliters from each nested PCR reaction containing amplified product were loaded onto gels of 1.5% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and the nested PCR products were visualized under UV light.

RESULTS

The experimentally infected birds developed inappetance, greenish diarrhea, depression, respiratory

embarrassment followed by onset of nervous signs by day six post infection. NDV was recovered in allantoic fluid from all tissues collected from experimentally infected chicks except the blood, liver.

The specific 356 bp PCR products, visualized on ethidium bromide-stained agarose gel, were obtained from all NDV RNA samples tested. The specific 356 bp PCR product was visualized from a variety of tissue samples from the experimentally infected chick including, lung, kidney, heart, brain, cloaca and trachea. The blood, liver, kidney, heart cloaca, trachea and spleen failed to demonstrate the primary 356 bp PCR product (Fig. 1). The brain tissue showed the highest intense band. However, the nested amplification increased the sensitivity of the RT-PCR and the nested 216 bp PCR product was detected from all tissue samples including blood, liver, kidney, heart, cloaca, trachea and the spleen (Fig. 2). Application of RNA from infectious bursal disease (IBD) virus and infectious bronchitis (IB) virus and blood samples from non infected chicks or total nucleic acid extracts from Vero cell controls failed to demonstrate the specific 356 bp PCR product (Fig. 3).

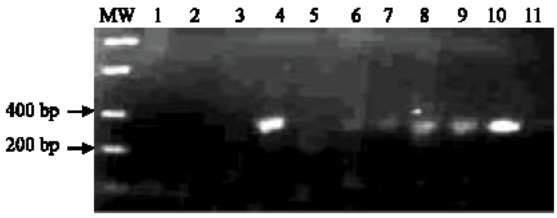

Fig. 1: Visualization of the specific 356 bp PCR product from tissue samples from experimentally infected chichs. Lane MW: Molecular weight marker; Lane 1: Blood; Lane 2: Blood; Lane 3: liver ; Lane 4; Lung; Lane 5; spleen; Lane 6: kidney; Lane7; heart; Lane8: cloacal; Lane 9: trachea; Lane 10; Brain

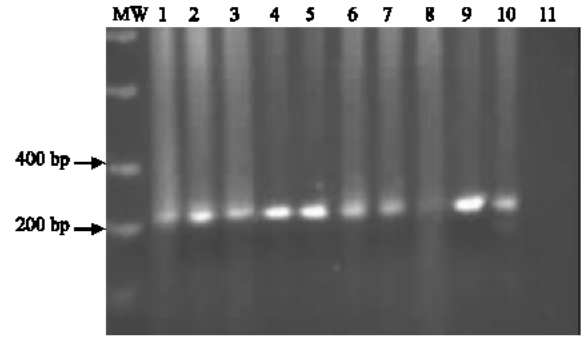

Fig. 2: Visualization of the nested 216 bp PCR products from all tissue samples tested. Lane MW: Molecular Weight marker; Lane 1: blood; Lane 2: blood; Lane 3: liver ; lane 4: lung; Lane 5: spleen; Lane 6: kidney; Lane7: heart; Lane8: cloaca; Lane 9: trachea; Lane 10: brain

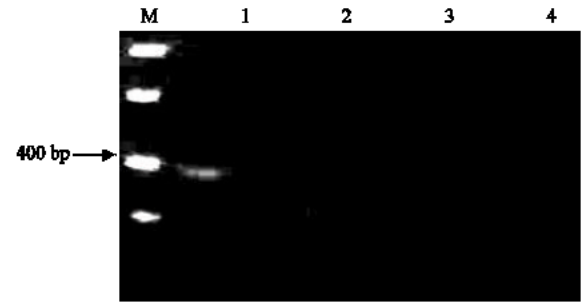

Fig. 3: Specificity of the RT-PCR for detection the specific 356 bp PCR product from NDV RNA. Lane MW: Molecular weight marker; Lane 1: NDV RNA (positive control); Lane 2: Infectious bursal disease (virus); Lane 3: Infectious bronchitis; Lane 4: Blood sample from non infected chick (negative control)

DISCUSSION

Newcastle Disease (ND) is a worldwide veterinary problem in poultry industry[16-21]. Very little information is available about field isolates originally recovered in Sudan. Further studies on these field isolates are necessary to determine their biology, ecology and molecular epidemiology. The NDV field isolates used in this study represented a range of topotype viruses, isolated from a diverse geographic location in Sudan including, Western, eastern, Northern and Central Sudan. The isolates were recovered from different avian species including chicken, pegion and wild bird.

The described NDV RT-PCR assay using primers derived from F gene of the virus reproducibly and specifically detected NDV RNA in infected cell cultures and clinical samples. Selection of the primers was based on the observation that the designed region of the F gene is highly conserved. The specific 356 bp PCR product was visualized from a variety of tissue samples from the experimentally infected chicks including, lung, kidney, heart, brain, cloaca and trachea. The blood, liver and spleen failed to demonstrate the primary 356 bp PCR product. The brain tissue showed the highest intense band compared to tissues from other organs. This is probably due to the neurotropic nature of this virus strain. However, the nested amplification increased the sensitivity of the RT-PCR and the nested 216 bp PCR

product was detected from all tissue samples including blood, liver and the spleen. The nested NDV RT-PCR assay was a simple procedure that efficiently detected all NDV isolates under the stringency condition used in this study. It is well documented that nested amplification increases the sensitivity of the PCR assay and confirms the identity of the primary PCR product[14-22]. In the present study, the use of nested amplification removes the hazardous and cumbersome hybridization assay with radilabled cDNA probes. In addition, hybridization confirmation assay is tedious, laborious and usually takes overnight.

The specificity studies indicated that the specific 356 bp PCR product was not amplified from 1.0 pg of RNA from Infectious Bursal Disease (IBD) and infectious bronchitis; total nucleic acid extracts from Vero cell controls; or total nucleic acid extract from blood of non infected chicks under the same stringency condition described in this study. Temperature and time for denaturation, primer annealing and extension, enzyme and $MgCl_2$ concentration and number of cycles of the three temperatures per time segments were very important for maintaining sensitivity and specificity of the PCR reaction.

The NDV RT-PCR assays provide supportive diagnostic techniques to the lengthy cumbersome conventional virus isolation procedures. The QIAamp kit provided a simple procedure that takes only one hour for viral RNA extraction. The thermal cycling profiles for reverse transcription and RT-PCR assay, including the primary and nested amplifications, required 6 hours. The time required from sample submission to interpretation of the final results was consistently 7 h. This means that confirmatory diagnosis of submitted samples, from NDV suspected bird could be made with in the same working day. The rapidity, sensitivity and specificity of the RT-PCR assay would greatly facilitate detection of NDV infection in an outbreak among susceptible birds.

This RT-PCR-based assay for detection of NDV field isolates provides the basis for future diagnosis of NDV. Further studies are in progress to determine the capability of the described NDV RT-PCR assay to detect additional isolates of NDV and to evaluate its potential as a sensitive and specific diagnostic assay through comparison with current diagnostic techniques used for detection of NDV infection.

In conclusion, the described NDV RT-PCR assay, using primers derived from F gene of NDV, should provide rapid detection of NDV infection during an epizootic of the disease among susceptible Birds.

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