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Evaluation of RT-PCR for Rapid Detection of Sudanese Isolates and Vaccine Strains of Newcastle Disease Virus

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Abstract: A Reverse Transcriptase (RT) Polymerase Chain Reaction (PCR) was developed to detect field isolates of Newcastle Disease Virus (NDV) in vero cell culture or Embryonated Chicken Eggs (ECE). Five Sudanese isolates of NDV designated (OB, KU, GR, A12 and A105) and five vaccine strains including Komarov, B1, LaSota, Clon30 and Clon79 were used in this study. A pair of primers (ND1 and ND2), targeting a fragment in the F gene of NDV, was designed for PCR amplification. The RT-PCR assay resulted in amplification of a 356 bp PCR product from RNAs of Sudanese and vaccine strains of NDV. However, nucleic acid extracts of Infectious Bursal Disease (IBD) virus, non infected vero cells or ECE failed to produce the specific 356 bp PCR product. The described RT-PCR assay was a simple procedure that involved a single amplification step. In addition, the developed RT-PCR assay provides a rapid, sensitive and specific method for detection of an out break of the disease in susceptible birds.

Key words: Newcastle disease virus, molecular diagnostics, RT-PCR

INTRODUCTION

Newcastle Disease Virus (NDV) is an important pathogen of domestic fowl, pigeon and other birds[1]. The virus can be readily grown in the allantoic cavity of Embryonated Chicken Eggs (ECE) and in many cell culture systems of chicken and mammalian origins[2-5]. Its complete genome is composed of six genes separated by conserved non coding regions[6]. The Fusion (F) gene codes for a precursor protein[7]. Routine laboratory diagnoses of ND necessitate primary isolation of the virus in Embryonated Chicken Eggs (ECE) or cell culture and subsequent characterization and identification by serum neutralization test. However, these conventional methods are time consuming and cumbersome. The surge of new techniques in molecular biology has revolutionized rapid detection and pathotyping of NDV worldwide.

Several molecular studies were conducted on NDV whole genome. However, nucleic acid sequence analysis of the F gene played an important role in virus infectivity and disease expression[8,9].

Reverse Transcriptase (RT) Polymerase Chain Reaction (PCR) for detection of NDVs was applied for the first time by Jestin and Jestin[10]. Subsequently, several studies were targeting other regions of the genome[11]. The identity of the PCR product was confirmed Restriction Fragment Length Polymorphism (RFLPs). Ballagi-Pordany *et al*.[10] and Kant *et al*.[12] used restriction enzyme endonuceases to differentiate between pathotypes. Jorecki Black *et al*.[13] and Aldous *et al*.[14] used a probe hybridization assay. Sequence analysis of the amplified region was done by Toyoda *et al*.[15], Collins *et al*.[16] and King and Seal[17]. Confirmation of the specificity of the PCR product by amplification of a region, internal to the annealing site of the first primers, was also described using nested RT-PCR[15].

The objective of the present study was to evaluate RT-PCR for detection of Sudanese isolates and vaccine strain of NDV in cell culture and ECE.

MATERIALS AND METHODS

Cell culture and virus propagation: The Sudanese isolates of NDV; OB, KU, GR, A12 and A105, vaccine strains, Komarov, B1, LaSota Clone30 and Clone 79 were obtained from the Central Veterinary Research Laboratory, Soba, Khartoum, as lyophilized allantoic harvest. All viruses were propagated in monolayers of vero cell line

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The infectious harvests were collected and centrifuged at 3000 rpm for 10 min and the cell free supernatant was used for ssRNA extraction.

Extraction of viral nucleic acid: The QIAamp viral RNA kit (Qiagen, Germany) was used to extract ssRNAs of the Sudanese isolates and vaccine strains following the mini spin protocol. 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 followed by incubation at room temperature for 10 min. Five hundred and sixty microliter of absolute ethanol were added and mixed by pulse-vortexing for 15 sec. Six hundred and thirty microliter of the mixture were loaded in the QIAamp spin column mounted on 2 mL collection tube and centrifuged at 6000 xg (8000 rpm) for 1 min. The column was then transferred to another collection tube and the other 630 µL of the mixture were passed through it. The column then washed twice by 500 µL of 2 washing buffers WB1 and WB2, respectively. Finally, ssRNAs were carefully eluted by 60 µL of buffer AVE equilibrated to room temperature.

Primer selection and synthesis of the probe: Primers (ND1 and ND2) were selected from the published F gene sequence of the velogenic NDV Miyadera strain[15] using the PrimerSelect programmer of the Lasergene software (DNASTAR Inc., Madison, WI, USA). Nd1 have the sequence of: 5'-GCAGCTCGAGGGATTGTGGT-3'nucleotide position 158-177. Nd2 have the reverse sequence of: 5'-TCTTTGAGCAGGAGGATGTTG-3' nucleotide position 513-493. This pair would result in 356 bp product[16].

Reverse transcription polymerase chain reaction: For the one step RT-PCR a mixture of 2 µL of dNTPs, 2.5 µL DDT, 0.5 µL Rnase inhibitor and 13 µL of dd H_2O was added to 5 µL of the RNA sample (RNA extract). Mixture 2 contains 10 µL of 5x PCR buffer, 4 µL magnesium chloride, 1 µL titan enzyme mix and 10 µL of ddH_2O then added to each reaction. All RT-PCR amplifications were performed at a total volume of 50 µL per reaction mixture.

Thermal profiles were done on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.). In the first cycle, the reverse transcription step was performed at 50°C for 30 min followed by inactivation at 94°C. Then 40 cycles of amplification were carried as follows; 94°C for 1 min, 52°C for 1 min and 68°C for 1 min. Final extension was carried at 68°C for 10 min. After amplification, 15 µL from each PCR reaction containing amplified product were stained with ethidium bromide and loaded onto 1.5% SeaKem agarose in TBE buffer and electrophoresis carried at 80 mVA for 30 min. The PCR products were visualized under UV light.

RESULTS AND DISCUSSION

The NDV RT-PCR based assay afforded sensitive and specific detection of all isolates of NDVs and vaccine strains. The specific 356 bp PCR products obtained from allantoic and vero cell culture extracts were visualized on ethidium bromide stained gel (Fig. 1). The specificity studies indicated that viral nucleic acid extract of IBD virus or total nucleic acid from Vero cell controls failed to produce the specific 356 bp PCR product (Fig. 2).

In Sudan, most of ND viruses were isolated from extensive disease outbreaks[19]. Attempts to isolate viruses of low virulence by a survey are very rare.

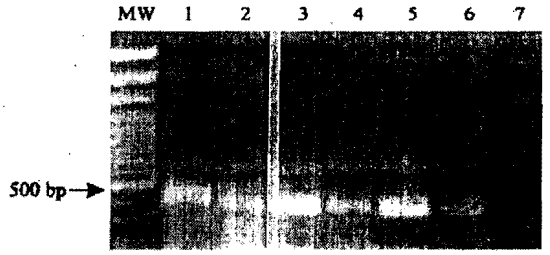

Fig. 1: RT-PCR amplification of the specific 356 bp PCR products from field isolates and vaccine strains of Newcastle disease virus
Lane MW: 100 base pair (bp) molecular weight marker; Lane 1-5: NDV field isolate, OB, KU, GR, A12 and A105, respectively; Lane 6-7: Vaccine strains, Kamarov and B1, respectively.

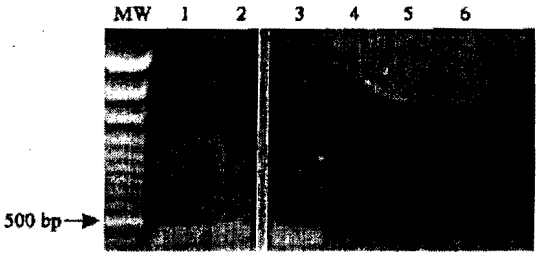

Fig. 2: Specificity of the RT-PCR assay for detection strains of Newcastle disease virus. The specific 356 bp PCR products were not detected from viral nucleic acid obtained from IBD and total nucleic acid from vero cells or Embryonated Chicken Egg (ECE)
Lane MW: molecular weight marker; Lane 1-3: Lassota, Clone 30 and Clone 79, respectively. Lane 4: Vero cell extract; Lane 5: Viral nucleic acid extracts from IBD; Lane 6: Embryonated Chicken Egg (ECE)

Diagnosis of Newcastle disease includes conventional virus isolation and identification. Serology is useful to identify a previous infection but is known to be laborious and time consuming[9]. The serological tests commonly used for detection of infection include. Haemagglutination Inhibition (HI) and Enzyme Linked Immunosorbent Assay (ELISA). However, these tests are less valuable as tools for detecting ND virus infection as they are complicated by cross reactions between other viruses[10]. Laboratory isolation of the virus from suspected field outbreaks necessitate the presence of sophisticated facilities, which are not available in most field diagnostic units.

All these viruses were isolated during outbreaks of the disease in large poultry farms over a period of 27 years from different geographical location in the Sudan. Three of these viruses were pathotyped as velogenic viruses. The remaining 2 virus isolates were not yet pathotyped. The viral RNA of the vaccine strain (Komarov strain) was incorporated in each RT-PCR assay as a positive control.

The specificity of the test indicated that the primers did not amplify any region in the culture extracts nor the genome of the IBD virus under the same stringency conditions described in this study.

In conclusion, this one tube RT-PCR technique could provide a means of rapid detection of NDV. In addition, the described RT-PCR assay could be used as a supportive or complementary method to the conventional methods currently used for diagnosis of the disease.

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