Molecular detection of NDV by Real-Time PCR in Baghdad

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Abstract

Newcastle disease virus (NDV) is widely spread in Iraqi chicken that is associated with notable economic losses. Current study was carried out to identify NDV in the North of Baghdad by employing a Molecular methods based on the Real-Time PCR technique, the primers and the probe also were designed based on the conserved fragment coding matrix protein(M gene).These genes of virus show the highest level of conservation, and therefore the test is adaptable for detection of NDV. In the current study, 14 clinically suspicious birds believed to be infected with ND, had been chosen from 14 different commercial poultry farms in various areas of North Baghdad brought to the Aurok and Al-Emad Lab. Baghdad/ Iraq. The infected birds were examined and the tissue samples collected from ceca tonsils, spleens, trachea, lungs and brains of each farm. These samples were submitted to examination directly by Real-time PCR. The Results of the samples collected from 14 poultry flocks were showed positive results in ten samples with the SYBR Green I real-time PCR and the other 4 flocks failed to show any positive results with both gene primers.

Key Words: Molecular, detection of NDV, Real-Time PCR.
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تشخيص جزيئي لمرض النيوكاسل بتقنية سلسلة انزم البلمرة الآني في بغداد

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الخلاصة

يعتبر مرض النيوكاسل من الأمراض واسعة الانتشار في العراق ويسبب خسائر اقتصادية كبيرة في قطعان الدواجن، هدفت هذه الدراسة إلى تشخيص مرض النيوكاسل في شمال بغداد باستخدام تقنية حديثة وسريعة هي تفاعل سلسة إنزيم البلمرة. تم تصميم البريمر المستخدم في التشخيص من المنطقة عالية الثبوتية والتي تسمى جين (M). أجريت هذه الدراسة على 14 طيرا أظهرت علامات سريرية للإصابة بمرض النيوكاسل، جلبت هذه الطيور من أربعة حقول مختلفة إلى مختبر العماد ومختبر أوروك في وسط بغداد. تم تشريح الطيور ومن ثم أخذت العينات من الدماغ والرئة والرغامي والطحال ولوز الأعورين، وتم استخلاص المادة الوراثية من الأنسجة المصابة وفحصها بجهاز تفاعل سلسلة إنزيم البلمرة الآني حيث أظهرت النتائج إصابة تلك القطعان بفايروس النيوكاسل لعشرة عينات في وقت قياسي وخلال ساعة فقط، لذلك هذه النتيجة تعطينا مؤشر جيد لاستخدام هذه التقنية في سرعة تشخيص الأمراض الوبائية الخطرة وتسهل إجراءات السيطرة والوقاية من هذه الأمراض.

الكلمات المفتاحية: تشخيص جزيئي، مرض النيوكاسل، سلسلة انزم البلمرة الآني.

Introduction

Real-Time PCR method is a very fast method of analysis with very high sensitivity to the little amount of genetic material, these phenomena make it appropriate for wide-ranging use in veterinary laboratories and for fast detection of viruses in the birds. Newcastle disease (ND) is caused by virulent strains of avian paramyxovirus type 1 (APMV-1) serotype of the genus Avulavirus belonging to the family Paramyxo viridae(1, 2) these virus belong to the avian species, which have been categorized by serological and phylogenetic analysis into ten subtypes nominated APMV-1 to APMV-10 (3). The virus is a single-stranded RNA negative-sense, non-segmented, the size of the genome reach to 15.2 kb, in addition the virus contains six structural and nonstructural proteins include; haemagglutinin-neuraminidase (HN) protein, RNA polymerase (L), fusion (F) protein, matrix (M) protein, phosphorus (P) protein and nuclear (N) protein (4). The HN protein of NDV plays an important role in promoting immune defense against virus infection and is consequently susceptible to immune pressure to produce antigenic difference more easily (5). Infection with NDV can lead to a high economic loss because high mortality rate reaches to 100% (6). Recently, the science has been increasing used of molecular methods to identify NDV in clinical samples(7), the advantage of these technologies for fast detection of the virus in comparison with another method (8). The real-time PCR methods have better further the significant benefits in comparison to conventional gel-based PCR assays, real-time PCR increased sensitivity and specificity in a rapid design, now real-time PCR is one of the most essential methods for the detection and checking of virus infections (9, 10). This work aimed to design a specific assay for NDV Iraqi isolates rapid detection using PCR technique for control this disease, early detection, and differentiation of NDV types are very important during NDV outbreaks. In this study, SYBR Green I real-time polymerase chain reaction (PCR) was developed for detection and differentiation of NDV path types. Velogenic-specific primers were designed to detect a specific sequence of velogenic strains. All the velogenic strains were only detected by using velogenic-specific primers (NDVIF2 & NPV2N) with threshold cycle (Ct) ranged from 12.92 to 22.76 and melting temperature between 85.8°C to 86.3°C.

Materials and Methods

- **Collection of samples:** A total number of 14 clinically suspicious birds believed to be infected with ND were chosen from 4 different commercial poultry farms in various areas of North Baghdad brought to the Aurok Lab by the owner, in Middle of Baghdad. The infected birds were observed and the samples collected from caeca tonsils, spleens, tracheas, lungs and brains of each farm (5 birds as a single sample) were surgically removed and transmitted into sterile tubes separately, Furthermore, stored the samples at -20°C until used. As furthermost of the herds mostly showed nervous signs of ND, the brain samples of birds were separately triturated for each farm, and homogenized using a sterilized mortared and pestle, after that added 5 ml tryptose phosphate broth solution treated with a high concentration of antibiotics (amikacin 5 mg/ml and streptomycin 10 mg/ ml). The suspension was incubated at 25°C for 30 minutes and centrifuged at 1000 g for 10 minutes.

Extraction of RNA, reverse transcriptase, real time RT-PCR (RT-qPCR): Extraction of viral RNA from clinical samples was done using a High Pure Viral Nucleic Acid Kit (Viral Gene-spin, 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. About 200 µL of supernatant has been added to the lysis buffer and, after incubation at room temperature, 70% ethanol was added. Then RNA was washed with buffers and, after final drying of the column, RNA

was eluted with 50 µL of RNA-ase free water. One step RT-qPCR for the detection of M gene has performed The primers for RT-PCR were designed by aligning multiple sequences of NDV M genes using the ApE software (http://biologylabs.utah.edu/jorgensen/wayned/ape/). The conserved regions in the M genes among the NDV isolates were selected for primer design. The primers were synthesized by (Biocorp company, Canada), five µL of RNA were added in 25 µL of final volume, The primers and hydrolysis probe used were (HEX APMV 1-LNA 5-HEX-gggaCrGChTgCtatCc-BHQ-3) PRIMER: APMV 1 F 5'-AGTGATGTGCTCGGACCTTC-3, and PRIMER: APMV1R 5'CCTGAGGAGAG GCATTTGCTA-3'. Synthesis and amplification of cDNA were performed with the master amplification reaction (Roche, Germany), the cDNA synthesis at 42 ºC for 3 minutes, Inactivate RT at 95ºC for 5 minutes and 40 cycles of 95 ºC for 10 seconds and 55 ºC for 30 seconds and annealing temperature of the primers of 58 ºC (11).

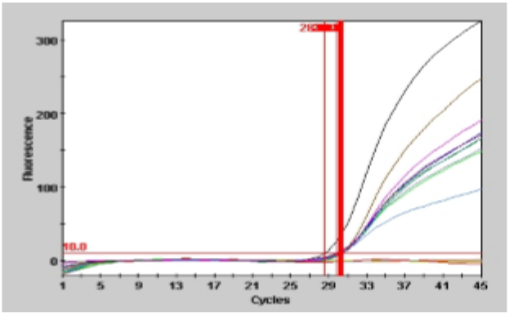
Results and Discussion

Results of the samples collected from 14 poultry flocks were showed positive results in ten sample with the SYBR Green I real-time PCR and the other 4 flocks failed to show any positive results with both gene primers. Fig. (1). On the other hand, were showed the viral load (Con.) and the threshold cycle (Ct.) in this figure In this work, we existing real-time PCR methods for detection of Newcastle Disease Virus, with the use of TaqMan probes. These techniques were a fast and sensitive method for detecting these viruses. The primers were designed to detect the conserved region of M proteins of NDV, these genes of virus show the highest level of conservation, and therefore the test is adaptable for detection of NDV. Fig (2) These results were agreement with (8) who showed that rapid detection and identification of the virus is crucial for an effective control and/or eradication of NDV because the disease is highly contagious. In this study, we Identified the virus 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 these results agreements with (12, 13) who showed the concentration and purification method will be of great help to enhance the detection sensitivity.

FAM

Site ID	Sample ID	Channel Result	Conc	Ct
A1	S1 T	POS	6.55E02	30.4
A2	S2 T	POS	8.15E02	30.1
A3	S3 L	NEG	No Result	0
A4	S4 CT	POS	7.88E02	30.1
A5	S5 T	NEG	No Result	0
A6	S6 T	POS	7.11E02	30.3
A7	S7 L	NEG	No Result	0
A8	S8 L	POS	6.43E02	30.5
A9	S9 T	POS	7.22E02	30.3
A10	S10 L	POS	6.40E02	30.5
A11	S11 CT	POS	7.05E02	30.3
A12	S12 T	POS	2.15E03	28.6
A13	S13 L	NEG	No Result	0
A14	S14 CT	POS	9.29E02	29.9

Fig. (1) Positive results in ten sample with SYBR Green I real-time PCR (S=sample, T=Trachea, L=Lung, CT= Cecal tonsil, POS=Positive, NEG=negative). FAM= (6-carboxy flurecent)- probe pigments.



Site Legend

Site	Assay	Sample ID
A1	NDV qPCR GPC	S1 T
A2	NDV qPCR GPC	S2 T
A3	NDV qPCR GPC	S3 L
A4	NDV qPCR GPC	S4 CT
A5	NDV qPCR GPC	S5 T
A6	NDV qPCR GPC	S6 T
A7	NDV qPCR GPC	S7 L
A8	NDV qPCR GPC	S8 L
A9	NDV qPCR GPC	S9 T
A10	NDV qPCR GPC	S10 L
A11	NDV qPCR GPC	S11 CT
A12	NDV qPCR GPC	S12 T
A13	NDV qPCR GPC	S13 L
A14	NDV qPCR GPC	S14 CT

Data Type	Line Type
Primary Curve	

Channel	Symbol
FAM	None

Fig. (2) Melting-curve analysis of M Gene fragment containing the F cleavage site amplified by M-gene primer which showed positive results of 10 samples

In conclusion, this work positively developed a SYBR Green I real-time PCR for detection and differentiation of NDV types. The virus can be detected directly from clinical samples without the need of virus propagation in chicken embryonated eggs. Due to these advantages, the developed methods will contribute significantly in the control and prevention of the spread of the disease, if the causal agent is detected at the early stage of the outbreak. Therefore, can be prevented the spread of the disease and avoid the economic losses.

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