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ORIGINAL RESEARCH

Detection and characterization of Newcastle disease virus in clinical samples using real time RT-PCR and melting curve analysis based on matrix and fusion genes amplification

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Abstract

Aim: Newcastle disease is still one of the major threats for poultry industry allover the world. Therefore, attempt was made in this study to use the SYBR Green I real-time PCR with melting curves analysis as for detection and differentiation of NDV strains in suspected infected birds.

Materials and Methods: Two sets of primers were used to amplify matrix and fusion genes in samples collected from suspectly infected birds (chickens and pigeons). Melting curve analysis in conjunction with real time PCR was conducted for identifying different pathotypes of the isolated NDVs. Clinical samples were propagated on specific pathogen free ECE and tested for MDT and ICIP.

Results: The velogenic NDVs isolated from chickens and pigeons were distinguished with mean T_m 85.03±0.341 and 83.78±0.237 respectively for M-gene amplification and for F-gene amplification the mean T_m were 84.04±0.037 and 84.53±0.223. On the other hand the lentogenic NDV isolates including the vaccinal strains (HB1 and LaSota) have a higher mean T_m (86.99±0.021 for M-gene amplification and 86.50±0.063 for F-gene amplification). The test showed no reaction with unrelated RNA samples. In addition, the results were in good agreement with both virus isolation and biological pathotyping (MDT and ICIP). The assay offers an attractive alternative method for the diagnosis of NDV that can be easily applied in laboratory diagnosis as a screening test for the detection and differentiation of NDV infections.

Conclusion: As was shown by the successful rapid detection and pathotyping of 15 NDV strains in clinical samples representing velogenic and lentogenic NDV strains, and the agreement with the results of virus isolation , biological pathotyping and pathogenicity indices. The results of this report suggests that the described SybrGreen I real-time RT-PCR assay in conjunction with Melting curve analysis used as a rapid, specific and simple diagnostic tools for detection and pathotyping of different NDVs in clinically infected birds.

Key words: lentogenic, melting temprature, Newcastle virus, syer green I, velogenic

Introduction

Newcastle disease is a highly contagious and fatal viral disease that affects all species of birds. The clinical signs seen in birds affected by this disease vary widely and are dependent on factors like the virus strain, host species, age, immune status, environmental stress and concurrent infection. In chickens, the disease may vary from sudden death with 100% mortality to subclinical disease. The disease has a worldwide distribution, and is a major threat to the poultry industries due to the huge economic loss associated with it specially in turkeys and chickens, [1]. Newcastle disease (ND), is caused by NDV, an enveloped virus that contains a liner, non segmented, single stranded negative sense RNA genome, in the genus Avulavirus of the family Paramyxoviridae. [2,3]. The RNA genome of NDV is ~15 kb follow the "role of six", the genome coding for six major proteins: a large RNA polymerase (L), hemagglutinin-neuraminidase protein (HN), fusion protein (F), matrix protein (M), phosphoprotein (P), and nucleoprotein (NP), in the order 3'-NP-P-M-F-HN-L-5', [4,5]. NDVs were classified into three major pathotypes based on the clinical signs induced in infected chickens: velogenic (highly virulent), mesogenic (intermediate virulent) and (avirulent) lentogenic strains [6,7,8].

The detection and differentiation of NDVs are based on virus isolation using embryonated chicken eggs, followed by an *in vivo* estimation of pathogenicity in chickens, such as the intracerebral pathogenicity index (ICPI) in 1-day-old chicks, the intravenous pathogenicity index (IVPI) in 6-week-old chickens, or the mean death time (MDT) in chicken embryos [8]**.** Studies comparing the deduced amino acid sequence at the cleavage site of NDV, varying in virulence for chickens, showed that virulent viruses usually have the motif ^{112}R/K-R-Q-K/R-R-F^{117} and avirulent viruses have ^{112}G/E-K/R-Q-G/E-R-L^{117} [8-11]**.** The advent of real-time PCR methods has improved further the significant benefits of RT-PCR in comparison

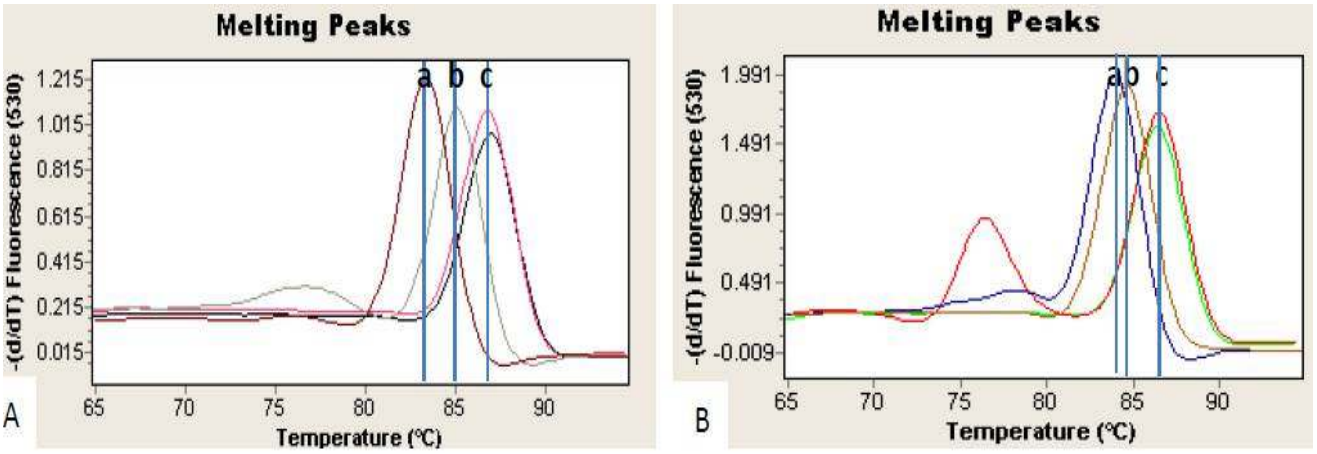

Figure-1. (A) Melting-curve analysis of a 101 bp fragment conaining the F cleavage site amplified by F-gene primer. (B) Melting curve analysis of a 121 bp fragment containing the F cleavage site amplified by F-gene primer a. Melting temperature of chicken velogenic strains (Fj939313). b. melting temperature of pigeon velogenic strain (NDV/Pg) c. melting temperature of lentogenic strain (LaSota and HB1).

to conventional gel- based PCR assays, real-time PCR offers increased sensitivity and specificity in a rapid format. Owing to these features, real-time PCR is now one of the most important techniques for the detection and monitoring of virus infections [12-14]**.** There are different formats available for real-time PCR. The intercalating dyes SYBR Green I assay with the melting curve analysis are the most cost effective and easier to establish as compared to other real-time detection methods. Melting curve generated by using Light Cycler instrument with SYBR Green I dye has been applied for rapid detection and differentiation of NDV[15].

In this study we used SYBR green assay with the melting curve analysis for rapid detection and differentiation of NDV in clinical samples using set of primers designed for amplification of F (Fusion) and (Matrix) M genes. Our results were confirmed by conventional methods of pathotyping and Real Time PCR with fluorogenic hydrolysis probes.

Materials and Methods

Samples: Organs (brain, conjunctiva, trachea, lung, and caecal tonsil) and swabs (Tracheal and cloacal) collected from birds (chickens and pigeons) showing nervous signs and/or respiratory signs over 21 poultry flock. Samples were prepared for virus propagation, isolation, pathotyping and Real Time PCR. Organs were weighed and homogenized manually with sterile sand in sterile PBS containing 0.01% antibiotic to make 1:10 (w/v) dilution. Oral and cloacal swab samples were suspended in 1 ml sterile PBS containing the antibiotics as described by [8].

Ethical approval: The experiments and sample collection procedures were consistent with the rules of the Animal Ethics and Monitoring Committee.

Primers and probes: Two sets of primer combinations designed for amplification of matrix and fusion genes were used in real time RT-PCR. A velogenic specific primer probe designed to amplify a wide range of Velogenic NDVs was used to detect the vNDVS in this study (Table-1).

RNA extraction and real time RT-PCR: The RNA was extracted from 140 µl of swab fluid or 140 µl of homogenized organs aliquot using QIAamp® Viral RNA mini kits, Qiagen (USA) following the manufacturer's recommended procedure.

Complementary cDNA was synthesized using 4µl of eluted RNA with transcriptor first strand cDNA Synthesis kits®, Roche, (USA) following producer recommendation.

The amplification reaction was performed in LightCycler 2.0 (Roche, USA). The reaction mixture per 20 µl reaction was (for M gene) 10 pmol of primers (1 µl/each), 5µl cDNA, 5µl of Master mix (Light Cycler® Fast Start DNA Master^{PLUS} SYBR Green I, Roche, USA), up to 20 µl of dd-H_2O. For F gene amplification, same procedures were used except that the concentration of forward primer was 30 pmol. The cycling conditions for matrix gene amplification primers were performed as follows: initial denaturation step at 95 °C for 15 min, 50 cycles of 94 °C for 10 s, 52 °C for 5 s, and 72 °C for 10 s. Same conditions were used for F gene except the annealing temperature was 58 °C. for Melting Curve analysis internal temperature of the LightCycler was rapidly increased to 95 °C then decreased to 65 °C for 15 s then raised to 95 with continuous acquisition mode and the lightCycler 2.0 software automatically converts them to melting peaks. For detection and characterization of velogenic NDVs a set of primer-probe designed for amplification of F gene was used as described and evaluated by [10]**,** using LightCycler® Fast Start DNA Master^{PLUS} HybProbe (Roch, USA).

Biological pathotyping: To confirm our results, virus isolation and virulence level of each isolated NDVs was measured by MDT of nine days old chicken SPF embryos and by ICPI in one day old SPF chicks, as described by[8]**.**

Results

After establishing the optimum condition of Real Time RT-PCR, the lentogenic LaSota and HB1 (Vaccines) and Velogenic NDV strains (FJ939313 and

Table-1. Primers and probes used in the study

Gene	Primer-Probe	Sequence	Size bp
Matrix	M+4100	5'- AGTGATGTGCTCGGACCTTC-3'	121
	M- 4220	5'- CCTGAGGAGAGGCATTTGCTA-3'	
Fusion	F+ 4839	5'-TCCGGAGGATACAAGGGTCT-3'	101
	F- 4939	5'-AGCTGTTGCAACCCCAAG-3'	
Fusion	F- 4894 (probe)	5'-[FAM]AAGCGTTTCTGTCTCCTTCCTCCA[BHQ-1]-3'	---

Primers-Probe sequences were used as previously described, and validated [16].

Table-2. Detection and pathotyping of NDV using SYBR Green I real-time PCR, MDT and ICIP

Sample	Species	T_m (M gene)	T_m (F gene)	Hybprobe Real Time PCR detection (F gene)	MDT (hr)	ICIP	Pathotype	Reference
FJ939313	Chickens	85.28	84.10	+	44	1.85	Velogenic	[20]
Pg/NDV	Pigeon	83.94	84.75	+	-	-	Velogenic	(*)
LaSota	-	87.10	86.55	-			Lentogenic	Commercial vaccine[a]
HB1	-	86.93	86.62	-			Lentogenic	Commercial vaccine[a]
AIV Antigen	NA	-	-	-	NA	NA	NA	
Pg/910	Pigeon	83.66	84.38	+	48	1.7	Velogenic	
Pg/313	Pigeon	84.19	84.18	+	55	1.82	Velogenic	
Pg/718	Pigeon	-	-	-	-	-	-	
Ch/1000	Chickens	85.30	83.85	+	50	1.6	Velogenic	
Ch/530	Chickens	85.28	83.99	+	48	1.7	Velogenic	
Ch/554	Chicken	86.98	86.55	-	>90	0.8	Lentogenic	
Ch/513	Chickens	84.62	84.29	+	50	1.6	Velogenic	
Ch/444	Chickens	-	-	-	-	-	-	
Ch/397	Chickens	-	-	-	-	-	-	
Ch/330	Chickens	84.64	83.79	+	48	1.8	Velogenic	
Ch/507	Chickens	84.75	83.80	+	48	1.85	Velogenic	
Ch/558	Chickens	-	-	-	-	-	Velogenic	
Ch/817	Chickens	85.35	84.56	+	50	1.69	Velogenic	
Ch/334	Chickens	-	-	-	-	-	-	
Ch/856	Chicken	87.01	86.46	-	>90	0.85	Lentogenic	
Ch/445	Chickens	85.28	83.98	+	50	1.6	Velogenic	
Pg/518	Pigeon	83.68	84.75	+	48	1.75	Velogenic	
Pg/923	Pigeon	83.94	84.49	+	48	1.7	Velogenic	
Pg/966	Pigeon	83.66	84.65	+	48	1.7	Velogenic	
Pg/1021	Pigeon	-	-	-	-	-	-	
Pg/891	Pigeon	83.56	84.73	+	50	1.56	Velogenic	

NV = Not available, - = Negative, T_m = Melting temperature, ICIP = Intra cerebral pathogenicity index, MDT = Mean Death Time, [a] Commercial vaccine produced by Veterinary Vaccine Production Center, Riyadh, Saudia Arabia. * Local NDV isolated from pigeon and characterized by Prof.Dr Saad Sharwi, Professor of Virology, Faculty of Veterinary Medicine, Banha university, Egypt

Pg/NDV) were detected and differentiated using both the F- gene primer amplifying the region around the cleavage site and the M- gene primers. The NDV vaccines LaSota and HB1 (lentogenic strains) were amplified with melting temperature (T_m) 87.10ºC and 86.93ºC respectively using the M-gene primer and melting temperature (T_m) was 86.55 ºC and 86.62 ºC respectively using the F- gene primer (Fig. 1). On the other hand the NDV strain FJ939313 that isolated from chickens and NDV/Pg isolated from pigeon were amplified with melting temperature (T_m) was 85.28 ºC and 83.94 ºC.

Samples collected from 21 poultry flock subjected to The SYBR Green I real-time PCR using both F-gene and M-gene primers. Results showed that all samples collected from 16 poultry flocks were positive with the SYBR Green I real-time PCR and the other 5 flocks failed to show any positive results with both gene primers.

Melting curve analysis revealed that all positive samples were divided into 3 groups based on the distinct melting peaks (Table-2). All the chicken velogenic isolates were grouped together with T_m between 84.62 °C to 85.30°C (85.03±0.341°C) and 83.79°C to 84.56°C (84.04±0.037) for M-gene and F-gene amplification respectively. While all pigeon velogenic isolates were grouped together with T_m ranged from 83.56°C to 83.94°C (83.78±0.237°C) and 84.18°C to 84.73°C (84.53±0.223°C). On the other hand all lentogenic isolates were grouped together with T_m ranged from 86.98°C to 87.01°C (86.99±0.021°C) and 86.55 to 86.64 (86.50±0.063) for both M-gene and F-gene amplification respectively (Table-2).

Our results were confirmed using virus isolation, MDT, ICIP and amplification of Velogenic cleavage site using fluorogenic probe. Results showed that samples collected from 15 suspected NDV flock were positive for SYBR Green I real-time PCR and virus isolation respectively. All isolates that fall in the T_m of velogenic category showed chicken embryo MDT test results ranged between 48 and 55 hours. and ICPI values between 1.56 and 1.85, respectively (Table 2). On the other hand all isolates that fall in the T_m of lentogenic category showed chicken embryo MDT test results ranged between >90 hours. and ICPI values ranged from 0.8 and 0.85, respectively (Table-2). All velogenic isolates were positive for fluorogenic probe detection and amplification.

Discussion

The objective of this study was to assess a diagnostic method for the rapid detection and differentiation of NDV strains using SYBR Green I melting-curve analyses. To accomplish this aim we use PCR primers that can amplify all APMV-1 focused on F-gene and M-gene [10]. This assay based on using the LightCycler system with SYBR Green I fluorescence dye, the PCR products could be differentiated during amplification by analysis of melting curves whose shape is a function of GC content, length, and

sequence. Melting curve analysis can distinguish products of the same length but different GC/AT ratio that differed in Tm less than 2 °C [11]**.** In this study the mean T_m values of lentogenic strains are 1.96 and 3.21 higher than velogenic NDV isolates of chickens and pigeon respectively regarding the M-gene primers, and 1.72 to 1.97 higher than velogenic NDV isolated of chickens and pigeons respectively regarding the F-gene primers (Table-1). SYBR Green I real-time PCR assay based on the F gene for rapid detection and differentiation of NDV strains. The assay was able to identify 38 NDV strains based on the melting temperature profiles exhibited by different NDV pathotypes: 89.23±0.27 °C for velogenic strains, 90.17 ±0.35 °C for pigeon mesogenic strains and 91.25±0.14 °C for two lentogenic strains B1 and Ishii. The melting temperatures differences among the NDV pathotypes ranged from0.94 to 2.02 °C. [16]. Previous studies have showed that a discriminatory melting temperature of 1 °C in SYBR Green I real-time PCR was sufficient to distinguish different serotypes or strains of viruses [17,18].

To confirm our result we used hybridization probes of higher specifity to the cleavage site of velogenic and mesogenic NDV strains [15] and results showed that all velogenic strains amplified and give fluorescein signals with the designed probe that come on conformity with the results of melting curves analysis. The assays based on hybridization probes reported previously[16] are of higher specificity than the SYBR Green I analysis, but they require labeled oligonucleotide and unique probes for each target. Like other RNA viruses, NDV strains are known to undergo genomic variations, and spontaneous random mutations. If a variation or mutation occurred in the region which primers or probes hybridize region, no reaction could occur and a false negative would be recorded [16]. Traditionally, the pathogenicity of any NDV isolate has been assessed by biologic methods such as ICPI and MDT [6,7,8]. MDT and ICPI results correlated with severe clinical signs reported in the field cases NDVs with an ICPI value above 0.7 are considered virulent [19]. In this study, the ICPI values ranged from 1.59 to 1.94, indicating the presence of a severe threat for the poultry industry due to circulation of highly virulent NDV viruses.

Conclusion

It is concluded that ND is still a big threaten to poultry industry all over the world. In this study we used a SYBR Green I melting-curve analyses as an alternative method for rapid diagnosis and pathotyping of NDV isolates, it could detect the NDV in clinical samples directly without isolation and propagation that consumed a lot of time. Also this method succeeded in distinguishes between the lentogenic and velogenic NDV isolates directly from clinical samples with minimal time.

Authors' contribution

The work presented here was carried out in collaboration between all authors.

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Competing interests

Authors declare that they have no competing interest.

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