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Short communication

Detection and differentiation of virulent and avirulent strains of Newcastle disease virus by real-time PCR

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A rapid diagnostic method based on the melting curve SYBR Green I real-time PCR analysis was developed to detect and differentiate Newcastle disease virus (NDV) strains. Degenerated primers based on the cleavage site sequence of the F0 gene were designed to detect specific sequences characteristic of virulent and avirulent strains of NDV.

Eighteen strains of NDV from four lineages were identified and grouped into virulent and avirulent strains. Peaks on the melting temperature graph with melting temperature values between 80.00 and 83.80 °C were observed for lentogenic (avirulent) strains. T_m values higher than 83.80 were observed for virulent (mesogenic and velogenic) strains. The detection limit of real-time PCR was 2×10^2 plasmid copies per reaction or 10^2 EID_{50} for velogenic strains and 10^3 EID_{50} for lentogenic strains.

The results obtained in this study demonstrate the possible applications for melting curve real-time PCR analysis in laboratory practice for the diagnosis and differentiation of avirulent and virulent strains of Newcastle disease virus.

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1. Introduction

Belonging to the *Mononegavirales* order, *Paramyxoviridae* family and *Avulavirus* genus, Newcastle disease virus (NDV) is an enveloped virus containing linear, non-segmented single-stranded RNA (Lamb et al., 2005). NDV strains are classified into one of three major pathotypes: velogenic, mesogenic and lentogenic (Beard and Hanson, 1984). Highly virulent velogenic strains cause acute infections with high mortality, mesogenic (intermediately virulent) strains cause respiratory disease in young chickens and decreased egg production, whereas lentogenic (avirulent) strains cause mild respiratory infection or give no symptoms in poultry (Seal et al., 1995). Studies conducted by Rott and Klenk (1988) revealed the key role of a small amino acid motif at the cleavage site of the F0 protein of NDV in the molecular determination of the pathotype. The presence of two pairs of basic amino acids at positions 112, 113, 115 and 116 and phenyloalanine at position 117, at fusion protein cleavage site, makes this site susceptible to cleavage by common subtilisin-like protease and easier spread throughout the host. The sequence (C-^{112}R/K-R-Q-K/R-R-F^{117}-N) is observed in highly virulent strains. In the low virulence strains, there are only two basic amino acids at position 113 and 116, along with leucine at position 117 (C-^{112}G/E-K/R-Q-G/E-R- L^{117}-N). This motif is recognized only by trypsin-like proteases, which are present mainly in the respiratory tract (Collins et al., 1996; Nagai et al., 1976). Because of the role of the cleavage site in pathogenicity, the F gene is the major target for differentiation of Newcastle disease virus.

The variability in the virulence of NDV causes diagnostic difficulties because only viral infections with an ICPI of 0.7 or greater are notifiable (OIE, 2009). Therefore, detection of the virus only is not a sufficient for notification and should be followed by methods that evaluate the pathogenicity of isolates.

The sensitivity and short time analysis of real-time PCR are its two most important advantages (Higuchi et al., 1992). Several methods for the detection and differentiation of NDV have been previously described. Most assays are based on the detection of the fusion gene (Pham et al., 2005) or the matrix and fusion genes (Wise et al., 2004; Steyer et al., 2010). However, other regions are also used for general detection and differentiation (Fuller et al., 2010; Tan et al., 2009). The first SYBR Green I assay for the detection of NDV was described by Tan et al. (2004), but this assay could not be used for differentiation of pathotypes of the virus. Pham et al. (2005) described a SYBR Green I real-time PCR melting curve analysis assay for differentiation, although the differences in the Tm values between the three genotypes were not very significant and could cause false characterization of the virus. Many real-time PCR assays for NDV detection and differentiation using different TaqMan probes were developed but degenerated primers were not used very often. Also, false negative results were reported

Abbreviations: AIV, avian influenza virus; APMV, avian paramyxovirus; CSFV, classical swine fever virus; EID, egg infective dose; ICPI, intercerebral pathogenicity index; NDV, Newcastle disease virus; PCR, polymerase chain reaction.

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(Cattoli et al., 2009; Wise et al., 2004). In some cases, to differentiate between pathotypes of NDV, it was necessary to use two or three pairs of primers and several different TaqMan probes in one reaction were necessary (Wise et al., 2004; Farkas et al., 2009).

Degenerated primers have been proven to be a good tool for the detection of the highly variable Newcastle disease virus (Farkas et al., 2009; Li et al., 2009; Steyer et al., 2010). In this study, a one tube diagnostic method is described for the detection and differentiation of Newcastle disease virus using degenerated primers.

2. Materials and methods

2.1. Virus strains and RNA extraction

The NDV strains that were analyzed in this study are listed in Table 1. All of the strains were derived from the collection of the National Veterinary Research Institute (Pulawy, Poland) and were classified into three major pathotypes based on the intracerebral pathogenicity index (ICPI) and comparison with reference strains. Avian influenza virus (AIV) subtype H5N2, Coronavirus (165/08 5–8), rotavirus (G036/10 1–5), Gumboro virus (131/92), and classical swine fever virus (CSVF) Cellpest strain were from the National Veterinary Research Institute (Pulawy, Poland). APMV strains were bought from x-OvO Limited (Dunfermline, United Kingdom). Reovirus (Reo S1133) and Infectious Bronchitis virus (IB K+ 4/91) were vaccine components of Nobilis REO 1133 and Nobilis IB 4-91 (Intervet/Schering-Plough Animal Health, Holland).

RNA was extracted from allantoic fluid of SPF embryonated eggs (Lohman, Germany), and inoculated with NDV strains in accordance with the OIE Diagnostic Manual (OIE, 2009) using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) protocol. Additionally, RNA from oral and cloacal swabs and homogenized tissues was isolated using the same kit.

2.2. Primer design

A set of degenerated primers based on the F0 gene of NDV was used in this study:

NDV-F (position 4826–4842) 5′-CTT GGT GAY TCY ATC CG-3′
NDV R (position 4901–4917) 5′-ACA CYG CCD ATA ATG GC-3′

where Y = C or T; D = A or G or T.

The amplified cDNA fragment was 92 base pairs (bp) long and contained the region encoding the F cleavage site.

2.3. cDNA synthesis and real-time PCR

Synthesis of the first strand of cDNA was carried out according to the procedure described for the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The reaction mixture contained: viral RNA, random hexamer primers and water that was incubated for 10 min at 65 °C. Subsequently, the reaction buffer, RNase inhibitor, dNTPs, DTT, and reverse transcriptase were added. The final mixture contained: 1× reaction buffer [250 mM Tris–HCl, 150 mM KCl, 40 mM $MgCl_2$; pH 8.5], 20 U of RNase inhibitor [in: 20 mM Hepes–KOH, 50 mM KCl, 8 mM DTT, 50% glycerol; pH 7.6], 60 μM of random hexamer primers,

Table 1
Melting-curve analysis of viral strains. Lineages by Aldous et al. (2003).

No	NDV strain	Lineage	ICPI	Pathotype	Cleavage site (position 4877–4894)	Cleavage site (position 112–117)	T_m [°C]	
1	Ulster 2C – reference	1	<0.7	L	GGGAGACAGGGGCGCCTT	GRQGRL	81.98	Group A: 80.00÷83.80
2	LaSota – reference AF077761	2	<0.7	L	GGGAGACAGGGGCGCCTT	GRQGRL	81.62	
3	Clone 30 – commercial vaccine	2	<0.7	L	GGGAGACAGGGGCGCCTT	GRQGRL	81.63	
4	APMV – 1/chicken/Poland/111/90 (1990)	2	<0.7	L	GGGAGACAGGGGCGCCTT	GRQGRL	82.35	
5	APMV – 1/chicken/Poland/89/90 (1990)	2	<0.7	L	GGGAGACAGGGGCGCCTT	GRQGRL	82.07	
6	APMV – 1/chicken/Poland/299/92 (1992)	2	<0.7	L	GGGAGACAGGGGCGCCTT	GRQGRL	82.16	
7	APMV – 1/chicken/Poland/117/90 (1990)	2	<0.7	L	GGGAGACAGGGGCGCCTT	GRQGRL	82.05	
8	APMV – 1/chicken/Poland/548/04 (2004)	2	<0.7	L	GGGAGACAGGGGCGCCTT	GRQGRL	82.09	
9	APMV – 1/turkey/Poland/549/04 (2004)	2	<0.7	L	GGGAGACAGGGGCGCCTT	GRQGRL	82.02	
10	APMV – 1/chicken/Poland/18/91 (1991)	2	<0.7	L	GGGAGACAGGGGCGCCTT	GRQGRL	81.95	
11	APMV – 1/pigeon/Poland/AR2/95 (1995)	4	>0.7	M	AGGAGGAAGAAGCGCTTT	RRKKRF	85.22	Group B: >83.80
12	APMV – 1/pigeon/Poland/AR7/98 (1998)	4	>0.7	M	AGGAGGAAGAAGCGCTTT	RRKKRF	85.28	
13	Italy/2736/00 – reference AY562989	4	>0.7	V	AGGAGGAAGAAACGCTTT	RRKKRF	87.44	
14	APMV – 1/pigeon/Poland/AR1/95 (1995)	4	>0.7	V	AGGAGGCAGAAGCGCTTT	RRQKRF	87.20	
15	APMV – 1/pigeon/Poland/AR3/95 (1995)	4	>0.7	V	AGGAGGCAGAAGCGCTTT	RRQKRF	86.89	
16	APMV – 1/pigeon/Poland/AR5/92 (1992)	4	>0.7	V	AGGAGGCAGAAGCGCTTT	RRQKRF	87.22	
17	APMV – 1/pigeon/Poland/AR8/92 (1992)	4	>0.7	V	AGGAGGCAGAAGCGCTTT	RRQKRF	87.52	
18	APMV – 1/chicken/Poland/Radom/70 (1970)	3	>0.7	V	AGGAGACAGAGACGCTTT	RRQRRF	86.55	
19	CSFV – Cellpest strain	NA	–			–	–	
20	AIV (H5N2)	NA	–			–	–	
21	APMV – 2	NA	–			–	–	
22	APMV – 3	NA	–			–	–	
23	APMV – 4	NA	–			–	–	
24	APMV – 6	NA	–			–	–	
25	APMV – 7	NA	–			–	–	
26	APMV – 8	NA	–			–	–	
27	APMV – 9	NA	–			–	–	
28	Coronavirus 165/08 5–8	NA	–			–	–	
29	Rotavirus G036/10 1–5	NA	–			–	–	
30	Gumboro virus 131/92	NA	–			–	–	
31	Reovirus Reo S1133 (Nobilis REO 1133, Intervet)	NA	–			–	–	
32	IB K+ 4/91 (Nobilis IB 4–91, Internet)	NA	–			–	–	

L, lentogenic; M, mesogenic; V, velogenic; NA, not applicable.

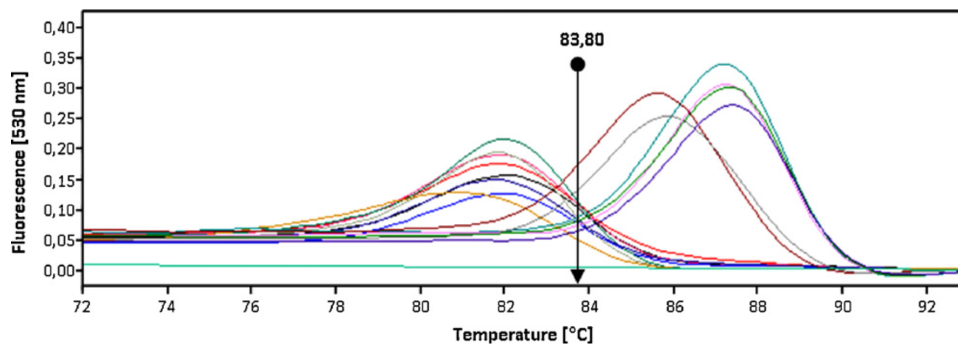

Fig. 1. Melting curve analysis of a 92 bp fragment of the F0 gene containing a cleavage site amplified by real-time PCR assay. Peaks in the range of T_m = 80.00–83.80 correspond to avirulent strains; peaks with T_m values higher than 83.80 correspond to virulent strains (mesogenic or lentogenic). Peaks with T_m values lower than 80.00 could be caused by dimers of primers.

1 mM of each dNTPs, 5 mM of DTT, and 10 U of reverse transcriptase (in: 200 mM potassium phosphate, 2 mM DTT, 0.2% Triton X-100, 50% glycerol, pH 7.2). It was then incubated for 30 min at 47 °C, followed by 5 min. incubation at 85 °C and chilled on ice.

SYBR Green I real-time PCR was performed using a LightCycler 2.0TM (Roche Diagnostics, Mannheim, Germany). 10 μl of the final mixture contained 9 μl of reaction mixture: 1× LC DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany), $MgCl_2$ (4 mM), 0.2 μM of each primer, and 1 μl of cDNA. A negative control lacking cDNA was also prepared. The samples were initially denatured at 95 °C for 10 min, followed by 50 cycles of the following: 95 °C (denaturation) for 10 s, 48 °C (annealing) for 10 s, and 72 °C (extension) for 11 s. To confirm the specificity of the amplified product, a melting curve analysis step was included. The internal temperature of the LightCycler was increased rapidly to 95 °C, then decreased at 0.1 °C/s to 70 °C, and then the sample was incubated for 15 s. The fluorescence at 530 nm was measured continuously. The melting peaks were generated using LightCycler software by plotting the first negative derivative of the fluorescence over the temperature versus the temperature ($-dF/dT$).

2.4. Detection limit, cloning, sequencing and specificity of the assay

The detection limit was determined using the target copy number and virus titer (50% egg infective dose – EID_{50}) as reference units. (i) A fragment (320 bp) of the F gene of the LaSota strain was cloned into pGEM-T easy vector (Promega, Madison, WI, USA). Plasmid DNA was extracted from the bacteria by using a Plasmid mini Kit (A&A Biotechnology, Gdansk, Poland). The concentration of the plasmid DNA was determined spectrophotometrically. The DNA copy number was calculated using the formula described by Ke et al. (2006). The plasmid was serially 10-fold diluted to serve as a standard for determining the sensitivity of the real-time PCR assay. (ii) Serial 10-fold dilutions of the known EID_{50} stock solution of lentogenic (LaSota) and velogenic (Italy/2736/00) strains were prepared using sterile dH_20. RNA was extracted and transcribed as described above. cDNA was added to the reaction mixture as a template.

The SYBR Green I melting peaks analysis assay was compared with the method described by Mia Kim et al. (2008).

The sequencing was carried out by Genomed Ltd. (Warsaw, Poland) using a Roche GS FLX/Titanium sequencer. MultAlin software (Corpet, 1988) was used to align the sequences to check for homology with reference NDV sequences.

The specificity of the SYBR Green I real-time PCR assay was investigated using the RNA extracted from NDV strains and 14 other avian viruses shown in Table 1.

2.5. Phylogenetic analysis

Phylogenetic analysis of members of 6 lineages of Newcastle disease virus (Aldous et al., 2003) was performed utilizing the neighbor-joining method (Saitou and Nei, 1987) in MEGA 4 software. Strains of NDV were derived from GenBank or from the Polish strains sequenced previously.

3. Results

3.1. Detection and differentiation of NDV strains by melting peak analysis

The representatives of 4 lineages of NDV strains were tested. Using the SYBR Green I real-time PCR melting peak analysis, it was possible to detect and differentiate virulent and avirulent strains of Newcastle disease virus. Eighteen NDV strains (including ten lentogenic, two mesogenic and six velogenic isolates) were analyzed. Melting curve analysis revealed that all 18 strains were divided into two distinct groups (Fig. 1). T_m values for lentogenic (avirulent) strains were estimated to be between 80.00 and 83.80 °C, and the T_m values for mesogenic and velogenic (virulent) strains were higher than 83.80 °C (Table 1). The boundary line was calculated as the medium between the highest T_m value for avirulent strains and the lowest for virulent strains. The phylogenetic relationship of the NDV strains used in this study is depicted in Fig. 2.

3.2. Specificity of real-time PCR assay and detection limit

The specificity of this real-time PCR assay was investigated by analysing 18 NDV strains and 14 samples of other viruses (Table 1). No amplification products were detected when templates originating from these fourteen unrelated viruses were used, while all templates from NDV strains gave distinctive products.

The detection limit of this assay was assessed by analysing the serial dilutions of a plasmid carrying s 320 bp fragment of the F gene of NDV and the serial dilutions of the known stock of NDV. In the real-time PCR assay, the detection limits were 2×10^2 copies of DNA plasmid per reaction or 10^2 EID_{50} concentration range for the velogenic strain (Italy/2736/00) and 10^3 EID_{50} for the lentogenic strain (LaSota). The results were comparable with the results described by Wise et al. (2004). Any results with a Ct value higher than 45 were treated as unconvincing and were rejected. The new assay was compared with the method described previously by Mia Kim et al. (2008) – both gave similar results (Table 2A) in oral and cloacal swabs. The difference in Ct values resulted from the use of different methods in the assays (TaqMan and SYBR Green I, respectively). The new assay detected NDV in three tissue samples while the one described previously detected NDV only in one sample (Table 2B).

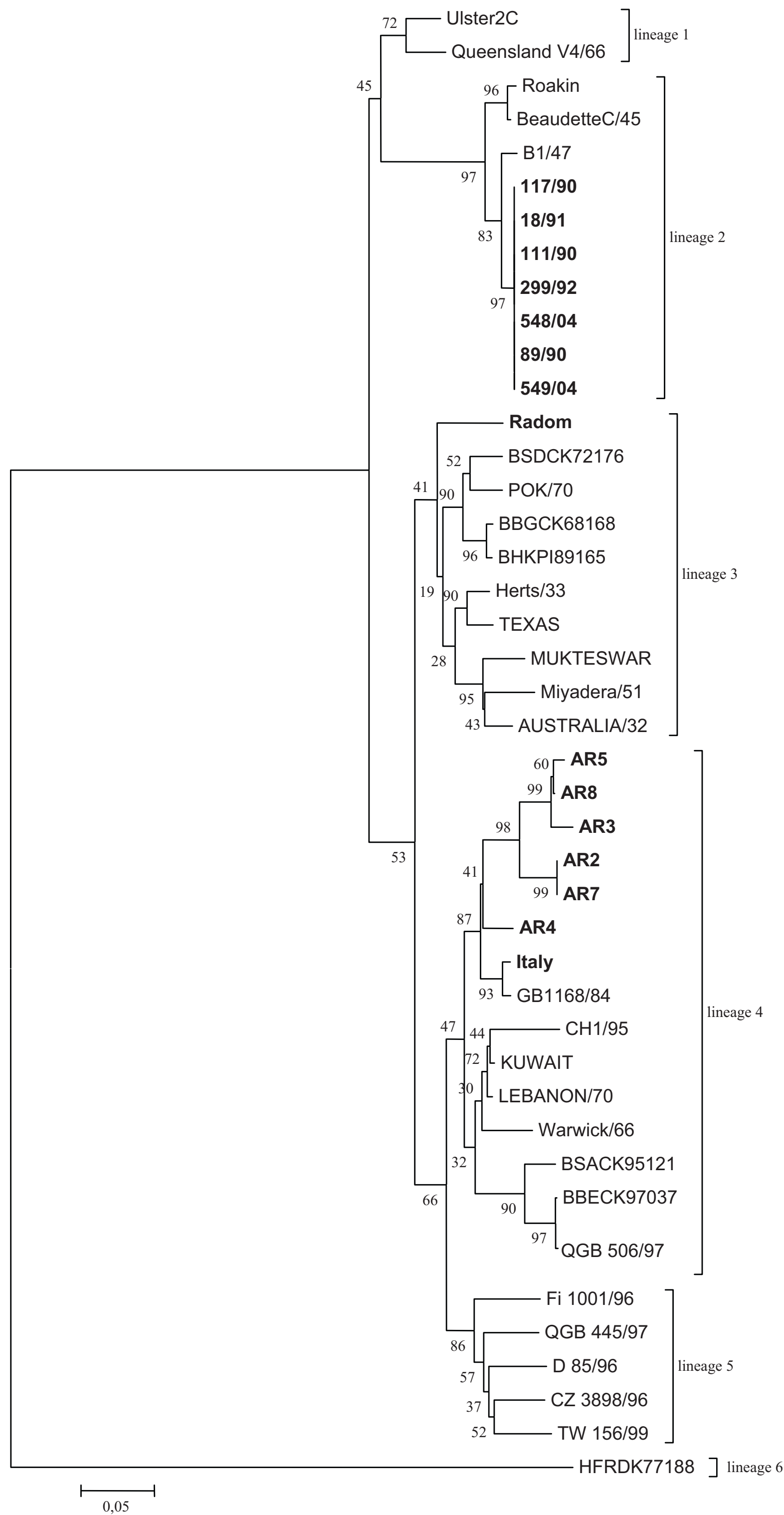

Fig. 2. Phylogenetic analysis of nucleotide sequences of the F gene (nucleotides 47–420) of NDV belonging to 6 known lineages (Aldous et al., 2003). Polish isolates are in bold. The Phylogenetic tree was generated using the neighbor-joining method (Saitou and Nei, 1987). *Ulster 2C* (Table 1); *Queensland V4/66* (M24693); *Roakin* (AY289000); *Beaudette C/45* (M24697); *B1/47* (M24695); *117/90* (Table 1); *18/91* (Table 1); *111/90* (Table 1); *299/92* (Table 1); *548/04* (Table 1); *549/04* (Table 1); *89/90* (Table 1); *Radom* (Table 1); *BSDCK72176* (AY135752); *POK/70* (Y19014); *BBGCK68168* (AY175669); *BHKPI89165* (AY175672); *Herts/33* (M24702); *TEXAS* (M33855); *Mukteswar* (AY175676); *Miyadera/51* (M24701); *Australia/32* (M21881); *AR5* (Table 1); *AR8* (Table 1); *AR3* (Table 1); *AR2* (Table 1); *AR7* (Table 1); *AR4* (Table 1); *Italy* (Table 1); *GB1168/84* (AF109885); *CH1/95* (AF001132); *Kuwait* (AF001109); *Lebanon/70* (AF139160); *Warwick/66* (U52457); *BSACK95121* (AY175694); *BBECK97037* (AY135741); *QGB506/97* (AF109887); *Fi1001/96* (AF091623); *QGB445/97* (AF109886); *CZ3898/96* (AF109883); *TW156/99* (AF234031); *HFRDK77188* (AY135758).

Table 2
A comparison of the sensitivity of SYBR Green I real-time PCR melting peaks analysis assay (SYBR MPA) with the previously published system by Mia Kim et al. (2008). (A) Three chickens were infected with a lentogenic strain (LaSota) with a dose of $10^{7.5}$ EID_{50} of the virus. Oral (O) and cloacal (C) swabs were collected 3 and 5 days post infection (dpi). (B) One chicken was killed 5 days post infection, and tissues were prepared. RNA was extracted as described above from a homogenate of tissues.

		Chicken no 1		Chicken no 2		Chicken no 3	
		Mia Kim	SYBR MPA	Mia Kim	SYBR MPA	Mia Kim	SYBR MPA
(A)							
3 dpi	O	26.69	36.52	23.67	40.05	25.17	39.49
3 dpi	C	n.d.	41.22	34.87	n.d.	n.d.	39.95
5 dpi	O	24.93	n.d.	25.01	41.84	23.15	43.51
5 dpi	C	34.89	n.d.	n.d.	n.d.	n.d.	n.d.

Tissue	Mia Kim	SYBR MPA
(B)		
Lung	–	–
Liver	–	–
Brain	–	–
Trachea	+	+
Spleen	–	–
Duodenum	–	+
Caecum	–	+
Jejunum	–	–

n.d. – not detected.

4. Discussion

In this study, a method for the rapid detection and differentiation of Newcastle disease virus by SYBR Green I melting-curve analysis was described. F gene-specific, degenerated primers were used in the assay. The use of degenerated primers made it possible to avoid the previously reported difficulties in the design of primers (Aldous and Alexander, 2001) and, additionally, it allowed for the use of only one set of primers for the differentiation of virulent and avirulent strains of Newcastle disease virus. This is the first assay based on SYBR Green I that uses degenerated primers to detect virulent and avirulent strains of NDV in one tube reaction with high resolution. The correlations between virulent (ICPI > 0.7) and non-virulent (ICPI < 0.7) strains and melting temperature values are shown in Table 1. The distinction between lentogenic and mesogenic (3.26 °C) or lentogenic and velogenic (5.15 °C) NDV strains was more simple than previously described by Pham et al. (2005), which yielded values of 1.22 °C and 2.15 °C, respectively. Additionally, such a high difference in melting temperature values makes it possible to calculate a safe boundary line (83.80 °C) for differentiation of NDV strains. This in turn allows for a straightforward method to monitor and classify virulent and avirulent strains of NDV in agreement with European Union directive (CEC, 1992), which calls for the full control of isolates with an intercerebral pathogenicity index (ICPI) greater than 0.7.

The sensitivity of the method described in this report is similar to the method described by Mia Kim et al. (2008) and Wise et al. (2004) and by Pham et al. (2005). Differences in Ct values are cause by differences across methods. In real-time PCR using the TaqMan method (Mia Kim et al., 2008), probes are released and degraded during each cycle and fluorescence increases cumulatively during the reaction. When the SYBR Green I method is used fluorescence increases proportionally. That explains why higher Ct values were obtained while using this method. The advantages of using this SYBR Green I method are that its operation costs are lower in comparison to either methods using TaqMan, and it is possible to perform the reaction in a single tube. The previous methods employed two or three pairs of primers (Wise et al., 2004), and their products for lentogenic and velogenic strains (Farkas et al., 2009), in some cases, were different in length, which excludes the possibility of using the real-time PCR SYBR Green I assay.

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