# DETECTION OF NEWCASTLE DISEASE VIRUS USING A SYBR GREEN I REAL TIME POLYMERASE CHAIN REACTION

S.W. TAN[1], A.R. OMAR[1*], I. AINI[1], K. YUSOFF[2], W.S. TAN[2]

[1]Faculty of Veterinary Medicine, [2]Faculty of Science and Environmental Studies, University Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

**Summary.** – A two-step SYBR Green I real time polymerase chain reaction (PCR, real time PCR) for the detection of Newcastle disease virus (NDV) was developed. A melting curve analysis was performed to distinguish specific from non-specific products and primer dimers. Regardless of different virus pathotypes the melting temperature ($T_m$) ranged from 86°C to 87°C. The sensitivity of the real time PCR was compared with the reverse transcription (RT)-nested PCR enzyme-linked immunosorbent assay (ELISA, RT-nested PCR ELISA). Whereas the detection limit of the real time PCR was 10 pg DNA, the RT-nested PCR ELISA and conventional PCR could only detect up to 1 ng and 10 ng DNA, respectively. Thus the real time PCR offers a sensitive, rapid and convenient method for screening large number of NDV specimens.

**Key words:** ELISA; Newcastle disease virus; SYBR Green I; real time PCR; RT-nested PCR ELISA

## Introduction

Newcastle disease (ND) is a highly contagious viral disease of domestic poultry, cage, aviary and wild birds with worldwide distribution (Yusoff and Tan, 2001). The etiological agent of the disease, NDV or avian paramyxovirus type 1 is a member of the genus *Rubulavirus*, the subfamily *Paramyxovirinae,* the family *Paramyxoviridae*, the order *Mononegavirales* (Van Regenmortel *et al.,* 2000). Based on the clinical symptoms induced in infected chickens, Beard and Hanson (1984) have classified NDV into five pathotypes: (i) viscerotropic velogenic – high mortality with intestinal lesions; (ii) neurotropic velogenic – high mortality with nervous symptoms; (iii) mesogenic – low mortality with respiratory and nervous symptoms; (iv) lentogenic – mild or inapparent respiratory infection, and (v) asymptomic enteric – inapparent intestinal infection. However, even under constant controlled laboratory conditions, this grouping may not be obvious but it serves as a guide to suspected ND. The lack of pathognomonic clinical signs, even in the infections with the most virulent viruses, requires usually a confirmatory diagnosis for further identification and characterization of the virus.

The use of RT-PCR followed by ethidium bromide staining of electrophoretically separated amplified product provides a convenient method for detecting various infectious viruses including NDV (Aldous and Alexander, 2001). Alternatively, PCR-ELISA may be used to capture an amplicon onto a solid phase using biotin- or digoxigenin-labeled primers, oligoprobes or amplicons (Dekonenko *et al.*, 1997; Kho *et al.*, 2000; Watzinger *et al.*, 2001).

The recent development of a fluorogenic PCR-based format, termed as a real-time PCR promises a wide application to diagnosis of viruses. Recently, Aldous *et al.* (2001) have reported a TaqMan-based real time PCR able to detect and differentiate different pathotypes of NDV. In this study, we report development of a sensitive and rapid detection of NDV using the real time PCR.

*Corresponding author. Fax: +603-89486317; E-mail: aro@vet.upm.edu.my.

**Abbreviations:** AMV = Avian myeloblastosis virus; $C_t$ = threshold cycle; ELISA = enzyme-linked immunosorbent assay; ND = Newcastle disease; NDV = ND virus; RT-PCR = reverse transcription–polymerase chain reaction; SPF = specific-pathogen-free; SD = standard deviation; $T_m$ = melting temperature

## Materials and Methods

*Virus isolates and vaccine strains.* A total of 12 NDV isolates, namely 7 velogenic (001/KS, 01/C, 01/TM, 01/GNS, AF2240, VRI2444, and VRI2478), 1 mesogenic (Mukteswar S) and 4 lentogenic (V4 UPM, F, B1, and LaSota) were tested in this study. Whereas the velogenic isolates 001/IKS, 01/C, 01/TMS, and 01/GNS were isolated from different broiler farms in 2001 (Tan *et al.,* 2001), AF2240 was isolated from a local field outbreak in 1960s. The remaining 2 velogenic, the mesogenic Mukteswar S and the lentogenic F isolates were kindly provided by the Veterinary Research Institute, Ipoh, Perak, Malaysia. The V4 UPM was a heat-resistant variant of V4 (QUE) (Aini *et al.,* 1990), while B1 and LaSota were vaccine strains obtained from the Malaysian Vaccine Pharmaceutical Sdn Bhd (Malaysia).

*Virus propagation.* All the isolates were propagated in the allantoic cavity of 9–10 day-old specific-pathogen-free (SPF) embryonated chicken eggs according to Blaskovic and Styk (1967). Allantoic fluids were clarified by centrifugation at 5,000 x g for 15 mins at 4°C and stored at -80°C.

*Extraction of viral RNA* was performed with Tri Reagent® (Life Technologies, USA) according to the manufacturer's instructions. The allantoic fluid (250 µl) was mixed with 750 µl of Tri Reagent solution and left to stand for 5 mins at room temperature. Then 200 µl of choloroform was added and the mixture was shaken vigorously for 15 secs followed by incubation at room temperature for 15 mins and centrifugation at 12,000 x g for 15 mins. The upper aqueous phase was withdrawn, precipitated with 500 µl of isopropanol at room temperature for 10 mins and centrifuged at 12,000 x g for 10 mins. The pellet was washed with 1 ml of 75% ethanol at 7,500 x g for 10 mins, air-dried and dissolved in distilled water for immediate use or dissolved in 100% ethanol for storage at -20°C.

*The primers* used in this study were designed from a conserved region of the fusion gene of a velogenic isolate of NDV, AF2240 (Table 1, Salih *et al.,* 2000). The outer (FOP1 and FOP2) and inner (FIP1 and FIP2) primers for the RT-nested PCR ELISA have been described previously by Kho *et al.* (2000). The forward (FIP1) and reverse (FIP2) inner primers were labeled at their 5'-ends with biotin and dioxigenin, respectively. The SYBR Green I real time PCR was performed using unlabeled FIP1 and FIP2 primers.

*Real time PCR.* Synthesis of the first strand cDNA was carried out in total volume of 20 µl using the Promega Reverse Transcription System according to the manufacturer's instructions. The reaction mixture contained 0.8 µmol/l FI1 and FI2, 1.0 mmol/l dNTPs, 2 U of Avian myeloblastosis virus (AMV) reverse transcriptase, 5 U of recombinant RNasin (ribonuclease inhibitor), 100 ng of RNA, 5 mmol/l $MgCl_2$ and 1x reaction buffer. The mixture was initially incubated at 42°C for 45 mins (denaturation) and then at 99°C for 5 mins (inactivation of the reverse transcriptase) in a PTC-200 DNA Peltier Thermal Cycler (MJ Research, USA). The cDNA was then chilled in ice for 5 mins and used immediately in PCR. A total volume of 50 µl of PCR mixture containing 3 mmol/l $MgCl_2$, 0.4 mmol/l dNTPs, 0.32 µmol/l FIP1 and FIP2, 5 U of *Taq* polymerase (Promega), 1 µl of SYBR Green I (Molecular Probes, USA), 1X reaction buffer and 1–5 µl of cDNA was prepared in low-profile 0.2 ml tube stripes. In order to determine the opti-

mum concentration of SYBR Green I for PCR, the velogenic NDV isolate 001/IKS was used. Briefly, the SYBR Green I was serially diluted in sterile nuclease-free water from $10^{-1}$ to $10^{-5}$ and used in PCR as described above. The PCR was performed in a DNA Engine Opticon™ System (MJ Research, USA). Three negative controls without cDNA were used. The cycle conditions were as follows: one cycle at 95°C for 3 mins followed by 40 cycles at 95°C for 10 secs, 55°C for 30 secs, and 72°C for 20 secs. Upon completion of the amplification a melting curve analysis was performed.

*Melting curve analysis* was carried out by raising the incubation temperature from 72°C to 99°C in 0.4°C increments with a hold of 1 secs at each increment. The SYBR Green I fluorescence (F) was measured continuously during the heating period and the signal was plotted against temperature (T) to produce a melting curve for each sample. The melting peaks were then generated by plotting the negative derivative of F over T versus T (-dF/dT versus T).

*RT-nested PCR ELISA.* The assay was carried out in two steps in one tube. The cDNA synthesis was performed as described in the SYBR Green I real time PCR with some modifications. Briefly, the PCR was performed in a 20 µl mixture containing 0.5 µmol/l outer primers FOP1 and FOP2, 2.5 mmol/l $MgCl_2$, 1X reaction buffer, 2.5 U of *Taq* polymerase (Promega) and 1–5 µl of cDNA. The cycle conditions were as follows: one cycle at 94°C for 3 mins, 20 cycles at 94°C for 1 min, 67°C for 30 secs, and 72°C for 30 secs (extension). The final extension step at 72°C was prolonged to 7 mins. The second PCR amplification step was carried out in the same tube by adding 30 µl of reaction mixture which consisted of 0.2 µmol/l inner labeled primers FIP1 and FIP2, 1x reaction buffer, and 1.0 mmol/l $MgCl_2$. The reaction mixture was subjected to another 30 cycles consisting of 94°C for 30 secs, 55°C for 30 secs, and 72°C for 15 secs followed by a final extension at 72°C for 7 mins in a PTC-200 DNA Peltier Thermal Cycler (MJ Research, USA). The detection of PCR product by ELISA was carried out according to the methods described by Kho *et al*. (2000).

*Agarose gel electrophoresis.* PCR products of the real time PCR and RT-nested PCR ELISA were also detected on a 1.5% (w/v) agarose gel electrophoresis in 1xT BE buffer at 60 V for 50 min. The gel was then stained with ethidium bromide and photographed over UV transillumination.

*Comparisons of sensitivity of PCR assays.* To evaluate the sensitivity of real-time PCR, a velogenic NDV 001/IKS was used. The detection limits of the real-time PCR and RT-nested PCR ELISA were compared by amplification of serial ten-fold dilutions of the cDNA product. The concentrations of the cDNA products of each PCR assay was estimated spectrophotometrically (Ausubel *et al.,* 1999). A regression analysis of the threshold cycle ($C_t$) values of the serially diluted cDNA samples was used to determine the amplification efficiency.

## Results

*Optimization of the real time PCR*

The real time PCR was optimized using serially diluted SYBR Green I to exclude low-emission signals caused by

**Table 1. Primers used in the RT-nested PCR ELISA and in the SYBR Green I real time PCR**

| Primer | Sequence (5'-3') | Position (nts)[a] | Size of product (bp) | Application |
|---|---|---|---|---|
| FOP1 | TACACCTCATCCCAGACAGGGTC | 203–225 | | |
| FOP2 | AGGCAGGGGAAGTGATTTGTGGC | 712–734* | 532 | Outer primers in RT-nested PCR ELISA |
| FIP1 | TACTTTGCTCACCCCCCTT | 313–331 | | |
| FIP2 | CATCTTCCCAACTGCCACT | 574–592* | 280 | Inner primers in RT-nested PCR ELISA |
| FIP1[b] | TACTTTGCTCACCCCCCTT | 313–331 | | |
| FIP2[c] | CATCTTCCCAACTGCCACT | 574–592* | 280 | SYBR Green I real time PCR |

Primers were designed according to the conserved region of fusion gene of a velogenic NDV isolate AF2240 (Salih *et al.,* 2000).
[a]Position in the fusion gene. [b]Biotin-labeled, [c]Digoxigenin-labeled. *Denotes antisense orientation
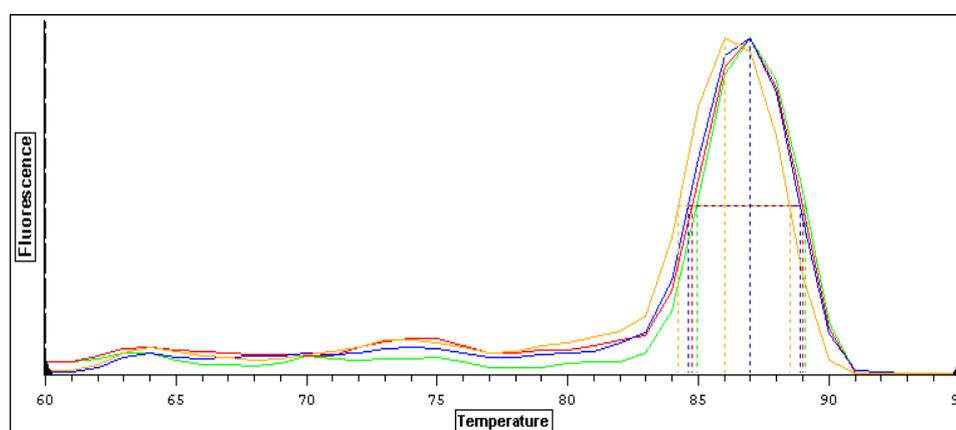


**Fig. 1**

**Melting curve profiles of SYBR Green I real time PCR in detecting NDV**

$T_m$ of NDV pathotypes ranged from 86°C to 87°C. No primer dimer or non-specific product was detected after amplification.

low dye concentration or inhibitory effects of high SYBR Green I concentration. In this study, the optimal dilution of SYBR Green I was $10^{-4}$. All the 12 isolates were successfully detected at this condition and showed a typical sigmoid curve of the amplification. No amplification was detected when a too high concentration (a too low dilution, namely dilutions $10^{0}$–$10^{-3}$) of the dye was used, while the fluorescent signal was very low and non-detectable when a too low concentration (a too high dilution, for example $10^{-5}$) of the dye was used.

*Melting curve analysis*

As SYBR Green I dye binds to any double-stranded DNA, product specificity and the absence of non-specific amplification or primer dimer were determined by melting curve analysis. In dF/dT vs T plots the $T_m$ of each isolate appeared as a single peak. Regardless of the virus isolate the $T_m$ ranged from 86 to 87°C (Fig. 1).

*RT-nested PCR ELISA*

In this assay 10 negative control samples (without template) were tested to obtain the cut-off level between positive and negative $A_{405}$ values. The cut-off value was calculated as the mean $A_{405}$ of 10 negative control samples plus 3SD, namely 0.0986 + 0.021 (0.007 x 3) = 0.1196. All samples with an $A_{405}$ above this cut-off value were considered positive.

*Comparison of sensitivities of the real time PCR and the RT-nested PCR ELISA*

The concentrations of undiluted cDNA in the real time PCR and the RT-nested PCR ELISA were 1.0 µg/µl and 0.2 µg/µl, respectively. In order to standardize this comparison, 1 µl of cDNA from the real time PCR and 5 µl of cDNA from the RT-nested PCR ELISA were considered the undiluted cDNA concentrations. When ten-fold dilutions
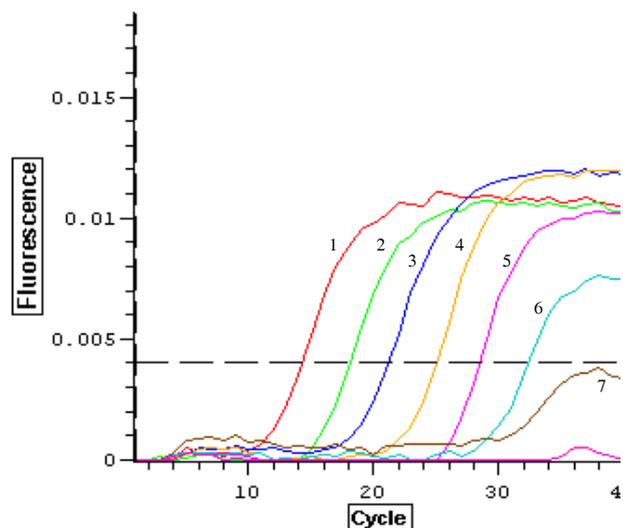
**Fig. 2**

**Performance of SYBR Green I real time PCR with ten-fold dilutions of cDNA**

The detection of the assay was done with the $10^{-5}$ dilution. cDNA undiluted (1), diluted $10^{-1}$ (2), $10^{-2}$ (3), $10^{-3}$ (4), $10^{-4}$ (5), $10^{-5}$ (6), $10^{-6}$ (7).

**Table 2. Comparison of sensitivity of the SYBR Green I real time PCR, RT-nested PCR ELISA and agarose gel electrophoresis detection methods**

| Dilution of cDNA | Detection method | | |
|---|---|---|---|
| | SYBR Green I | RT-nested PCR ELISA | Agarose gel electrophoresis |
| $10^{0}$ (undiluted) | + | + | + |
| $10^{-1}$ | + | + | + |
| $10^{-2}$ | + | + | +[c] |
| $10^{-3}$ | + | +[b] | – |
| $10^{-4}$ | + | – | – |
| $10^{-5}$ | +[a] | – | – |
| $10^{-6}$ | – | – | – |

(+), (–) = positive, negative result.
[a] 10 pg, [b] 1 ng, [c] 10 ng.

of cDNA were amplified by the real-time PCR, this assay detected up to the $10^{-5}$ dilution of cDNA (Fig. 2). The detection limits of the real time PCR, RT-nested PCR ELISA and agarose gel electrophoresis are shown in Table 2. The ELISA assay detection limit was 1 ng with an $A_{405}$ of 0.204 (Fig. 4), while the agarose gel electrophoresis could detect only up to 10 ng of cDNA with a faint band (data not shown). $C_t$ is used as a unit to indicate the PCR cycle at which the fluorescence signal first exceeds the background noise level. As shown in Fig. 3, whereas the undiluted sample showed the lowest $C_t$ value the $10^{-5}$ dilution of cDNA showed the highest $C_t$ value. A linear relationship was observed between the amount of input cDNA and the $C_t$ values over five ten-fold dilutions, namely from a dilution of $10^{-1}$ ($C_t$ value of 14.29) to $10^{-5}$ ($C_t$ value of 32.29) (Fig. 3). No $C_t$ value was produced with a dilution of $10^{-6}$. Regression analysis of the $C_t$ values generated by the serial ten-fold dilutions produced a correlation coefficient over 0.998 for the reaction.

## Discussion

The sensitivities of the real time PCR, RT-nested PCR ELISA and conventional PCR in the detection of NDV pathotypes were compared. The real time PCR was found to be specific and sensitive. The primers used in the real-time PCR were designed on a highly conserved region of the F gene of a velogenic NDV isolate AF2240 (Salih *et al.,*

2000). However, regardless of the virus pathotypes, all the isolates showed positive results with $T_m$ ranging from 86°C to 87°C. The specificity of the primers was proven by lack of amplification of RNAs of other avian viruses such as Infectious bronchitis virus and Infectious bursal disease virus. In addition, lack of additional peaks indicated the absence of non-specific products or primer dimers in the melting curves. Several studies have been carried out to explore the advantage of the melting curve analysis for the strain and pathotype differentiation (Beuret, 2004; Helps *et al.,* 2002; Limor *et al.*, 2002; Nicolas *et al.*, 2002), based on the GC/AT ratio, length and nucleotide sequences of the amplified product. In this study, no distinguishing melting peaks were observed between the velogenic, mesogenic and lentogenic isolates of NDV. Recently Aldous and coworkers (2001) have reported a rapid pathotyping of NDV using six pathotype-specfic TaqMan probes, which recognized the nucleotide sequence at the F cleavage site of NDV pathotypes.

In the sensitivity test, there was a good correlation between the $C_t$ values from the real-time PCR and the template concentration used in the amplification. It was observed that by using ten-fold dilutions of cDNA the $C_t$ value increased consistently, whereby the larger the amount of template, the fewer the cycles were needed to reach a detectable threshold. However, in the RT-nested PCR ELISA, proportionally decreased $A_{450}$ with increased dilution of cDNA was observed only with a diluted cDNA template. The actual reason for this observation is unknown. Probably the undiluted cDNA, which was associated with the formation of heterodimers and homodimers of the primers binds to the microtiter plate, thus blocking the binding site and thereby reducing the dynamic range of the ELISA reaction. However, a low cut-off value (0.1196) was obtained with the negative control samples indicating that the labeled primers used in the assay were specific.
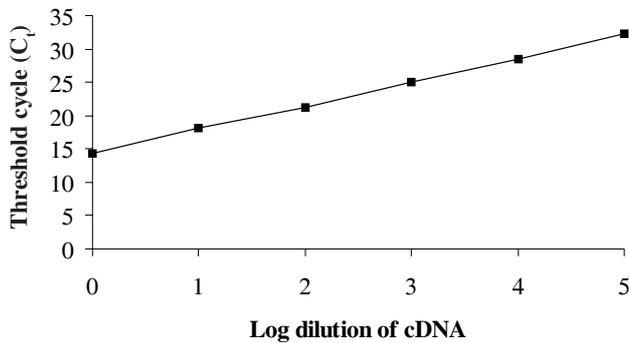
**Fig. 3**

**A linear relationship between $C_t$ values and the dilution of the cDNA used as template**

No $C_t$ value was obtained with the $10^{-6}$ dilution of template. The regression equation was $C_t = 3.566$ (log dilution) $+ 14.29$ and $R^2 = 0.998$.



**Fig. 4**

**Detection limit of the RT-nested PCR ELISA**

The optimal dilution of the SYBR Green I in the ELISA assay was $10^{-4}$ (1:10,000) of the stock solution (the final dilution of 1:500,000). With this dilution, the SYBR Green I generated sufficient signal intensity for detection and analysis. As this dye intercalates dsDNA and may interfere with the amplification reaction, an excessive dye concentration may lead to an inhibition of the reaction whilst an insufficient dye concentration may not provide enough dye to label the amplicon (Arezi *et al*., 2003; Lipsky *et al*., 2001). The real time PCR was found to be 100-fold more sensitive than the RT-nested PCR ELISA for NDV detection. The calculated detection limit of the optimized real time PCR was 10 pg compared to 1 ng and 10 ng for ELISA and agarose gel electrophoresis stained with ethidium bromide, respectively. The lower detection limit of the RT-nested PCR ELISA compared to 10–100 fg as reported by Kho *et al.* (2000) was associated probably with different PCR conditions. The RT-nested PCR ELISA carried out in this study consisted of 2 steps in 1 tube and the sensitivity of the assay was based on serial dilution of cDNA while the RT-nested PCR ELISA described by Kho *et al.* (2000) consisted of 1 step in 1 tube and the serial dilution of the template was based on viral RNA.

The SYBR Green I real time PCR provides a rapid and sensitive method for NDV detection. The turnaround time for the real time PCR was less than 6 hrs compared to less than 10 hrs for the RT-nested PCR ELISA (Kho *et al.,* 2000). It is also considered simpler and cheaper than the fluorescently labeled probes such as TaqMan , Molecular Beacons and Scorpions. The labeled probes are expensive and introduce additional complexity to both the design and the parameters of the amplification reaction. This study has successfully established a SYBR Green I real time PCR,
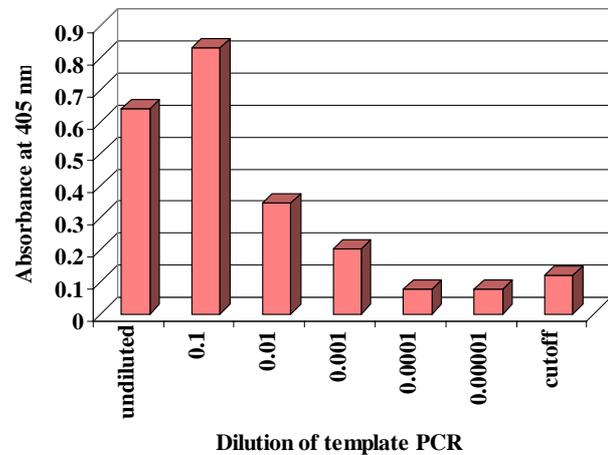
which has the potential to be a powerful tool in screening for NDV and for the diagnosis of ND outbreaks. However, the application of the SYBR Green I-based real time PCR to differentiation of NDV pathotypes remains to be studied.

## References

Aini I, Ibrahim AL, Spradbrow PB (1990): Field trials of a food-based vaccine to protect village chickens against Newcastle disease. *Res. Vet. Sci.* **49**, 216–219.

Aldous EW, Alexander DJ (2001): Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). *Avian Pathol.* **30**, 117–128.

Aldous EW, Collins MS, McGoldrick A, Alexander DJ (2001): Rapid pathotyping of Newcastle disease virus (NDV) using fluorogenic probes in a PCR assay. *Vet. Microbiol.* **80**, 201–212.

Arezi B, Xing W, Sorge JA, Hogrefe HH (2003): Amplification efficiency of thermostable DNA polymerase. *Anal. Biochem.* **321**, 226-235.

Ausubel FM, Brent R, Kingston, RE, Moore DD, Seidman JG, Smith JA, Struhl K (1997): *Current Protocols in Molecular Biology*. John Wiley & Sons Inc., pp. A3D1–A3D8.

Beard CW, Hanson RP (1984): Newcastle disease. In Hofstad MS, Barnes HJ, Calnek BW, Reid WM, Yoder HW (Eds): *Diseases of Poultry*. 8th ed., Iowa State University Press, Ames, pp. 452–470.

Beuret C (2004): Simultaneous detection of enteric viruses by multiplex real-time PCR. *J. Virol. Methods* **115**, 1–8.

Blaskovic D, Styk B (1967): Laboratory methods of virus transmission in multicellular organisms. In Maramorasch K, Koprocvski H (Eds): *Virology*. Vol. 1, Academic Press, New York, pp. 194–197.

Dekonenko A, Ibrahim MS, Schmaljohn CS (1997): A colorimetric PCR-enzyme immunoassay to identify hantaviruses. *Clin. Diagn. Virol.* **8**, 113–121.

Helps C, Lait P, Tasker S, Harbour D (2002): Melting curve analysis of feline calicivirus isolates detected by real-time reverse transcription PCR. *J. Virol. Methods* **106**, 241–244.

Kho CL, Mohd-Azmi ML, Arshad SS, Yusoff K (2000): Performance of an RT-nested PCR ELISA for Newcastle disease virus. *J. Virol. Methods* **86**, 71–83.

Limor JR, Lal AA, Xiao L (2002): Detection and differentiation of *Crytosporidium* parasites that are pathogenic for humans by real-time PCR. *J. Clin. Microbiol.* **40**, 2335–2338.

Lipsky RH, Mazzanti CM, Rudolph JG, Xu K, Vyas G, Bozak D, Radel MQ, Goldman D (2001): DNA melting analysis for detection of single nucleotide polymorphisms. *Clin. Chem.* **47**, 635–644.

Nicolas L, Milon G, Prina E (2002): Rapid differentiation of Old World *Leishmania* species by LightCycler polymerase chain reaction and melting curve analysis. *J. Microbiol. Methods* **51**, 295–299.

Salih O, Omar AR, Ali AM, Yusoff K (2000): Nucleotide sequence analysis of F protein gene of a Malaysian velogenic NDV strain AF2240. *J. Mol. Biol. Biochem. Biophys.* **4**, 51–57.

Tan SW, Aini I, Omar AR, Yusoff K, Tan WS (2001): Characterisation of Newcastle disease virus (NDV) isolated from outbreaks of NDV in commercial chickens in Peninsular Malaysia. *Proceedings of the 2nd International Congress/13th VAM Congress and CVA-Australasia/Oceania Regional Symposium*, Kuala Lumpur, Malaysia, pp. 84–85.

Van Regenmortel MHV, Fauquet CM, Bishop DHL (2000): *Virus taxonomy. Classification and Nomenclature of Viruses*. The Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego-San Francisco-New York-Boston-London-Sydney-Tokyo.

Watzinger F, Horth E, Lion T (2001): Quantitation of mRNA expression by competitive PCR using non-homologous competitors containing a shifted restriction site. *Nucleic Acids Res.* **29**, 52–55.

Yusoff K, Tan WS (2001): Newcastle disease virus: macromolecules and opportunities. *Avian Pathol.* **30**, 439–455.