[BIO24] Detection of infectious bursal disease virus using SYBR Green 1 based real-time polymerase chain reaction

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Introduction

Infectious bursal disease (IBD) is an acute contagious viral disease of young chickens known as Gumboro disease (Lasher and Shane, 1994). The etiological agent, IBD virus (IBDV), has a predilection for the cells of the bursa of Fabricius where the virus infects lymphocytes of the B-cell lineage (Burkhardt *et al*., 1987). Thus, IBD is a fatal immunosuppressive disease causing heavy losses to the poultry industry.

The current method to detect and differentiate very virulent and vaccine strains of IBDV is by restriction fragment length polymorphism of (RFLP) VP2 gene. However, this method is time consuming, prone to error and less sensitive. The newly developed TaqMan real-time PCR is very sensitive but not suitable as routine test since the test is expensive (Jackwood, *et al.,* 2003). Moreover, application of the assay in detecting very virulent and vaccine strains of IBDV has not been reported. Thus, SYBR Green 1 based real-time PCR assays were developed for the detection and differentiation of very virulent and vaccine strains of IBDV.

Materials and Methods

IBDV isolates

Two field isolates, UPM94/273 and UPM97/61 were used in this study. The viruses were classified as very virulent IBDV (vvIBDV) (Hoque *et al.,* 2001, Kong *et al.,* 2004). A total of five vaccine strains of IBDV; TAD Gumboro (Lohman, Germany), V877 (MVP, Malaysia), Cevax® IBD L (Ceva, France), Delvax Gumboro LZD (Mycofarm, Holland) and IBDVAC (MVP, Malaysia) were also used in the study. These strains were classified as mild to intermediate classical IBDV vaccine strains.

Extraction of viral RNA

Viral RNA was performed with TRI Reagent® (Life Technologies, USA) according to the manufacturer's instruction.

Primers

Two pairs of nested primer were designed based on the conserved region of VP2 gene of IBDV. The external primers (G3 and G4) have been described previously by Lin *et al*. (1993), while the internal primers (G5 and G6) were designed within the flanking region. The internal primers G5 and G6 were labeled at their 5’ ends with biotin and digoxigenin (DIG), respectively. These primer pairs were used to compare the performance of real-time PCR, conventional agarose and ELISA detection methods in detecting IBDV.

For the differentiation of IBDV strains, outer primers FVVC and RVVC were designed from the conserved region of both very virulent and vaccine strains and the inner primer IF was also designed from the conserved region whilst inner primers IVIR and RCLA were designed based on conserved sequences of very virulent and vaccine strains, respectively. The primers IF and IVIR were considered as match primer combination for very virulent strains but as mismatch primer combination for vaccine strains. Meanwhile, primers IF and RCLA were considered as match primer combination for vaccine strains but as mismatch primer combination for very virulent strains.

Nested real-time PCR and RT-nested PCR ELISA

Synthesis of the first strand cDNA was carried out in total of 20 µl using the Promega Reverse Transcription System according to the manufacturer's instructions. The reaction mixture contained 25 ρmol G3 and G4, and 1 µl of 90% dimethyl sulfoxide (DMSO) was incubated at 99°C for 5 min to denature the RNA. The mixture was chilled on ice then mixed with a reaction mixture contained 20 µl

of 10 mM of dNTP mixture, 5.0 U of AMV reverse transcriptase, 20 U of recombinant RNasin ribonuclease inhibitor, 5 mM of $MgCl_2$ and 1x of reaction buffer. The final reaction mixture was incubated at 42°C for 1 hour and then denatured at 99°C for 1 min to inactivate the reverse transcriptase. A total volume of 50 ul of PCR mixture containing 2 mM $MgCl_2$, 1 µl of 10 mM dNTP mixture, 25 pmole of each primer (G3 and G4), 2.5 U of *Taq* DNA polymerase and 0.8x reaction buffer and 5 µl of cDNA. The amplification was performed in MiniCycler™, MJ Research. The protocol was developed as follows: one cycle at 95ºC for 1 min followed by 30 cycles of 94ºC for 1 min, 48ºC for 1 min and 72ºC for 2 mins. The reaction was terminated with a final extension at 72ºC for 5 mins. The second PCR amplification step was carried out in 50 µl volume containing 2 mM $MgCl_2$, 2 µl of 10mM dNTP mixture, 25 ρmole each of the labeled primers (G5 and G6), 2.5 U of *Taq* DNA polymerase, 0.8x reaction buffer and 1 µl of the first round amplification product. The second amplification profiles was setup as follows; 95ºC for 1 min followed by 30 cycles of 94ºC for 30 secs, 53ºC for 20 secs and 72ºC for 45 secs. The last cycle was incubated at 72ºC for 5 mins. The nested products were detected by ELISA reader according to methods previously described by Phong et al. (2003) with some modification.

The first round PCR product was used as template in SYBR Green 1 based real-time PCR detection using the internal primers. A total of 50 µl PCR mixture containing 3 mM $MgCl_2$, 1.0 mM dNTP mixture (Promega, USA), 25 ρmole of each internal primers (G5 and G6), 2.5 U of *Taq* DNA polymerase (Promega, USA), 1 µl of diluted SYBR Green 1 dye (Molecular Probes, USA) and 0.8x reaction buffer and 1.0 µl of the first round PCR product was prepared in low-profile 0.2 ml tube stripes (MJ Research, USA). The amplification was performed in DNA Engine Opticon™ System (MJ Research, USA). No template control (cDNA replaced by distilled water) was used as negative control. The cycle conditions were as follows: 95ºC for 5 mins then followed by 30 cycles of 94ºC for 30 secs, 53ºC for 20 secs and 72ºC for 45 secs.

Evaluation of the performances of nested real-time PCR and RT-nested PCR ELISA

In order to determine the detection limits of the RT nested PCR ELISA and real-time PCR detection methods, the cDNA obtained from UPM94/273 were diluted ten-fold. The undiluted concentration of cDNA was 2.5 ug/µl. Following a PCR reaction, the product was used as template for the nested PCR ELISA and real-time PCR. The performance of the PCR in detecting several different strains of IBDV was also evaluated. The specificity of the primers was assessed against viral RNA extracted from other infectious avian RNA viruses namely Newcastle disease virus (NDV) and infectious bronchitis virus (IBV).

Evaluation of the performances of real-time PCR for differentiation of IBDV strains

PCR reaction and programs were optimized using IBDV strains, UPM94/273 and D78 each represent the very virulent and vaccine strains, respectively. Briefly, a premix reaction containing 8 µl of total RNA, primers FVVC & RVVC, DMSO in a 10 µl volume was incubated at 99°C for 5 mins. The premix reaction was reverse transcribed with the methods recommended by the manufacturer (Promega, USA). The condition of the real-time PCR was optimized with a total volume of 50 µl volume. The mixture containing effective amount of $MgCl_2$, dNTP mixture, Primer IF & IVIR and Primer IF & RCLA, Taq DNA polymerase (Promega, USA), diluted SYBR Green 1 dye (Molecular probe, Eugene, USA) in deionised distilled water, 0.8x reaction buffer and cDNA template (undiluted to 1:10^5 dilution) in low-profile 0.2 ml tube stripes (MJ Research, USA). PCR was performed with the established protocol and conditions.

Melting curve analysis

The melting curve analysis was performed by raising the incubation temperature from 72ºC to 99ºC in 0.4ºC increments with a hold of 1 second at each increment. The SYBR Green 1 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).

Agarose gel electrophoresis

During the course of this study, agarose gel electrophoresis was used to verify the PCR and also to compare the sensitivities of the different detection methods. The amplified products were analyzed on agarose gel 1.7% (w/v) electrophoresis in TAE buffer at 60 V for 55 minutes. The gel was then stained with ethidium bromide (0.5ug/ml) and photographed under UV illumination.

DNA sequencing

The expected PCR products (~ 593 bp) generated from primers, FVVC and RVVC were purified by using GENECLEAN (BIO 101, USA) following the manufacturer's instructions. Sequencing was carried out using ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Perkin Elmer) in an automated DNA sequencer (ABI PRISM® 377 DNA Sequencer) following the instructions supplied by the manufacturer. The cycle sequencing was conducted with the following thermal cycle profiles; 30 cycles, each with 96ºC for 10 seconds, 50ºC for 5 seconds, and 60ºC for 4 minutes.

Sequence assembly and analysis

The sequencing data were initially aligned to the known DNA sequences using the basic BLAST (Basic Local Alignment Search Tool) search programme of National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST). The database searches were performed using the FASTA programme and the sequences data were assembled and analyzed using the Bio-Edit package (Version 3.75c) of the Cluster W Multiple alignment (Thompson *et al.,* 1994). The following IBDV strains were used for sequence comparisons; very virulent strains; UPM97/61 (AF247006), UPM94/273 (AF527039), OKYM (D49706), UK661 (X92760), IBDKS (L42284), D6948 (AF240686), BD3/99 (AF362776), Tasik94 (AF322444), Chinju (AF508176), HK46 (AF092943), SH95 (AY134874), Gx (AY444873), SDH1 (AY323952), TO9 (AY099456) and vaccine strains; D78 (AF499929), Cu-1M (AF362771), P2 (X84034), CT (AJ310185), CEF94 (AF194428), PBG-98 (D00868), JD1 (AF321055), HZ-2 (AF321054) and Edgar (AY462026).

Results

Comparison of RT Nested SYBR Green 1 real-time PCR and RT nested CR ELISA

The detections of nested PCR products by different assays were compared. The real-time PCR assay was able to detect up to $1{:}10^7$ (Table 1), which was 100 times more sensitive than RT nested PCR ELISA (Figure 1). Amplification beyond $1{:}10^7$ was nonspecific as proven by the melting temperature (Tm) curve analysis. The Tm from specific amplification was between 87.2°C and 87.6°C whilst nonspecific associated with Tm values ≥ 82.8°C (Table 1).

Specificity study

The developed methods were found to be specific to IBDV. No bands were detected on agarose gel from the other RNA virus (NDV and IBV) (Table 2)

Strain-specific primer pairs

Based on sequencing analysis, the primer IVIR was conserved to the known DNA sequences search from NCBI: (UPM97/61, UPM94/273, OKYM, UK661, IBDKS, D6948, BD3/99, Tasik94, Chinju, HK46, SH95, Gx, SDH and TO9) (data not shown) whilst the primer RCLA was conserved to the following vaccine IBDV strains ; (D78, Cu-1M, P2, CT, CEF94, PBG-98, JD1 and HZ-2, Edgar) The primers IVIR and RCLA have 3 nucleotide differences.

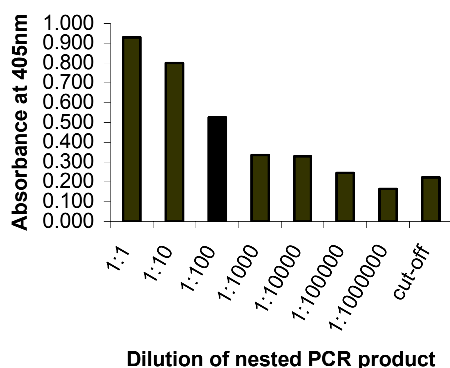


FIGURE 1 The detection limit of RT-nested PCR ELISA. The detection can be seen up to $1:10^5$ of the nested PCR product. The color development was read at the 405 nm of the OD by the ELISA reader. The detection value was positive if they were above the cut-off value reading, 0.221.

TABLE 1 Threshold cycle (C_T) and melting temperature (Tm) values of amplification of first round PCR product from serially diluted cDNA of vvIBDV UPM94/273. Data were collected from amplification curve and Tm analysis curve (not shown).

cDNA concentration (ug/ul)	C_T value	Tm value
2.50	8.396	87.6
$2.5 \times 1:10^1$	6.846	87.6
$2.5 \times 1:10^2$	7.477	87.2
$2.5 \times 1:10^3$	7.776	87.2
$2.5 \times 1:10^4$	9.993	87.2
$2.5 \times 1:10^5$	13.727	87.2
$2.5 \times 1:10^6$	18.103	87.2
$2.5 \times 1:10^7$	18.199	87.2
$2.5 \times 1:10^8$	18.814	82.8
$2.5 \times 1:10^9$	19.493	82.8
$2.5 \times 1:10^{10}$	19.057	82.8

TABLE 2 Threshold cycle (C_T) and melting temperature (Tm) values of amplification of different isolates of IBDV and other RNA viruses. No C_T values were detected from negative control and samples from IBV and NDV. Data were collected from amplification curve and Tm analysis curve (not shown).

Virus strains/Isolates	C_T value	Mean Tm
TAD Gumboro	3.043	87.2
V877	4.246	87.6
IBD VAC	3.083	86.8
IBD L	3.887	86.8
Delvax LZD	3.349	86.4
UPM97/61	2.244	86.4
UPM94/273	4.253	87.2
IBD	None	78.0
NDV	None	79.2
Negative control	None	78.4

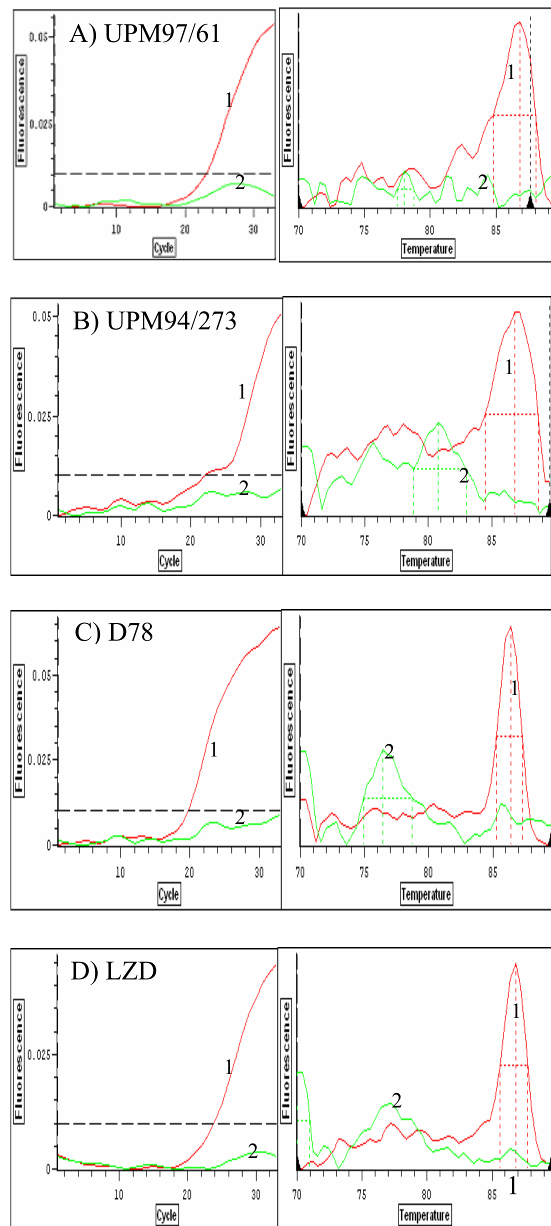
Evaluation of the real-time PCR

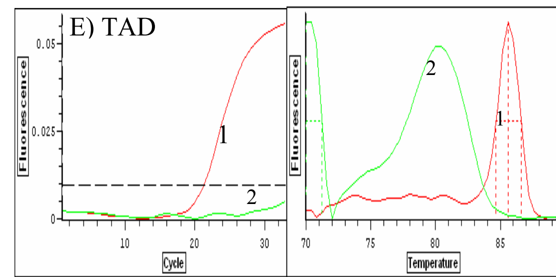
The real-time PCR was performed using fixed amount of cDNA and both the match and mismatch primer combinations The amplification profiles of the real-time PCR assay for the amplification of IBDV strains UPM97/61, UPM94/273, D78, LZD, TAD and IBDVAC were shown in Figure 2A, 2B, 2C, 2D, 2E and 2F, respectively. Regardless of the IBDV isolates, the specific amplification was detected only from match primer combination with the C_T value of the amplified products ranged from 19 to 28 and the Tm of the amplified products ranged from 86^oC to 88^oC for both cDNA obtained from very virulent and vaccine strains (Table 3). The very virulent strains, UPM94/273 and UPM97/61 were amplified only with the match primer (primer IF & IVIR) whilst the vaccine strains, D78, LZD, TAD and IBDVAC were amplified only with match primer (primer IF & RCLA). No amplification with C_T value 0 was detected for amplification of the IBDV using mismatch primer combinations.

Specific amplification of PCR product of the expected size (316 bp) was detected only from match primer but not mismatch primer combinations (data not shown).

Sequence analysis of the PCR amplified product

A total of 316 bp sequences encompassing the amplified product of UPM94/273, UPM97/61, D78, LZD, TAD and IBDVAC were also characterized (data not shown). The sequence analysis proved the amplified PCR product.





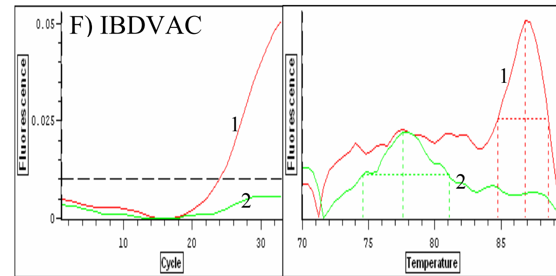


FIGURE 2 The performance of the real-time in detecting specific amplification of vvIBDV (UPM97/61 and UPM94/273) and attenuated vaccine (D78, LZD, TAD and IBDVAC) strains. Specific amplification was detected only from strain-specific primer combination (red line {1}) while no amplification from nonspecific primer combination (green line{2}). Regardless of strain of IBDV, amplification of the expected PCR product associated with C_T values ranged from 19 to 28 (left). The Tm of the amplified products ranged from 86 to 88°C for both cDNA obtained from very virulent and vaccine strains (right).

Discussion

The real-time PCR was both specific and sensitive to the targeted gene. The nested primer used in the real-time PCR was designed based on the conserved region of VP2 that amplified the hypervariable region of VP2 gene. Hence, the developed real-time PCR is applicable as a universal detection method for all IBDV strains. The nested SYBR Green 1 real-time PCR was 100 times more sensitive compared to the RT nested PCR ELISA, where the detection limit was up to $1{:}10^7$ dilution which was equivalent to 2.5×10^{-7} µg/µl or 0.25 ρg/µl of cDNA (Table 1).

In this study, the very virulent strains showed Tm ranging from 86.4˚C to 87.2˚C, whilst the vaccines stains with Tm ranging from 86.4˚C to 87.6˚C (Table 2). Hence, the variations at the Tm values were too small to differentiate the different strains of IBDV. Therefore, further improvements of the real-time PCR assay for the differentiation of IBDV strains were developed.

TABLE 3 Detection of signatory threshold cycle (C_T) values signatory melting temperature (Tm) values from very virulent and vaccine strains IBDV using different primer combinations. Data were pooled together from different isolates had been tested (figure 2) but with the same real-time PCR assay.

Isolates	**Strains**	**Threshold cycle (Ct) value**		**Melting temperature (Tm) values (^oC)**	
		Primer IF & IVIR	**Primer IF & RCLA**	**Primer IF & IVIR**	**Primer IF & RCLA**
UPM97/61	very virulent	19 to 28	> 29 or 0	86 to 88	< 80
UPM94/273	very virulent	19 to 28	> 29 or 0	86 to 88	< 80
D78	vaccine	> 29 or 0	19 to 28	< 80	86 to 88
TAD Gumboro	vaccine	> 29 or 0	19 to 28	< 80	86 to 88
Delvax Gumboro LZD	vaccine	> 29 or 0	19 to 28	< 80	86 to 88
IBDVAC	vaccine	> 29 or 0	19 to 28	< 80	86 to 88

The present study described for the first time development of a SYBR Green 1 based real-time PCR method for the detection of very virulent and vaccine strains of IBDV based on detection of signatory C_T and Tm values. This study relied on the use of novel primer combinations, whereby the match and mismatch primer combinations gave a certain PCR amplification characteristics in an optimized PCR condition. In the optimum conditions, real-time PCR assay gave consistent results in detecting very virulent and vaccine strains of IBDV. By using Primer IF & IVIR and Primer IF & RCLA, a RT product from very virulent IBDV strains has an early amplification (C_T value between 19 to 28 and Tm between 86.0 to 88.0^oC) and late specific amplification (C_T value > 29 and Tm < 82^oC) or no specific amplification (C_T value 0 and Tm < 82^oC), respectively (Figure 2 and Table 3). Meanwhile, by using Primer IF & RCLA and Primer IF & IVIR, a RT product from vaccine strain of IBDV has an early amplification (C_T value between 19 to 28 and Tm between 86.0 to 88.0^oC) and late specific amplification (C_T value >29 and Tm < 82.0^oC) or no specific amplification (C_T value 0 and Tm < 82.0^oC), respectively (Figure 2 and Table 3).

The RT-PCR assay followed by RFLP has been used to detect and differentiate IBDV strains (Hoque *et al.,* 2001). In other recent studies by Jackwood and Sommer (2002) and Jackwood *et al.* (2003), hybridization probe based real-time PCR was shown to be able to detect vaccine and wild type IBDV strains in infected chickens. Although, these method was able to differentiate different IBDV strains, they are not preferred to be tested on large number of clinical samples. The present study, offers an alternative format assay in detecting and differentiate of very virulent or vaccine IBDV strains. In addition, the developed technique is more efficient and accurate since the detection is based on the signatory C_T value and specific amplification was verified by Tm analysis. Therefore, the established SYBR Green 1 based real-time PCR assay has the potential to be commercialized as routine kit for detection and differentiation of IBDV strains. The established method is specific, simple, rapid and less expensive compared to the current available methods for detection and differentiation of IBDV strains.

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