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(54) **Method for detecting and distinguishing infectious bursal disease virus (IBDV) strains by fluorescence-based real time RT-PCR**

Verfahren zur Erkennung und Unterscheidung der Infektiöse-Bursitis-Virusstämme durch Fluoreszenz-basiertes real-time-RT-PCR

Méthode pour la détection et la caractérisation de souches du virus de la bursite infectieuse (VBI) au moyen d' une réaction RT-PCR en temps réel fondée sur la fluorescence

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Description

FIELD OF INVENTION

[0001] This invention relates to the detection and distinguishing Infectious Bursal Disease Virus (IBDV) strains by a Molecular Biology Method in chicken or other birds. More particularly, this invention relates to distinguishing different Infectious Bursal Disease Virus (IBDV) strains in chicken and other bird sample by Real-time Polymerase Chain Reaction (PCR) method.

BACKGROUND OF THE INVENTION

[0002] Infectious bursal disease (IBD) is an acute contagious viral disease of young chickens often known as Gumboro disease (Kibenge *et al*., 1988; Lasher *et al*., 1994). The etiological agent, IBD virus (IBDV), has a predilection for the cells of the bursa of Fabricius where the virus infects actively dividing and differentiating lymphocytes of the B-cell lineage (Burkhardt *et al*., 1987). Thus, IBD is a fatal immunosuppressive disease causing heavy losses to the poultry industry (Eterradossi *et al*., 1998).

[0003] The first outbreak of IBDV was reported in commercial chicken flocks in Delaware, USA (Cosgrove, 1962). The IBDV strains, which were isolated during the outbreak, now referred to as classical serotype I isolates. The disease was also first report in Europe in 1962 (Faragher, 1972). And from 1966 to 1974, IBD was reported in the Middle East, Southern and Western Africa, India, the Far East and Australia (Faragher, 1972; Firth 1974; Jones 1986; van den Berg, 2000). In most cases, the IBDV strains that associated with the outbreaks were of low virulence and caused only 1 to 2 % of specific mortality (van den Berg, 2000).

[0004] However, a new IBDV strain (antigenic variant) emerged and able to cause up to 5% specific mortality in USA (Rosenberger and Cloud, 1986). The antigenic variant was recovered from flocks with selection pressure of field vaccination against classical IBDV serotype I (Snyder, 1990). Although being antigenic variant these isolates have only minor amino acid changes and do not form a separate serotype.

[0005] Nevertheless, these changes occur at the VP2 conformation-dependent antigenic epitopes that are responsible for stimulating virus neutralizing antibodies (Bayliss *et al*., 1990). Currently, variant form of IBD has been reported outside Central America particularly in countries such as China (Cao *et al*., 1998), South America (Banda *et al*., 2003) and Australia (Sapats and Ignjatovic, 2000).

[0006] Since variant IBDV causes only changes at the bursa and depending on the immune status of the chickens, the disease is often manifested with subclinical signs, it is difficult to detect variant IBDV in commercial flocks. Hence, variant IBDV may be common in many countries in the world but remains undiagnosed. A second serotype - serotype II of IBDV was identified in 1987 (McNulty and Saif, 1988). Serotype II IBDV isolates are apathogenic and are recovered mainly from turkeys (Ismail *et al*., 1988).

[0007] In the 1990s, IBDV isolates, which were able to break through levels of maternal antibodies that normally were protective, were reported in Europe (Chettle *et al*., 1989). These isolates, the so called very virulent IBDV are causing more severe clinical signs during an outbreak which mortality approaching 100% in susceptible flocks, and are now found almost world-wide (van den Berg, 2000). The emergence of very virulent strains of IBDV has complicated the immunization programs against the disease.

[0008] Early vaccination may result in failure due to interference with the maternal antibody, whilst its delay may cause field virus infections. Currently, outbreaks of vvIBDV have been reported throughout various countries in the world (Branda *et al*., 2003; Cao *et al*., 1998; Chai *et al*., 2001; Chettle *et al*., 1989; Eterradossi *et al*., 1992; Hoque *et al*., 2001; Liu *et al*., 2002; Majo *et al*., 2002; Rudd *et al*., 2003; Scherbakova *et al*., 1998; Ture, *et al*., 1998; Zorman-Rojs *et al*., 2003).

[0009] In designing an effective disease control program one should consider the diagnostic methods use to diagnose disease caused by infectious agent. Currently, IBD can be diagnosed based on virus isolation, electron microscopy, immunofluorescence, virus neutralization, monoclonal antibody assays, and/or enzyme-linked immunosorbent assay (Jackwood *et al*., 1996; Liu *et al*., 1994; Lukert and Saif, 1997; Wu *et al*., 1992). However, these methods have one or more disadvantages such as time consuming, labour intensive, expensive and of low sensitivity (Wu *et al*., 1992).

[0010] Recently, the reverse transcriptase polymerase chain (RT-PCR) has been used to detect IBDV based on the amplification of the central hypervariable region of the VP2 region (Tham *et al*., 1995; Jackwood and Nielsen, 1997). Subsequently, RT-PCR assay followed by restriction fragment length polymorphism (RFLP) also has been used to detect and differentiate IBDV strains (Jackwood and Sommer, 1997; 1999; Hoque *et al*., 2001; Ture *et al*., 1998; Zierenberg *et al*., 2001).

[0011] Although, this method able to differentiate different IBDV strains, it is not automated and time consuming. Both radioactive and non-radioactive based nucleic acid probes that can differentiate IBDV strains have been used in the detection of IBDV (Akin *et al*., 1993; Davis and Boyle, 1990). However, apart for academic interest, their use in diagnosing IBD is uncommon.

[0012] Fluorescence-based real-time PCR assays have been developed to provide a rapid and sensitive method for quantifying nucleic acids (Gibson *et al.*, 1996; Heid *et al.*, 1996; Desjardin *et al.*, 1998). In this assay, reactions are monitored by the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. There are currently 2 general approaches in real-time PCR depending on the types of fluorescence dyes. The simplest method uses fluorescent dye, SYBR Green I that bind specifically to double stranded DNA (Morrison *et al.*, 1998). The major problem with SYBR Green I-based detection is that non-specific amplifications cannot be distinguished from specific amplifications. However, specific amplification can be verified by melting curve analysis (Ririe *et al.*, 1997).

[0013] The other dyes (TaqMan, Molecular Beacons, Scorpion) rely on the hybridization of fluorescence labeled probes to the correct amplicon (Wittwer *et al.*, 1997; Bonnet *et al.*, 1999). Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye. The threshold cycle (Ct) is defined as the fractional cycle number at which the reporter fluorescence generated by the accumulating amplicons passes a fixed threshold above baseline (Mackay *et al.* 2002).

[0014] Hence, a plot of the log of initial target copy number for a set of standards versus Ct is a straight line whereby the higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed (Higuchi *et al.*, 1993; Gibson *et al.*, 1996; Heid *et al.*, 1996; Desjardin *et al.*, 1998). It has also been established that primers combination playing an important role for the Ct value prediction, where the approach is similar to the analysis of single-nucleotide polymorphism (Frederique *et al.*, 2003; Christy *et al.*, 2002; Srinivas *et al.*, 2000). Thus, several recent studies have used SYBR Green I based real-time PCR to differentiate different serotypes or strains of organisms based on Ct and/or Tm values (Aldea *et al.*, 2003; Beuret, 2004; Nicolas *et al.*, 2002; Shu *et al.*, 2003). A quantitative real-time PCR assay based on TaqMan has been developed to detect IBDV (Moody *et al.*, 2000). In other recent studies by Jackwood and Sommer (2002) and Jackwood *et al.* (2003a), TaqMan based real-time PCR was shown to be able to detect vaccine and wild type IBDV strains in infected chickens. However, the assay is expensive and more complex compared to the method established in this study. In addition, the application of the method to differentiate very virulent and vaccine strains IBDV is not known.

[0015] Comparative analysis of viral RNA and apoptotic cells in bursae following infection with IBDV has been carried out using a SYBR™ Green based real-time reverse transcriptase reaction assay; the analysis is described in Kong et al. Comparative immunology, Microbiology & Infectious Diseases 27 (2004) 433-443. The findings indicate that the very virulent strain UPM94/273 resulted in lower pathogenicity compared to UPM97/61 and that UPM97/61 and UPM 94/273 have different efficiency of replication and percentage of apoptotic cells in bursae during the acute phase of IBDV infection. The present invention seeks to provide alternative methods for distinguishing between IBDV strains.

SUMMARY OF INVENTION

[0016] According to one embodiment of the invention there is provided a method of distinguishing between Infections Bursal Disease Virus (IBDV) as defined in claim 1.

[0017] Advantageously, the method further comprises the features as set out in dependent claims 2 to 21.

[0018] According to a further embodiment of the invention a PCR primer pair is provided as set out in claim 22.

[0019] Preferably, the PCR primer pair is for use as defined in claim 23.

[0020] In accordance with a still further embodiment of the invention, there is provided a diagnostic test kit as defined in claim 24.

[0021] The biological material, which is to be investigated, was obtained in some suitable manner and isolated. By means of standardized methods, RNA was isolated from the material. A defined amount of RNA was transcribed into cDNA by means of conserved primers in RT reaction. Subsequently, a defined amount of cDNA was used to generate specific products using PCR.

[0022] One of the most common dyes that bind to double-stranded DNA that is commonly used in real-time PCR is SYBR Green I. In real-time PCR, measurements are detected during the exponential phase of the reaction typically by obtaining the threshold cycle (Ct) value. Ct value can be defined as the fractional cycle number at which there is a significant increase in fluorescence above a specified threshold.

[0023] The Ct value is also proportional to the numbers of target copies present in the samples. In SYBR Green I based real-time PCR the specificity of the amplification is determined by measuring the melting temperature (Tm) of the product in melting curve analysis. Different IBDV strains can be differentiated based on the Ct values by using different primer combinations and the detection of expected Tm confirm the specificity of the amplification.

[0024] The PCR conditions are optimized in order to obtain effective PCR parameters on the ingredients and profiles using samples containing IBDV RNA in a SYBR Green I based real-time PCR. Hence, for differentiation of very virulent from vaccine strains of IBDV by using Primer IF & IVIR and Primer IF & RCLA, a PCR product from very virulent strain IBDV has an early amplification (Ct value between 19 to 28 and Tm between 86 to 88°C) and late amplification (Ct value > 29 and Tm < 82°C) or no amplification (Ct value 0 and Tm < 82°C), respectively.

[0025] Meanwhile, differentiation of vaccine from very virulent strains of IBDV by using Primer IF & IVIR and Primer IF & RCLA, the PCR product from vaccine strain of IBDV has an early amplification (Ct value between 19 to 28 and Tm between 86°C to 88°C) and late amplification (Ct value > 29 and Tm < 82°C) or no amplification (Ct value 0 and Tm < 82°C), respectively.

[0026] It is an objective of the invention to provide a new method for differentiating IBDV strains and for identifying IBDV strains based on the detection of signatory Ct and Tm values.

DETAILED DESCRIPTION OF THE INVENTION

[0027] The field of the invention is detection of IBDV in homogenate tissue samples using different primer combinations. Total RNA from test sample is reverse transcribed using a pair of conserved primer. The RT product is amplified using different primer combinations where by, different IBDV strains can be differentiated based the detection of Ct and Tm values. Based on these principals, a pair of primer, primers FVVC & RVVC that hybridized to the conserved region are designed and used in RT reaction.

[0028] The RT products are then used as template in cDNA amplification using 2 pairs of primers whereby only the sequences of the reverse primers are different depending on the strains of IBDV; primers IF & IVIR and primers IF & RCLA each are specific to very virulent and vaccine strains of IBDV, respectively. Since the concentration of cDNA influence the specificity of the assay, the optimum of cDNA concentration is optimized. In addition, other parameters including concentration of total RNA, primers, MgCl2, SYBR Green I, PCR profiles and standardization of the fluorescence threshold level of the real-time PCR machine were also optimized.

[0029] Hence, for differentiation of very virulent from vaccine strains of IBDV primer IF & IVIR and primers IF & RCLA are used. A PCR amplification product from very virulent strain IBDV has an early amplification (Ct value between 19 to 28 and Tm between 86 to 88°C) and late amplification (Ct value > 29 and Tm < 82°C) or no amplification (Ct value 0 and Tm < 82°C), respectively. This embodiment of the invention describes the development of a novel SYBR Green I based real-time PCR method for the detection of very virulent and vaccine strains of IBDV based on detection of signatory Ct and Tm values.

[0030] Thus, this invention also includes novel real-time PCR-based assays which do not require size determination of the PCR amplification product to confirm the specific amplification of the IBDV target nucleic acid sequence. Therefore, the invention includes simple format assays which obviate the need for complex molecular biology techniques such as restriction enzyme digestion and sequencing to confirm that the amplification product is, indeed, of IBDV strains of very virulent or vaccine strains. The methods of the invention are, therefore, less prone to operator error, faster, and may be fully automated. In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below.

[0031] As used herein, "nucleic acid," RNA", cDNA and similar terms also include nucleic acid analogs, i.e. analogs having other than a phosphodiester backbone. For example, the so-called "peptide nucleic acids," which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention.

[0032] The term "very virulent" strain of IBDV means samples containing IBDV that associated with high mortality in chickens and with the following characteristic amino acid residues at the positions 222 (alanine), 242 (isoleucine), 256 (isoleucine) and 294 (isoleucine) of the VP2 region, at the positions 680 (tyrosine), 685 (asparagine), 715 (serine) and 751 (aspartate) of the VP4 region and at the positions 990 (valine) and 1005 (alanine) of the VP3 region. Typically, a very virulent IBDV usually associated with mortality up to 100% in SPF chickens, 25% mortality in broilers and 60% in layers.

[0033] The term "vaccine" strain of IBDV means samples containing IBDV that do not cause mortality in non-vaccinated commercial chickens and with the following characteristic amino acid residues at VP2 region ; 253 (histidine), 279 (asparagine), and 284 (threonine) and associated with one or more changes at the serine residues of the heptapeptide region SWSASGS at the position 326 to 332. A vaccine strain also has characteristic amino acid residues at the positions 680 (cysteine), 685 (lysine), 715 (proline) and 751 (histidine) of the VP4 region and at the positions 990 (alanine) and 1005 (threonine) of the VP3 region. Typically, a vaccine strain is an attenuated classical IBDV derived from repeated passages in embryonated eggs and/or cell cultures.

[0034] As used herein, "effective amount" means an amount sufficient to produce a selected effect. For example, an effective amount of cDNA is an amount sufficient to amplify a segment of nucleic acid by PCR provided that a DNA polymerase, buffer, template, and other conditions, including temperature conditions, known in the art to be necessary for practicing PCR are also provided.

[0035] The term "test sample" as used herein, means anything suspected of containing a target sequence. The test sample can be derived from any biological source and can be used (i) directly as obtained from the source or (ii) following a pre-treatment to modify the character of the sample. The pre-treatment that can be applied for example, disrupting cells, preparing liquids from solid materials, diluting viscous fluids, filtering liquids, concentrating liquids, inactivating

interfering components, adding reagents, purifying nucleic acids, and the like. Typically, the test sample will be derived from bursal tissue samples.

[0036] A "target sequence" as used herein means a nucleic acid sequence that is detected, amplified, or otherwise is complementary to one of the primers herein provided.

[0037] A "match primer" as used herein means the nucleotide sequence of primer with at least the last 3 nucleotide sequences at the 3'end conserved to the template sequences. Typically, a match primer will has a conserved nucleotide sequences with template sequences.

[0038] A "mismatch primer" as used herein means the nucleotide sequence of primer with at least the last 1 nucleotide sequence at the 3'end differ to the template. Typically, a mismatch primer will has 1 to 3 nucleotide variation at the 3'end with the template sequences.

[0039] The term "bp" means base pair.

[0040] The term "signatory Ct and Tm values" means consistent readings that give selected or desirable effect; to distinguish different strains of IBDV, i.e., very virulent from vaccine strain. Typically, a match primer and template combination will give an early amplification (Ct value between 19 to 28 and Tm between 86 to 88°C) and late amplification (Ct value > 29 and Tm < 82°C) or no amplification (Ct value 0 and Tm < 82°C). A mismatch primer and template combination will give late amplification (Ct value > 29 and Tm < 82°C) or no amplification (Ct value 0 and Tm < 82°C).

DETAILED EXAMPLE OF APPLICATION

Isolation of very virulent and vaccine strains

[0041] A total of 6 IBDV which include 2 field isolates of very virulent strains (UPM97/61 and UPM94/273) and 4 vaccine strains (Table 1) were used. The very virulent viruses were in the form of bursal homogenate whilst the vaccine strains were distilled in 8 to 10 ml of deionised water and used for RNA extraction.

Table 1: Infectious bursal disease viruses used in the study

Isolates	Strains
UPM97/61	very virulent
UPM94/273	very virulent
D78	vaccine
TAD Gumboro	vaccine
Delvax Gumboro LZD	vaccine
IBDVAC	vaccine

Primer Design

[0042] Five different primers were used in this study (Table 2). All the primers were designed with the aid of Primer Premier 5.0 software. The primers were designed based on the following criteria for real-time PCR; primers should be designed to amplify short amplicons. Minimum length of the amplicons should be 80 bp and not exceed 400 bp. Ideally, primers should have about 50 % of G/C content. The nucleotide difference should be at the 3'end region of the primers and the region should have no more than two G and/or C bases.

Table 2: Primers used for amplification of different strains of IBDV. The nucleotide variations between the primers are indicated in bold.

Primer	Sequence (5'-3')	Positions[a]	SEQ ID NO
FVVC[d]	AGA GGG TGC CAC GCT ATT	1662-1679	SEQ ID NO; 1
RVVC[d]	GGT ACT GGC GTC CTG CAT T	2255-2237	SEQ ID NO; 2
IF[d]	ATG CTC CAG ATG GGG TAC TTC	1835-1855	SEQ ID NO; 3
IVIR[b]	TTG GAC CCG GTG TTC ACG	2150-2133	SEQ ID NO; 4

(continued)

Primer	Sequence (5'-3')	Positions[a]	SEQ ID NO
RCLA[c]	TTG GGC CCG GTG TTT ACA	2150-2133	SEQ ID NO; 5

[a]The numbering of the nucleotide position based on Bayliss *et al.* (1990).

[b] The sequences of the primer was conserved when compared to 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 (AF13474), Gx (AY 444873), SDH1 (AY323952) and TO9 (AY099456).

[c]The sequences of the primer was conserved when compared to vaccine (attenuated) strains, D78 (AF499929), Cu-1M (AF362771), P2 (X84034), CT (AJ310185), CEF94 (AF194428), PBG-98 (D00868), JD1 (AF321055), HZ-2 (AF321054) and Edgar (AF462026).

[d]The sequences of the primers were conserved when compared to both very virulent and attenuated vaccine strains of IBDV as stated above.

[0043] The primers FVVC and RVVC were designed from the conserved region of VP4 of both very virulent and vaccine strains to generate a 593 bp product. Similar to primers FVVC & RVVC, primer IF was also designed from the conserved region of VP4 of both very virulent and attenuated vaccine strains whilst primers IVIR and RCLA were designed based on conserved sequences of very virulent and attenuated vaccine strains, respectively, and expected to amplify a 316 bp product.

[0044] Primers IVIR and RCLA differed by 3 nucleotide differences at positions 2133, 2136 and 2146 (Table 2). The relationship between the primer combinations and IBDV isolates as template is indicated in Table 3. 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.

Table 3: Primer combinations and their relationship to template (IBDV isolates) used in real-time PCR.

Isolates	Origin	Strains	Primer combinations[a]	Relationship between primer combinations to IBDV
UPM97/61	UPM	very virulent	IF & IVIR	Match
			IF & RCLA	Mismatch
UPM94/273	UPM	very virulent	IF & IVIR	Match
			IF & RCLA	Mismatch
D78	Intervet	vaccine	IF & IVIR	Mismatch
			IF & RCLA	Match
TAD Gumboro	Lohman	vaccine	IF & IVIR	Mismatch
			IF & RCLA	Match
Delvax Gumboro LZD	Mycofarm	vaccine	IF & IVIR	Mismatch
			IF & RCLA	Match
IBDVAC	Veterinary Research Institute	vaccine	IF & IVIR	Mismatch
			IF & RCLA	Match

[a] The forward primer (IF) is conserved whilst the reverse primers (IVIR and RCLA) varies depending on the strains of IBDV.

[0045] As shown in Figure 1, the nucleotide difference at position 2146 associated with an amino acid substitution at position 715.

[0046] RNA Extraction. Total RNA was extracted from the prepared biological material using Tri Reagent® (Life Technologies, USA) following the method described by the manufacturer.

Reverse Transcriptase

[0047] The extracted RNA was transcribed into cDNA using primers FVVC and RVVC. In this study, two tubes format of real-time PCR was carried out. The optimum conditions of the 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 5,000 ng/μl to 13,000 ng/μl of total RNA, 25 pmole of primers FVVC & RVVC, 1 μl of DMSO (v/v) in a 10 μl volume was incubated at 99°C for 5 mins. The premix reaction was reverse transcribed at 42°C for 1 hour in a final volume of 20 μl containing 2x of reaction buffer (Promega, USA), 2 μl of 10mM dNTP mixture, 5.0 U of AMV reverse transcriptase, 20 U of recombinant Rnasin ribonuclease inhibitor. The reaction was then denatured at 99°C for 1 min to inactivate the reverse transcriptase. The cDNA was then chilled on ice for 5 mins and was used immediately or stored at -80°C.

Determination of the RNA and cDNA Concentration and Purity

[0048] The concentration and purity of the extracted total RNA and cDNA were measured at the wavelength of 260 nm and 280 nm using a spectrophotometer. Optimization of the real-time PCR

[0049] The condition of the real-time PCR was optimized by using different concentration of cDNA and SYBR Green I. Briefly, cDNA (undiluted to $1:10^5$ dilution) was used as template was made in 50 μl volume with the following ingredients; 3.0 mM $MgCl_2$, 2 μl of 10mM dNTP mixture, 25 pmole/μl of each primer (Primer IF & IVIR and Primer IF & RCLA), 2.5 U of Taq DNA polymerase (Promega, USA), 1 μl of SYBR Green 1 dye diluted $1:10^3$ (Molecular Probe, Eugene, USA) in deionised distilled water (Molecular Probes, USA), 0.8x reaction buffer and cDNA template in low-profile 0.2 ml tube stripes (MJ Research, USA).

[0050] The PCR reactions and conditions that amplified very virulent and vaccine strain, UPM94/273 and D78, respectively, was optimized. It was found that the optimum concentration of SYBR Green I as labeling dye was 1 μl of $1:10^3$ diluted stocks. Real-time PCR profiles obtained from 2μl of $1:10^4$ diluted SYBR Green I stock is not consistent (data not shown).

[0051] The amplification was performed in DNA Engine Opticon™ System (MJ Research, USA). No template control and tissue samples (bursa, thymus and ceacal tonsil) of uninfected SPF-chickens were used as negative control. PCR was performed with these conditions; 95°C for 5 min then followed by 33 cycles of 94°C for 30 sec, 60°C for 20 sec and 72°C for 40 sec then allowed the reaction to be incubated 82°C to 85°C before the fluorescence reading was taken. The fluorescence threshold limit of the DNA Engine Opticon™ System was set at 0.01.

[0052] A real-time PCR assay is also optimized by the number of cycle. It was found that amplification of mismatch PCR product occurred after 33 cycles. As shown in Figure 2, the melting temperature (Tm) of the mismatch product was 86°C to 87°C whilst the Tm for the dimer product was 77°C.

[0053] Based on agarose gel electrophoresis (Figure 2), a faint band of the expected size, 316 bp was observed with the formation of nonspecific dimer product. Lane M, 100 bp marker (Promega, USA), Lane 2, 3, 4 are triplicate of D78 with primer IF and IVIR.

Melting Curve Analysis

[0054] Upon completion of the amplification, the specificity of the amplified product was confirmed by melting curve analysis whereby the reaction was incubated by raising the incubation temperature from 72°C to 99°C in 0.4°C increments with a hold of 1 second 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 the fluorescence over temperature versus the temperature (-dF/dT versus T). Since SYBR Green I dye binds to any dsDNA product, specificity and the absence of non-specific amplification were determined by determining the Tm of the non-specific product.

Development of the real-time PCR to detect very virulent and vaccine strains of IBDV

[0055] After establishing the optimum condition of the real-time PCR, the assay was performed on very virulent and attenuated vaccine strains, UPM94/273 and D78, respectively. The cDNA obtained from both strains were serially diluted and used as template in PCR using both match and mismatch primer combinations. Figure 3 comprising figures 3A, 3B, 3C, 3D and 3E depicting the amplification from 10-fold dilutions from 10^0 to 10^{-5} of cDNA template of very virulent strain, UPM94/273 using match primer (IF & IVIR) and mismatch (IF & RCLA) primer combinations.

[0056] The RT product was generated from 12,000 ng/μl of total RNA. Amplification of specific PCR product using match primer was detected from undiluted (7,700 ng/μl) until 10^{-3} diluted (7.7 ng/μl) cDNA concentrations (Figure 3A). However, amplification using mismatch primer was detected only from undiluted cDNA (Figure 3C). The specificity of the amplification was also analyzed using melting curve analysis.

[0057] The melting temperature (Tm) of the amplicons obtained from undiluted cDNA until 10^{-3} diluted cDNA using match primer combinations was between 87.2°C to 87.6°C whilst the Tm for 10^{-4} to 10^{-5} diluted cDNA and negative control ranged from 78.0° C to 78.4°C (Figure 3B). The melting curve analysis also showed that the mismatch primers

produced a product with Tm of 87.6° C from undiluted cDNA whilst the Tm for 10^{-1} until 10^{-5} diluted cDNA and negative control was 81.2°C to 81.6°C (Figure 3D) (Table 4). Hence, the detection of specific amplification based on detection on Ct and Tm analysis was up to 10^{-3} diluted cDNA (7.7 ng/μl).

[0058] The correlation between the concentration of the cDNA and Ct values was analyzed by plotting a standard curve. As shown in Figure 3E, Ct values can only be detected from amplification of undiluted to 10^{-4} diluted cDNA using match primer combination with a linear relationship between the amount of input cDNA and the Ct value from the amplification. The regression equation was Ct = 3.7765 (log 10 dilution) + 16.393 and R^2 = 0.999 (Figure 3E). The Ct and Tm values obtained from amplification of serially diluted cDNA of very virulent IBDV strain UPM94/273 are summarized in Table 4 and 7, respectively.

[0059] The performance of the real-time PCR in detecting vaccine strain IBDV was also analyzed and showed very similar as found for very virulent strain UPM94/273 (Figure 4A to 4E). The RT product was generated from 6,500 to 7,000 ng/μl of total RNA. Amplification of PCR product using match primer was detected from undiluted (6,600 ng/μl) until 10^{-3} diluted (6.6 ng/μl) cDNA concentration (Figure 4A). However, amplification using mismatch primer was detected only from undiluted cDNA (Figure 4C).

[0060] However, the Tm of the amplicons obtained from match primer combinations was between 86.4°C to 86.8°C whilst the Tm for 10^{-4} and 10^{-5} diluted cDNA as well as negative control ranged from 80.4° C to 81.6° C (Figure 4B). The melting curve analysis also showed that the mismatch primers produced a product with Tm of 87.6° C from undiluted cDNA whilst the Tm for 10^{-1} until 10^{-5} diluted cDNA and negative control was 77.2°C to 77.6°C (Figure 4D) (Table 5). As shown in Table 5, Ct values can only be detected from amplification of undiluted to 10^{-4} diluted cDNA using match primers. However, the detection of specific amplification based on detection on Ct and Tm analysis was up to 10^{-3} diluted cDNA (6.6 ng/μl). A standard curve line was generated from amplification of the serially diluted vaccine strain, D78 by using match primer combination. As shown in Figure 4E, a linear standard curve line was also observed between the serially diluted cDNA and the Ct value with the regression equation of Ct = 5.1279 (log10 dilution) + 19.433 and R^2 = 0.9809 (Figure 4E). The Ct and Tm values obtained from amplification of serially diluted cDNA of vaccine IBDV strain D78 are summarized in Table 5 and 8, respectively.

Table 4: Threshold cycle (C_1) and melting temperature (Tm) values of amplification of serially diluted cDNA of vvIBDV UPM94/273 using match and mismatch primer combinations.

cDNA dilution concentration	/ Amplification using different primer combinations[a]			
	Primer IF & IVIR (Match)		Primer IF & RCLA (Mismatch)	
	Ct value	Tm value (°C)	Ct value	Tm value (°C)
undiluted / 7,700 ng/μl	16.40	87.6	19.59	87.6
$1:10^1$ /770 ng/μl	20.41	87.6	0	81.6
$1:10^2$ / 77 ng/μl	24.10	87.6	0	81.6
$1:10^3$ / 7.7 ng/μl	27.45	87.2	0	81.6
$1:10^4$ / 0.77 ng/μl	31.70	78.0	0	81.2
$1:10^5$ / 0.077 ng/μl	0	78.4	0	81.6

[a] The real-time PCR was performed using 1 μl of SYBR Green I (diluted $1:10^3$) as labeling dye and the fluorescence threshold limit set at 0.01.

Table 5: Threshold cycle (Ct) and melting temperature (Tm) values of amplification of serially diluted cDNA of vaccine IBDV D78 using match and mismatch primer combinations.

cDNA dilution concentration	/ Amplification using different primer combinations [a]			
	Primer IF & RCLA (Match)		Primer IF & IVIR (Mismatch)	
	Ct value	Tm value (°C)	Ct value	Tm value (°C)
undiluted / 6,660 ng/μl	19.43	86.8	19.42	87.2
$1:10^1$ / 660 ng/μl	24.27	86.8	0	77.2
$1:10^2$ / 66 ng/μl	27.72	86.8	0	77.2
$1:10^3$ / 6.6 ng/μl	33.34	86.4	0	77.6

(continued)

cDNA dilution concentration	/ Amplification using different primer combinations [a]			
	Primer IF & RCLA (Match)		Primer IF & IVIR (Mismatch)	
	Ct value	Tm value (°C)	Ct value	Tm value (°C)
1:10^4 / 0.66 ng/μl	40.53	81.6	0	77.6
1:10^5 0.066 ng/μl	0	80.4	0	77.2

[a] The real-time PCR was performed using 1 μl of SYBR Green I (diluted 1:10^3) as labeling dye and the fluorescence threshold limit was set at 0.01.

Agarose Gel Electrophoresis Analysis

[0061] Amplification of the real-time PCR products was also verified on 1.7 % agarose gel in TAE buffer. The size of the product was estimated using 100 bp DNA ladder (Promega, USA). The specificity and detection limit of the PCR using different primer combinations in detecting very virulent and vaccine strains IBDV were also confirmed by agarose gel electrophoresis. Specific amplification of PCR product of the expected size 316 bp was observed from 10^0 to 10^{-3} diluted cDNA of UPM94/273 with match primer combination only (Figure 5A). Nonspecific product was observed only from amplification of undiluted cDNA sample. In the case of mismatch primer combination, no amplification was detected except for nonspecific PCR products (~400 base pair and ~600 base pair) were observed from undiluted cDNA sample (Figure 5B).

[0062] Similar results were obtained when the PCR products from D78 were analyzed on 1.7 % agarose gel. A PCR product of the expected size (316 bp) was observed from 10° to 10^{-3} diluted cDNA using match primer combination only (Figure 6A). When the serially diluted cDNA were tested using mismatch primer combinations, nonspecific amplification was detected only from the undiluted cDNA whilst, no amplification was detected from the diluted cDNA samples (Figure 6B).

Evaluation of the real-time PCR

[0063] After establishing the real-time PCR condition and the detection limits for the detection of very virulent and vaccine strains, UPM94/273 and D78, respectively, the assay was also performed on other IBDV isolates as listed in Table 1. The real-time PCR was performed using both match and mismatch primer combinations and the total RNA concentration ranging from 4,500 to 12,800 ng/μl. The real-time PCR was also tested using tissue samples such as bursa, thymus and ceacal tonsil of control uninfected SPF-chickens. As showed in Table 6, no Ct values were generated from amplification of the control negative tissue samples and the Tm values were in the ranged of 72.0°C to 79.6°C.

[0064] However, as expected the match primer combinations amplified the UPM94/273 sample. No specific amplifications were detected from those tissue samples using both match and mismatch primers (Figure 9).However, the cDNA concentration was set at 4000 ng/μl. The amplification profiles of the real-time PCR assay for the amplification of IBDV strains UPM97/61, UPM94/273, D78, Delvax LZD, TAD Gumboro and IBDVAC were shown in Figure 7A, 7B, 7C, 7D, 7E and 7F, 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°C to 88°C for both cDNA obtained from very virulent and vaccine strains.

[0065] The very virulent strains, UPM94/273 and UPM97/61 were amplified only with the match primer (primer IF & IVIR) whilst the vaccine strains, D78, Delvax LZD, TAD Gumboro and IBDVAC were amplified only with match primer (primer IF & RCLA). No amplification with Ct value 0 was detected for amplification of the IBDV using mismatch primer combinations.

[0066] The specific amplification of the vaccine strains, Delvax LZD, TAD Gumboro and IBDVAC using match and mismatch primer combination were also verified using 1.7 % agarose gel. As shown in Figure 8, specific amplification of PCR product of the expected size (316 bp) was detected only from match primer but not mismatch primer combinations.

Table 6: Threshold cycle (Ct) and melting temperature (Tm) values of amplification of negative control samples.

Samples[a]	Primer IF & IVIR		Primer IF & RCLA	
	Ct	Tm(°C)	Ct	Tm (°C)
UPM94/273 (positive control)	19.158	88.0	None	72.0
Bursa	None	78.8	None	72.0

(continued)

Samples[a]	Primer IF & IVIR		Primer IF & RCLA	
	Ct	Tm(°C)	Ct	Tm (°C)
Thymus	None	79.6	None	79.6
Cecal tonsil	None	79.2	None	79.6

[a] The concentration of the undiluted cDNA was 4000 ng/μl.

Table 7: Detection of signatory threshold cycle (Ct) values of very virulent and vaccine strains IBDV using different primer combinations.

Isolates	Strains	Threshold cycle (Ct) value[a]	
		Primer IF & IVIR	Primer IF & RCLA
UPM97/61	very virulent	19 to 28	> 29 or 0
UPM94/273	very virulent	19 to 28	> 29 or 0
D78	vaccine	> 29 or 0	19 to 28
TAD Gumboro	vaccine	> 29 or 0	19 to 28
Delvax Gumboro LZD	vaccine	> 29 or 0	19 to 28
IBDVAC	vaccine	> 29 or 0	19 to 28

[a]The real-time PCR was performed using 4000 ng/μl of cDNA, 1 μl of SYBR Green I (diluted 1:10^3) as labeling dye and the fluorescence threshold limit was set at 0.01.

[0067] The SYBR Green I based real-time PCR detects both very virulent and vaccine strain IBDV as detected based on the Ct and Tm values. However, the detection based on Tm has lower CV value, 0.87 compared to Ct with a CV of 9.58 (Table 9). This suggests that Tm is a consistent parameter in detecting specific amplification. The high CV for Ct values are expected since the absolute quantity of the viral RNA in the samples may be different. It has been showed that the initial copy number of targeted sequences in the template significantly influencing the Ct values (Mackay *et al*., 2002).

Table 8: Detection of signatory melting temperature (Tm) values from very virulent and vaccine strains IBDV using different primer combinations.

Isolates	Strains	Melting temperature (Tm) values (°C)[a]	
		Primer IF & IVIR	Primer IF & RCLA
UPM97/61	very virulent	86 to 88	< 80
UPM94/273	very virulent	86 to 88	< 80
D78	vaccine	< 80	86 to 88
TAD Gumboro	vaccine	< 80	86 to 88
Delvax Gumboro LZD	vaccine	< 80	86 to 88
IBDVAC	vaccine	< 80	86 to 88

[a] The real-time PCR was performed using 4000 ng/μl of cDNA, 1 μl of SYBR Green I (diluted 1:10^3) as labeling dye and the fluorescence threshold limit set at 0.01.

Table 9: Intra-assay variation of Ct and Tm values of real-time PCR using match primer combination in detecting very virulent and vaccine strains of IBDV

Threshold cycle (Ct)				Melting temperature (Tm)			
Interval	Range	Mean ± SD	CV	Interval	Range	Mean ± SD	CV
19.57-25.66	6.09	22.775 ± 2.18	9.58	85.6 - 87.6	2.00	86.8 ± 0.759	0.87

CV = coefficient variations
SD = standard deviations

Purification of PCR Products

[0068] 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. Briefly, the PCR products were cut from the agarose gel by scalpel blade and the weight of the each excided band was measured. Three volumes (three times the weight of the gel) of Sodium Iodine (NaI) was added and incubated at 45°C - 55°C water bath for 5 min until all of the gel dissolved. The glass milk (containing insoluble silica matrix) was vortex vigorously for 1 min. Approximately 5 μl of glass milk was added for each tube (solution containing 5 μg or less DNA) and mixed well, incubated for 5 min at room temperature and mixed every 1-2 min to allow binding of the DNA to the silica matrix.

[0069] The silica matrix with the bound DNA was then pelleted in a microcentifuge for 5 secs at full speed. The pellet was then washed three times with 10-15 volume (approximately 500 μl) New Wash buffer. The pellet was resuspended in the wash by pipetting back and forth while digging into the pellet with the pipet tip and centrifuged at 13000 rpm for 5 sec at 4°C. After the third wash, the last bit of liquid was removed by centrifuged the tube again for a few seconds, the pellet was allowed to dry at RT (5 to 10 min). The DNA was eluted from the glass milk by resuspending the white pellet with 10 μl of sterile dH2O, centrifuged at 13000 rpm for 1 min. The yield of the eluted DNA was estimated by agarose gel electrophoresis.

DNA Sequencing

[0070] The yield of the eluted DNA was estimated by agarose gel electrophoresis. The purified products obtained from PCR amplification from very virulent and vaccine strains were sequenced using primers IF & IVIR and primers IF & RCLA, respectively (Table 2). Each PCR product was sequenced twice from both directions. The sequencing was carried out using ABI PRISM (RTM) BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Perkin Elmer, USA) in an automated DNA sequencer (ABI PRISM (RTM) 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 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes.

Sequence analysis of the PCR amplified product

[0071] As shown in Figure 10, a total of 316 bp sequences encompassing the VP4 gene from position 1835 to 2133 of the PCR products of UPM94/273, UPM97/61, D78, Delvax LZD, TAD Gumboro and IBDVAC were characterized. The nucleotide sequences of primer FVVC, VVC, IF, IVIR and RCLA were also given. The sequences of the match primers were conserved when compared to the respective isolates except for the vaccine strain, IBDVAC. IBDVAC has two nucleotide variations each on primer IF and RCLA. At primer IF, the nucleotide variation is at position 1851 from A to T whilst at primer RCLA, the variation is at position 2141 from C to T (Figure 10).

[0072] References: Bayliss CD et al., J Gen Virol. 1990 Jun;71 (Pt 6):1303-12; PMID: 2161902

Figure 1: Nucleotide sequences and the deduced amino acids translation of primers IVIR and RCLA. The IVIR primer matched to very virulent meanwhile primer RCLA matched to vaccine strains.

[0073] [a] The nucleotide sequences of the primers that associated with amino acid variations are bold.

[0074] [c] The primer conserved to 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 (AF13474), Gx (AY 444873), SDH1 (AY323952) and TO9 (AY099456)

[0075] [d] The primer conserved to vaccine (attenuated) strains, D78 (AF499929), Cu-1M (AF362771), P2 (X84034), CT (AJ310185), CEF94 (AF194428), PBG-98 (D00868), JD1 (AF321055), HZ-2 (AF321054) and Edgar (AF462026).

Figure 2: Influence of number of cycles in real-time PCR assay using template from vaccine strain D78 and mismatch primer combinations.

[0076] Evidence of faint PCR product of the expected size (316 bp) (arrow) and primer-dimer from amplification of D78 cDNA with mismatch primer combinations (IF & IVIR) after 33 cycles. The Tm of the mismatch product was 86 °C to 87 °C whilst the Tm for the dimer product was 77°C. Lane M, 100 bp marker (Promega, USA), Lane 1, 2 and 3 are triplicate of D78 with primers IF & IVVR.

Figure 3: The performance of the real-time in detecting very virulent strain UPM94/273.

[0077] Reverse transcription was generated from 12,000 ng/μl of total RNA. The real-time PCR was performed using 10-fold dilutions from 10° to 10^{-5} (7,700 ng/μl to 0.077 ng/μl) of cDNA template (UPM94/273), match primer combination (primer IF & IVIR), mismatch primer combination (primer IF & RCLA), 1 μl of SYBR Green I (diluted 1:10^3) as labeling dye and fluorescence threshold limit set at 0.01.

[0078] Amplification of PCR product using match primer was detected from undiluted until 10^{-3} diluted cDNA whilst amplification using mismatch primer was detected only from undiluted cDNA (Figure 3A and 3C). With match primer combinations, the Tm of amplicons from undiluted to 10^{-3} diluted cDNA was between 87.2°C to 87.6°C whilst the Tm for 10^{-4} to 10^{-5} diluted cDNA and negative control ranged from 78°C to 78.4°C.

[0079] Melting curve analysis also showed that the mismatch primer combinations produced a product with Tm of 87.6°C from undiluted cDNA whilst the Tm for 10^{-1} until 10^{-5} diluted cDNA and negative control was 81.6° C (Figure 3D). A standard curve derived from 10-fold diluted cDNA of very virulent IBDV strain indicated a linear relationship was observed between the amount of input cDNA and the Ct value. The regression equation was Ct = 3.7765 (log 10 dilution) + 16.393 and R^2 = 0.999 (Figure 3E).

Figure 4: The performance of the real-time in detecting vaccine strain D78.

[0080] Reverse transcription was generated from 6,500 to 7,000 ng/μl of total RNA. The real-time PCR was performed using 10-fold dilutions from 10^0 to 10^{-5} (6,600 ng/μl to 0.066 ng/μl) of cDNA template (D78), match primer combination (primer IF & RCLA), mismatch primer combination (primer IF & IVIR), 1 μl of SYBR Green I (diluted 1:10^3) as labeling dye and fluorescence threshold limit was set at 0.01. Amplification of PCR product using match primer was detected from undiluted until 10^{-3} diluted cDNA whilst amplification using mismatch detected only from undiluted cDNA (Figure 4A and 4C). With match primer combinations, the products obtained from undiluted until 10^{-3} diluted cDNA produced a Tm ranged from 86° C to 88°C.

[0081] The Tm for 10^{-4} to 10^{-5} diluted cDNA and negative control was between 79° C to 81°C (Figure 4B). Melting curve analysis also showed that the mismatch primers produced a product with Tm of 87.6° C from undiluted cDNA whilst the Tm for 10^{-1} until 10^{-5} diluted cDNA and negative control was between 76°C to 78°C (Figure 4D). A standard curve derived from 10-fold diluted cDNA of vaccine IBDV strain showed a linear relationship was observed between the amount of input cDNA and the Ct value. The regression equation was Ct = 5.1279 (log10 dilution) + 19.433 and R^2 = 0.9809 (Figure 4E).

Figure 5: Agarose gel electrophoresis showing the specificity and detection limit of the PCR assay for cDNA from very virulent strain UPM94/273.

[0082] The cDNA was diluted 10-fold from 10° (7,700 ng/μl) to 10^{-5} (0.077 ng/μl) and used in amplification with match and mismatch primer combinations. Lane 1, 100 bp marker (Promega, USA); Lane 2, undiluted cDNA; Lane 3, 10^{-1} diluted cDNA; Lane 4, 10^{-2} diluted cDNA; Lane 5, 10^{-3} diluted cDNA; Lane 6, 10^{-4} diluted cDNA; Lane 7, 10^{-5} diluted cDNA; Lane 8, non-template negative control. Specific amplification of PCR product of the expected size 316 bp was observed from 10^0 to 10^{-3} diluted cDNA with match primer combination only (arrow) (Figure 5A). A faint of nonspecific amplification were observed from amplification of undiluted cDNA sample using mismatch primer (Figure 5B). No specific amplification was observed from the serially diluted cDNA and non-template negative control when amplification was performed using mismatch primer combination (Figure 5B).

Figure 6: Agarose gel electrophoresis showing the specificity and detection limit of the PCR assay for cDNA from vaccine strain D78.

[0083] The cDNA was diluted 10-fold from 10^0 (6,600 ng/μl) to 10^{-5} (0.066 ng/μl) and used in amplification with match and mismatch primer combinations. Lane 1, 100 bp marker (Promega, USA); Lane 2, undiluted cDNA; Lane 3, 10^{-1} diluted cDNA; Lane 4, 10^{-2} diluted cDNA; Lane 5, 10^{-3} diluted cDNA; Lane 6, 10^{-4} diluted cDNA; Lane 7, 10^{-5} diluted

cDNA; Lane 8, non-template negative control. Specific amplification (arrow) was observed from 10^0 to 10^{-3} diluted cDNA with match primer combination only (Figure 6A). A faint band of the nonspecific amplification was also observed from amplification of undiluted cDNA sample using mismatch primer (Figure 6B). No specific amplification was observed from the serially diluted cDNA and non-template negative control when amplification was performed using mismatch primer combination (Figure 6B).

Figure 7: 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.

Figure 8: Agarose gel electrophoresis showing the specificity of the real-time PCR assay in detecting vaccine strains; TAD, LZD and IBDVAC using both match and mismatch primer combinations.

[0084] The real-time PCR was performed using 4,000 ng/μl of cDNA. Lane 1, 100 bp marker (Promega, USA); Lane 2, TAD Gumboro with mismatch primer; Lane 3, Delvax LZD with mismatch primer; Lane 4, IBDVAC with mismatch primer; Lane 5, TAD Gumboro with match primer; Lane 6, Delvax LZD with match primer and Lane 7, IBDVAC with match primer. PCR product with the expected size 316 bp (arrow) was observed only with match primer combination.

Figure 9: The specificity of real-time PCR using IF & IVIR and IF & RCLA primer combinations on control uninfected tissue samples.

[0085] No specific amplifications were detected using IF & IVIR and IF & RCLA primers combinations on ~ 4,000 ng/μl of total RNA extracted from bursa, thymus and cecal tonsil of control uninfected SPF-chickens. As expected the positive control sample, UPM94/273 showed specific amplification using match primer IF and IVIR. No amplification from the negative control tissue and the Tm values ranged between 72.0 °C to 79.6°C.

Figure 10: Nucleotide sequences (316 bp) of UPM94/273, UPM97/61, D78, LZD, TAD and IBDVAC used in this study.

[0086] The nucleotide sequences of primer FVVC, RVVC, IF, IVIR and RCLA were also given. The nucleotide sequences encompassed from position 1835 to 2133 of the VP4 region.

Claims

1. A method of distinguishing between Infectious Bursal Disease Virus (IBDV) strains in a chicken or other bird sample, wherein said method comprises:

   a) providing a sample from a chicken or other bird that is infected with IBDV strains to be distinguished;
   b) amplifying said IBDV strains by means of a polymerase chain reaction (PCR) using at least one oligonucleotide primer pair selected from the group consisting of:

      (i) SEQ ID NO: 3 (ATG CTC CAG ATG GGG TAC TTC) and SEQ ID NO: 4 (TTG GAC CCG GTG TTC ACG); and
      (ii) SEQ ID NO: 3 and SEQ ID NO: 5 (TTG GGC CCG GTG TTT ACA); and

   c) distinguishing said IBDV strains.

2. The method according to claim 1, wherein said PCR comprises real-time (RT)-PCR.

3. A method according to claim 2, wherein said PCR comprises SYBR Green I based real-time PCR.

4. The method according to any of the preceding claims, wherein said distinguishing between IBDV strains comprises distinguishing between very virulent and vaccine IBDV strains.

5. The method according to claim 1, wherein said IBDV strains are selected from the very virulent strain UPM 94/273 and other IBDV strains that have nucleotide sequence complementary to the SEQ ID NO: 3 and SEQ ID NO: 4.

6. The method according to claim 5, wherein said IBDV strains are selected from the very virulent strains UPM97/61 and UPM 94/273.

7. The method according to claim 1, wherein said IBDV strains are selected from the vaccine strain D78 and other IBDV strains that have nucleotide sequence complementary to the SEQ ID NO: 3 and SEQ ID NO: 5.

8. The method according to claim 7, wherein said IBDV strains are selected from the vaccine strains D78, TAD Gumboro, Delvax Gumboro LZD, and IBDVAC.

9. The method according to any of the preceding claims, comprising the step of detecting a 316 base pair amplification product.

10. The method according to any one of claims 2 to 9, wherein step b) of amplifying said IBDV strains by real time polymerase chain reaction (PCR) comprises:

i) an amplification step of at least 33 cycles, each cycle comprising:

(1) at least 94°C for at least 30 seconds;
(2) at least 60°C for at least 20 seconds; and
(3) at least 72°C for at least 40 seconds; and

ii) melting temperature of at least 82°C to 88°C.

11. The method according to any of claims 1 to 3, wherein said IBDV strains are further isolated.

12. A method according to any of the preceding claims, said sample comprising RNA and said amplification step (b) being preceded by a reverse transcription step to transcribe said RNA into cDNA.

13. A method according to claim 12, said reverse transcription step being performed using the primer pair consisting of SEQ ID NO: 1 (AGA GGG TGC CAC GCT ATT) and SEQ ID NO: 2 (GGT ACT GGC GTC CTG CAT T).

14. The method according to claim 12, further comprising diluting cDNA from said IBDV strain(s).

15. The method according to claim 14, wherein cDNA from very virulent strains is diluted from at least 10° to 10^{-5} fold using said at least one oligonucleotide primer pair.

16. The method according to claim 14, wherein cDNA from vaccine strains is diluted from at least 10^{0} to 10^{-5} fold using said at least one oligonucleotide primer pair.

17. A method according to claim 9, wherein the detection of a 316 base pair amplification product when using the primer pair consisting of SEQ ID NO: 3 and SEQ ID NO: 4 is indicative of the presence of a very virulent IBDV strain.

18. A method according to claim 9, wherein the detection of a 316 base pair amplification product when using the primer pair consisting of SEQ ID NO: 3 and SEQ ID NO: 5 is indicative of the presence of a vaccine IBDV strain.

19. A method according to any of the preceding claims, further comprising the step of determining a threshold cycle (Ct) value and a melting temperature (Tm) for any PCR product, wherein a Ct value of 19-28 and a Tm of 86-88 °C indicate early amplification by said at least one primer pair, and a Ct value 0 and Tm <82 °C indicate no amplification.

20. A method according to claim 19, said at least one primer pair consisting of SEQ ID NOs: 3 and 4, wherein a Ct value of 19-28 and a Tm of 86-88 °C indicate the presence of a very virulent IBDV strain.

21. A method according to claim 19, said at least one primer pair consisting of SEQ ID NOs: 3 and 5, wherein a Ct value of 19-28 and a Tm of 86-88 °C indicate the presence of a vaccine IBDV strain.

22. A PCR primer pair selected from the group consisting of:

(a) SEQ ID NO: 3 and SEQ ID NO: 4; and
(b) SEQ ID NO: 3 and SEQ ID NO: 5.

23. The use of a PCR primer pair according to claim 22 for detecting and/or differentiating Infectious Bursal Disease

Virus (IBDV) in chicken and bird samples.

24. A diagnostic test kit for detecting Infectious Bursal Disease Virus (IBDV) strains, comprising a PCR primer pair according to claim 22.

Patentansprüche

1. Verfahren zur Unterscheidung zwischen Virenstämmen der infektiösen Bursitis (IBDV-Stämmen) in einer Probe von einem Huhn oder einem anderen Vogel, wobei das Verfahren aufweist:

a) Bereitstellen einer Probe von einem Huhn oder einem anderen Vogel, das (der) mit zu unterscheidenden IBDV-Stämmen infiziert ist;
b) Amplifikation der IBDV-Stämme mittels einer Polymerase-Kettenreaktion (PCR) unter Verwendung mindestens eines Oligonucleotid-Primerpaars, das aus der Gruppe ausgewählt ist, die aus den folgenden besteht:

(i) SEQ ID Nr.: 3 (ATG CTC CAG ATG GGG TAC TTC) und SEQ ID Nr.: 4 (TTG GAC CCG GTG TTC ACG); und
(ii) SEQ ID Nr.: 3 und SEQ ID Nr.: 5 (TTG GGC CCG GTG TTT ACA);
und

c) Unterscheiden der IBDV-Stämme.

2. Verfahren nach Anspruch 1, wobei die PCR Echtzeit- bzw. (RT)-PCR beinhaltet.

3. Verfahren nach Anspruch 2, wobei die PCR SYBR-Green I-basierte Echtzeit-PCR beinhaltet.

4. Verfahren nach einem der vorstehenden Ansprüche, wobei die Unterscheidung zwischen IBDV-Stämmen eine Unterscheidung zwischen sehr virulenten und Impfstoff-IBDV-Stämmen beinhaltet.

5. Verfahren nach Anspruch 1, wobei die IBDV-Stämme unter dem sehr virulenten Stamm UPM 94/273 und anderen IBDV-Stämmen ausgewählt sind, die eine zu SEQ ID Nr.: 3 und SEQ ID Nr.: 4 komplementäre Nucleotidsequenz aufweisen.

6. Verfahren nach Anspruch 5, wobei die IBDV-Stämme unter den sehr virulenten Stämmen UPM97/61 und UPM94/273 ausgewählt sind.

7. Verfahren nach Anspruch 1, wobei die IBDV-Stämme unter dem Impfstoff-Stamm D78 und anderen IBDV-Stämmen ausgewählt sind, die eine zu SEQ ID Nr.: 3 und SEQ ID Nr.: 5 komplementäre Nucleotidsequenz aufweisen.

8. Verfahren nach Anspruch 7, wobei die IBDV-Stämme unter den Impfstoff-Stämmen D78, TAD Gumboro, Delvax Gumboro LZD und IBDVAC ausgewählt sind.

9. Verfahren nach einem der vorstehenden Ansprüche, das den Schritt zum Nachweis eines Amplifikationsprodukts von 316 Basenpaaren aufweist.

10. Verfahren nach einem der Ansprüche 2 bis 9, wobei Schritt b) zur Amplifikation der IBDV-Stämme durch Echtzeit-Polymerase-Kettenreaktion (PCR) aufweist:

i) einen Amplifikationsschritt von mindestens 33 Zyklen, wobei jeder Zyklus aufweist:

(1) mindestens 30 Sekunden bei mindestens 94°C;
(2) mindestens 20 Sekunden bei mindestens 60°C; und
(3) mindestens 40 Sekunden bei mindestens 72°C; und

ii) eine Schmelztemperatur von mindestens 82°C bis 88°C.

11. Verfahren nach einem der Ansprüche 1 bis 3, wobei die IBDV-Stämme ferner isoliert werden.

12. Verfahren nach einem der vorstehenden Ansprüche, wobei die Probe RNA aufweist und dem Amplifikationsschritt (b) ein Umkehrtranskriptionsschritt vorausgeht, um die RNA in cDNA zu transkribieren.

13. Verfahren nach Anspruch 12, wobei der Umkehrtranskriptionsschritt unter Verwendung des Primerpaars durchgeführt wird, das aus SEQ ID Nr.: 1 (AGA GGG TGC CAC GCT ATT) und SEQ ID Nr.: 2 (GGT ACT GGC GTC CTG CAT T) besteht.

14. Verfahren nach Anspruch 12, das ferner das Verdünnen von cDNA aus dem einen oder den mehreren IBDV-Stämmen aufweist.

15. Verfahren nach Anspruch 14, wobei cDNA aus sehr virulenten Stämmen unter Verwendung des mindestens einen Oligonucleotid-Primerpaars mindestens 10°- bis 10^5-fach verdünnt wird.

16. Verfahren nach Anspruch 14, wobei cDNA aus Impfstoff-Stämmen unter Verwendung des mindestens einen Oligonucleotid-Primerpaars mindestens 10°- bis 10^5-fach verdünnt wird.

17. Verfahren nach Anspruch 9, wobei der Nachweis eines Amplifikationsprodukts von 316 Basenpaaren bei Verwendung des Primerpaars SEQ ID Nr.: 3 und SEQ ID Nr.: 4 auf die Anwesenheit eines sehr virulenten IBDV-Stamms schließen lässt.

18. Verfahren nach Anspruch 9, wobei der Nachweis eines Amplifikationsprodukts von 316 Basenpaaren bei Verwendung des Primerpaars SEQ ID Nr.: 3 und SEQ ID Nr.: 5 auf die Anwesenheit eines Impfstoff IBDV-Stamms schließen lässt.

19. Verfahren nach einem der vorstehenden Ansprüche, das ferner den Schritt zur Bestimmung eines Schwellwertzyklus (Ct)-Werts und einer Schmelztemperatur (Tm) für jedes PCR-Produkt aufweist, wobei ein Ct-Wert von 19-28 und ein Tm-Wert von 86-88°C frühe Amplifikation durch das mindestens eine Primerpaar anzeigen und ein Ct-Wert 0 und ein Tm-Wert <82°C keine Amplifikation anzeigen.

20. Verfahren nach Anspruch 19, wobei das mindestens eine Primerpaar aus SEQ ID Nr.: 3 und SEQ ID Nr.: 4 besteht, wobei ein Ct-Wert von 19-28 und ein Tm-Wert von 86-88°C die Anwesenheit eines sehr virulenten IBDV-Stamms anzeigen.

21. Verfahren nach Anspruch 19, wobei das mindestens eine Primerpaar aus SEQ ID Nr.: 3 und SEQ ID Nr.: 5 besteht, wobei ein Ct-Wert von 19-28 und ein Tm-Wert von 86-88°C die Anwesenheit eines Impfstoff-IBDV-Stamms anzeigen.

22. PCR-Primerpaar, das aus der Gruppe ausgewählt ist, die aus

    (a) SEQ ID Nr.: 3 und SEQ ID Nr.: 4; und
    (b) SEQ ID Nr.: 3 und SEQ ID Nr.: 5 besteht.

23. Verwendung von einem PCR-Primerpaar nach Anspruch 22 zum Nachweis und/oder zur Unterscheidung von Viren der infektiösen Bursitis (IBDV) in Huhn- und Vogelproben.

24. Diagnostischer Testkit zum Nachweis von Virenstämmen der infektiösen Bursitis (IBDV), wobei das Kit ein PCR-Primerpaar nach Anspruch 22 aufweist.

Revendications

1. Méthode pour faire une distinction entre des souches du virus de la bursite infectieuse (VBI) dans un échantillon de poulet ou d'un autre oiseau, où ladite méthode comprend:

    a) la fourniture d'un échantillon provenant d'un poulet ou d'un autre oiseau qui est infecté avec des souches de VBI devant être distinguées;
    b) l'amplification desdites souches de VBI au moyen d'une réaction en chaîne de la polymérase (PCR) en utilisant au moins une paire d'amorces oligonucléotidiques choisie dans le groupe constitué de:

(i) la SEQ ID NO:3 (ATG CTC CAG ATG GGG TAC TTC) et la SEQ ID NO:4 (TTG GAC CCG GTG TTC ACG); et
(ii) la SEQ ID NO:3 et la SEQ ID NO:5 (TTG GGC CCG GTG TTT ACA); et

c) la distinction desdites souches de VBI.

2. Méthode selon la revendication 1, dans laquelle ladite PCR comprend une PCR en temps réel (RT).

3. Méthode selon la revendication 2, dans laquelle ladite PCR comprend une PCR en temps réel basée sur le vert SYBR I.

4. Méthode selon l'une quelconque des revendications précédentes, dans laquelle ladite distinction entre des souches de VBI comprend une distinction entre des souches de VBI très virulentes et de vaccin.

5. Méthode selon la revendication 1, dans laquelle lesdites souches de VBI sont choisies parmi la souche très virulente UPM 94/273 et d'autres souches de VBI qui possèdent une séquence de nucléotides complémentaire à la SEQ ID NO:3 et la SEQ ID NO:4.

6. Méthode selon la revendication 5, dans laquelle lesdites souches de VBI sont choisies parmi les souches très virulentes UPM 97/61 et UPM 94/273.

7. Méthode selon la revendication 1, dans laquelle lesdites souches de VBI sont choisies parmi la souche de vaccin D78 et d'autres souches de VBI qui possèdent une séquence de nucléotides complémentaire à la SEQ ID NO:3 et la SEQ ID NO:5.

8. Méthode selon la revendication 7, dans laquelle lesdites souches de VBI sont choisies parmi les souches de vaccin D78, TAD Gumboro, Delvax Gumboro LZD et IBDVAC.

9. Méthode selon l'une quelconque des revendications précédentes, comprenant l'étape de détection d'un produit d'amplification de 316 paires de bases.

10. Méthode selon l'une quelconque des revendications 2 à 9, dans laquelle l'étape b) d'amplification desdites souches de VBI par une réaction en chaîne de la polymérase (PCR) en temps réel comprend:

i) une étape d'amplification d'au moins 33 cycles, chaque cycle comprenant:

(1) au moins 94°C pendant au moins 30 secondes;
(2) au moins 60°C pendant au moins 20 secondes; et
(3) au moins 72°C pendant au moins 40 secondes; et

ii) une température de fusion d'au moins 82°C à 88°C.

11. Méthode selon l'une quelconque des revendications 1 à 3, dans laquelle lesdites souches de VBI sont en outre isolées.

12. Méthode selon l'une quelconque des revendications précédentes, ledit échantillon comprenant un ARN et ladite étape d'amplification (b) étant précédée par une étape de transcription inverse pour transcrire ledit ARN en un ADNc.

13. Méthode selon la revendication 12, ladite étape de transcription inverse étant effectuée en utilisant la paire d'amorces constituée de la SEQ ID NO:1 (AGA GGG TGC CAC GCT ATT) et la SEQ ID NO:2 (GGT ACT GGC GTC CTG CAT T).

14. Méthode selon la revendication 12, comprenant en outre la dilution d'un ADNc à partir de ladite ou desdites souches de VBI.

15. Méthode selon la revendication 14, dans laquelle un ADNc provenant de souches très virulentes est dilué au moins 10^0 à 10^{-5} fois en utilisant ladite au moins une paire d'amorces oligonucléotidiques.

16. Méthode selon la revendication 14, dans laquelle un ADNc provenant de souches de vaccin est dilué au moins 10^0

à 10^{-5} fois en utilisant ladite au moins une paire d'amorces oligonucléotidiques.

17. Méthode selon la revendication 9, dans laquelle la détection d'un produit d'amplification de 316 paires de bases lorsque l'on utilise la paire d'amorces constituée de la SEQ ID NO:3 et la SEQ ID NO:4 est indicatrice de la présence d'une souche de VBI très virulente.

18. Méthode selon la revendication 9, dans laquelle la détection d'un produit d'amplification de 316 paires de bases lorsque l'on utilise la paire d'amorces constituée de la SEQ ID NO:3 et la SEQ ID NO:5 est indicatrice de la présence d'une souche de VBI de vaccin.

19. Méthode selon l'une quelconque des revendications précédentes, comprenant en outre l'étape de détermination d'une valeur de cycle seuil (Ct) et d'une température de fusion (Tm) pour chaque produit PCR, dans laquelle une valeur Ct de 19-28 et une Tm de 86-88°C indiquent une amplification précoce par ladite au moins une paire d'amorces et une valeur Ct 0 et Tm < 82°C indiquent qu'il n'y a pas d'amplification.

20. Méthode selon la revendication 19, ladite au moins une paire d'amorces étant constituée des SEQ ID NOs:3 et 4, dans laquelle une valeur Ct de 19-28 et une Tm de 86-88°C indiquent la présence d'une souche de VBI très virulente.

21. Méthode selon la revendication 19, ladite au moins une paire d'amorces étant constituée des SEQ ID NOs:3 et 5, dans laquelle une valeur Ct de 19-28 et une Tm de 86-88°C indiquent la présence d'une souche de VBI de vaccin.

22. Paire d'amorces PCR choisie dans le groupe constitué de:

(a) la SEQ ID NO:3 et la SEQ ID NO:4; et
(b) la SEQ ID NO:3 et la SEQ ID NO:5.

23. Utilisation d'une paire d'amorces PCR selon la revendication 22 pour la détection et/ou de différenciation du virus de la bursite infectieuse (VBI) dans des échantillons de poulet et d'oiseau.

24. Kit de test diagnostique pour la détection de souches du virus de la bursite infectieuse (VBI), comprenant une paire d'amorces PCR selon la revendication 22.

Figure 1: Nucleotide sequences and the deduced amino acids translation of primers IVIR and RCLA.

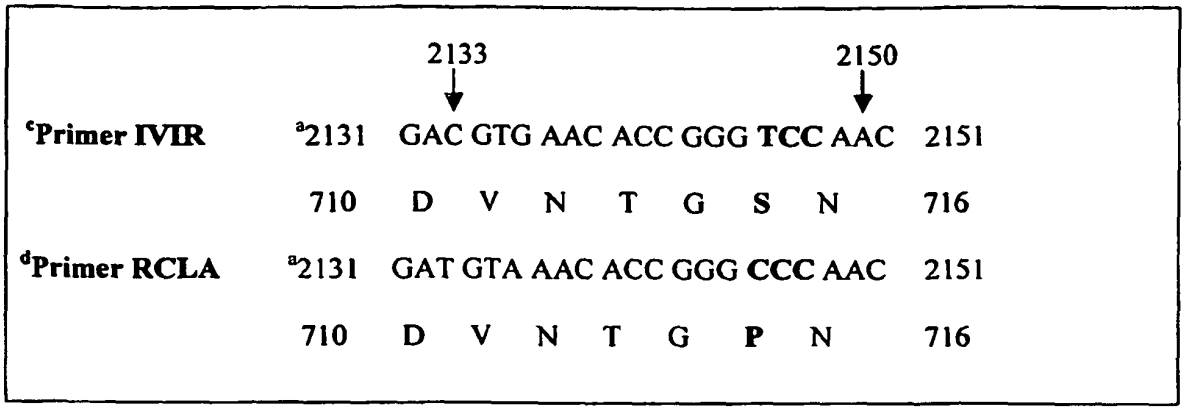
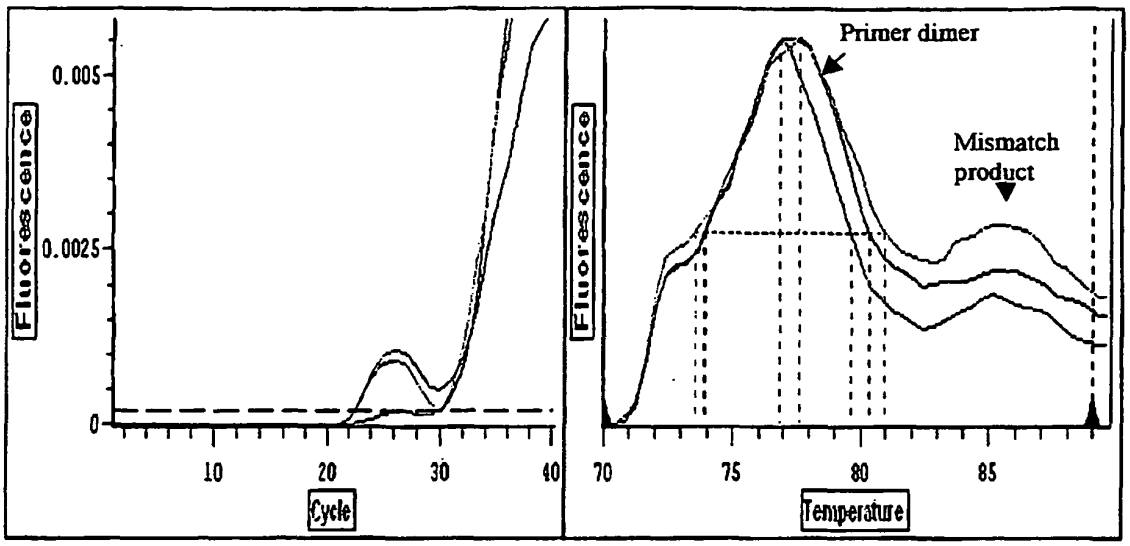


Figure 2: Influence of number of cycles in real-time PCR assay using template from vaccine strain D78 and mismatch primer combinations.



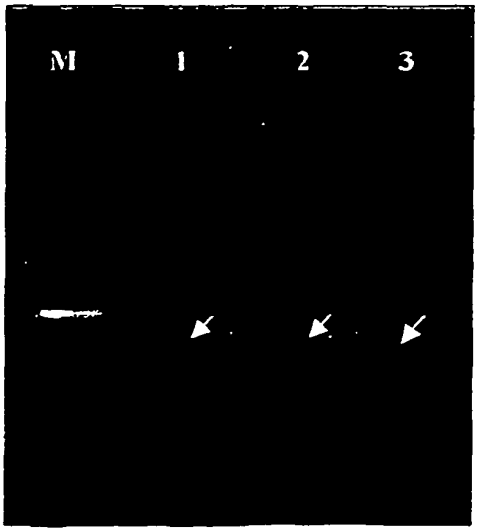
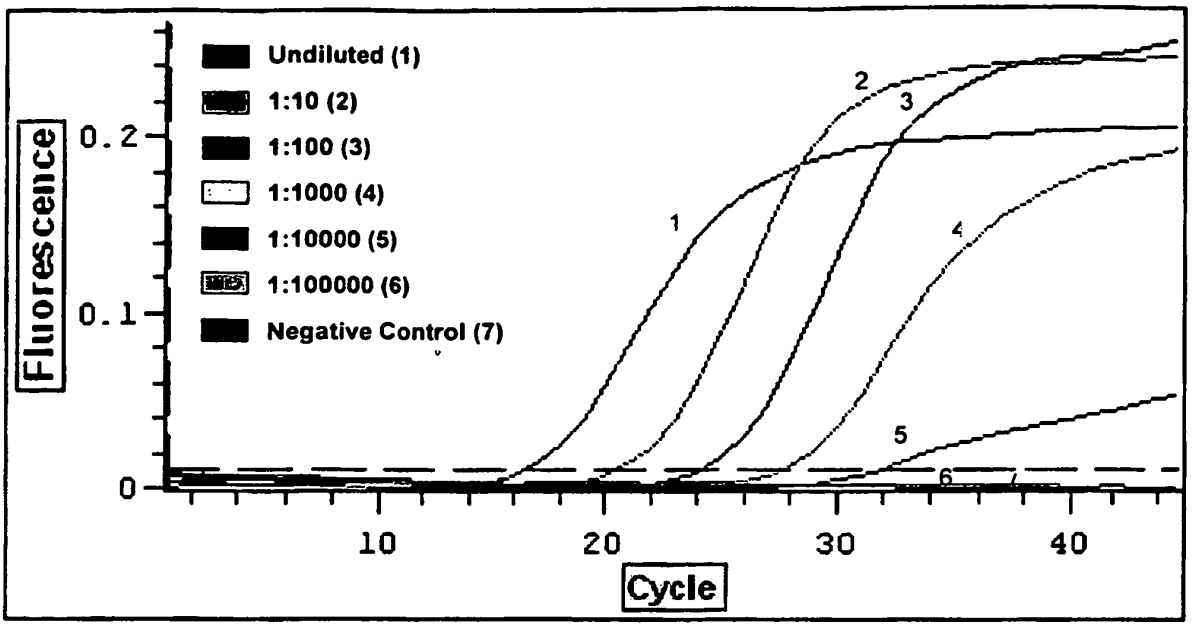


Figure 3A


Undiluted (1)
1:10 (2)
1:100 (3)
1:1000 (4)
1:10000 (5)
1:100000 (6)
Negative Control (7)
Fluorescence
0.2
0.1
0
10
20
30
40
Cycle
1
2
3
4
5
6

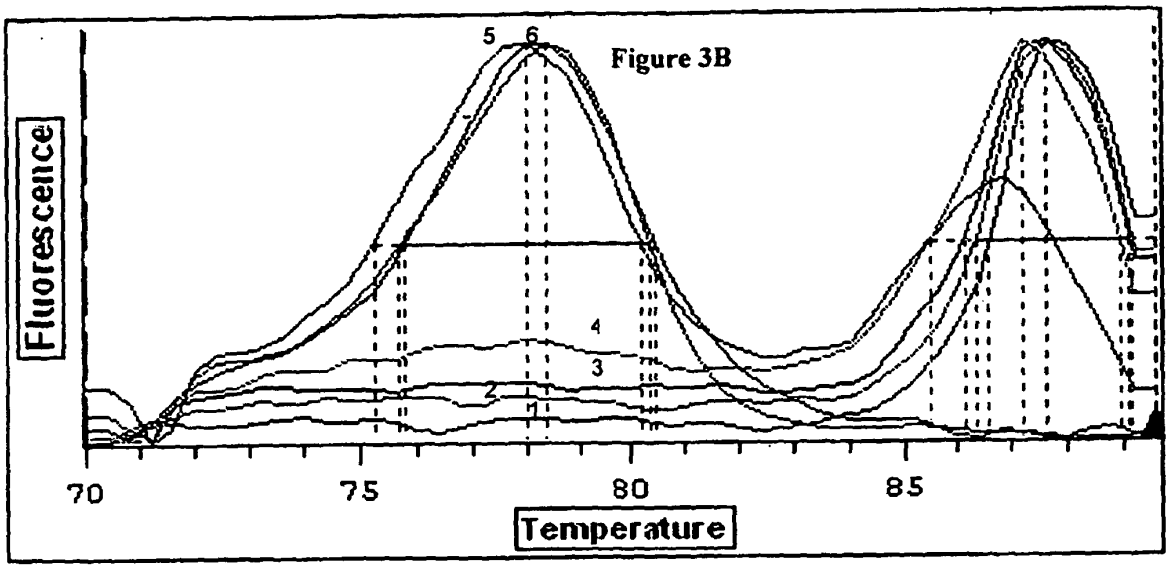
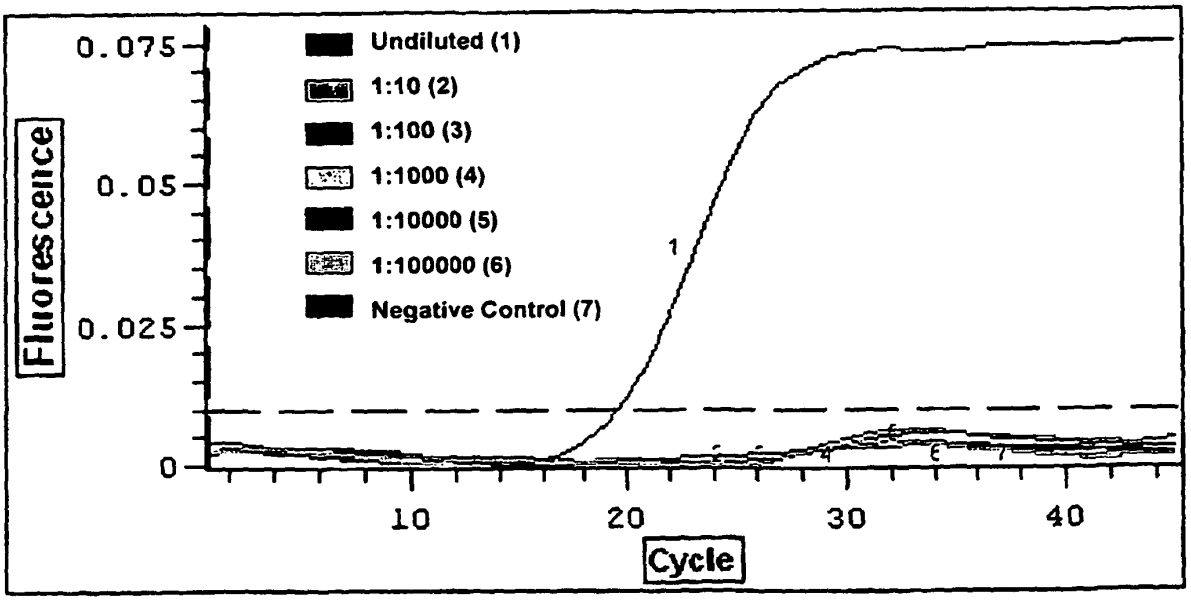


Figure 3B
Fluorescence
Temperature
70
75
80
85
1
2
3
4
5
6

Figure 3C


Fluorescence
Cycle
0.075
0.05
0.025
0
10
20
30
40
Undiluted (1)
1:10 (2)
1:100 (3)
1:1000 (4)
1:10000 (5)
1:100000 (6)
Negative Control (7)
1

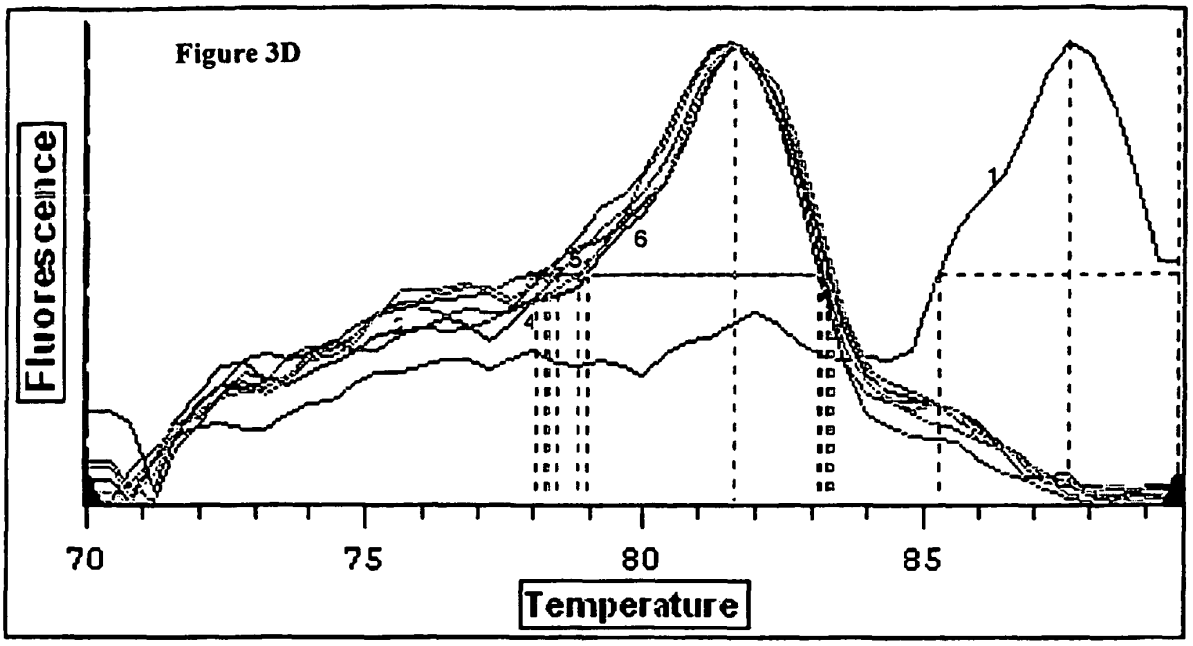


Figure 3D
Fluorescence
70
75
80
85
Temperature
1
6
5
4

Figure 3E

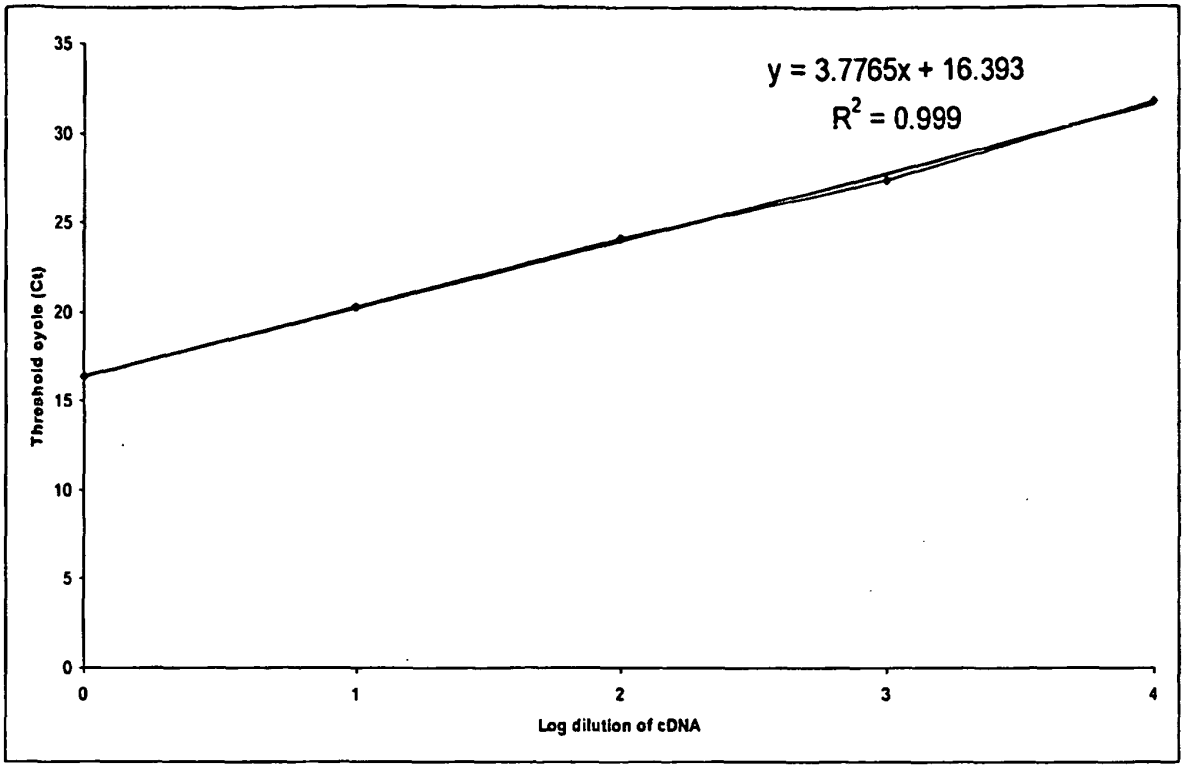


Figure 3: The performance of the real-time in detecting very virulent strain UPM94/273.

Figure 4A

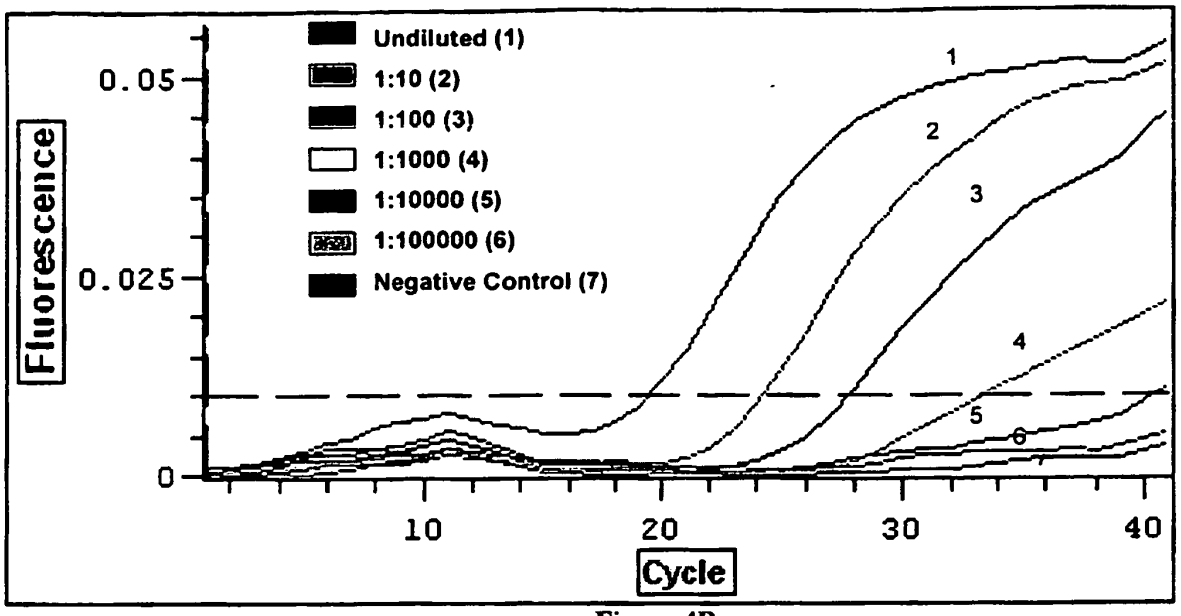


Figure 4B

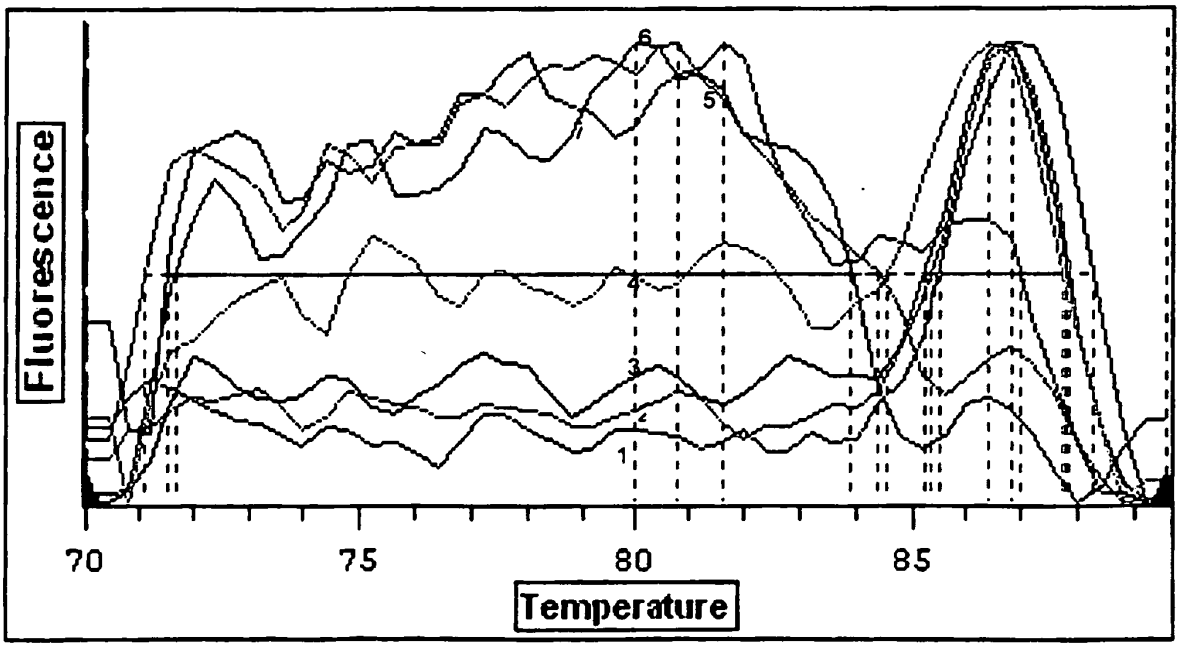


Figure 4C

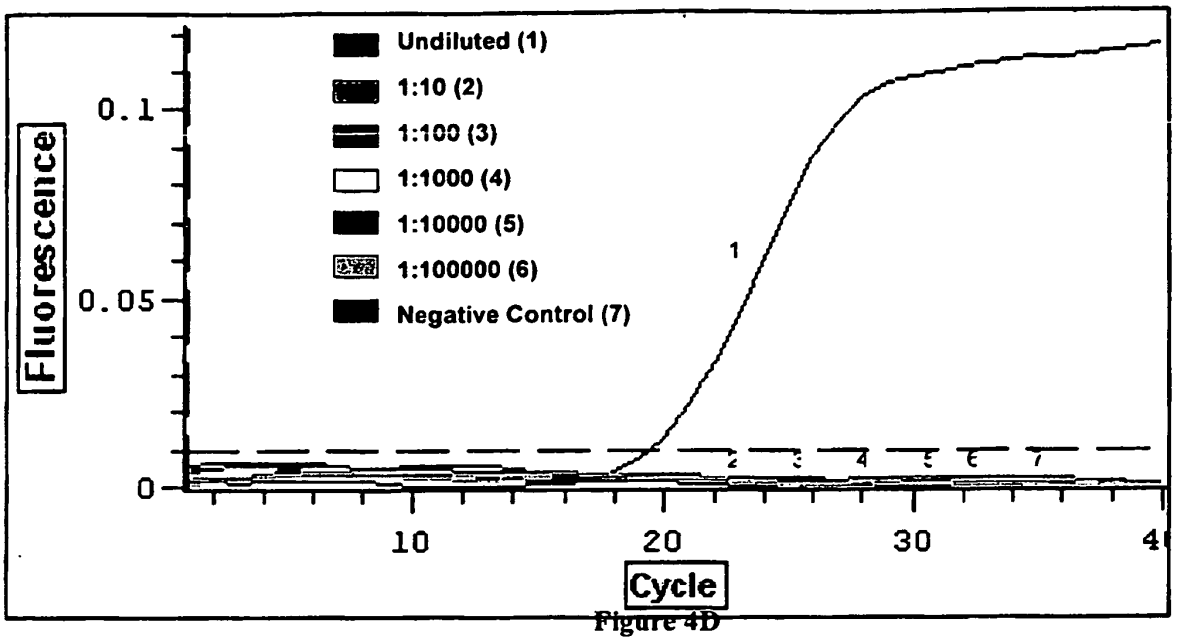


Figure 4D

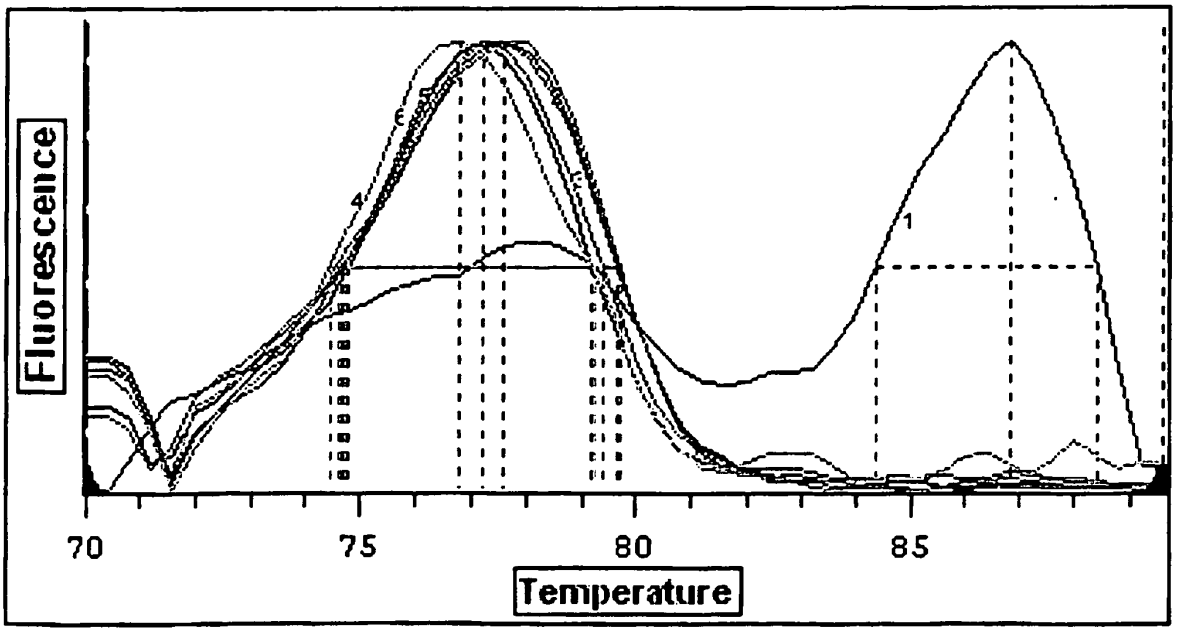


Figure 4E

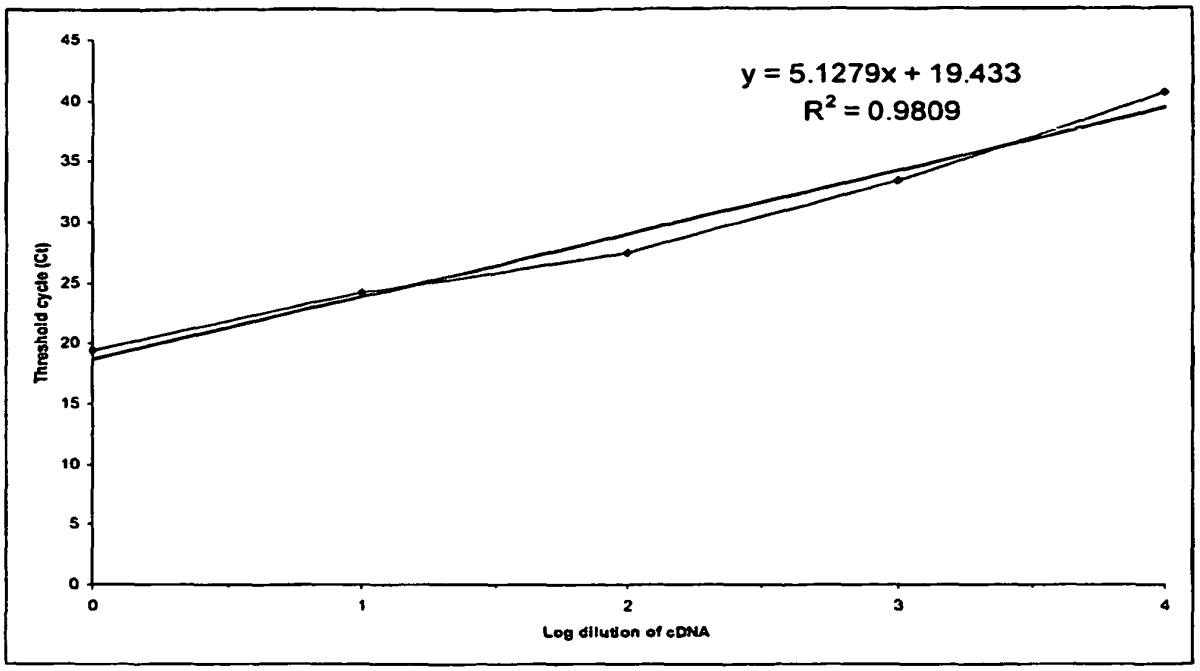


Figure 4: The performance of the real-time in detecting vaccine strain D78.

Figure 5A

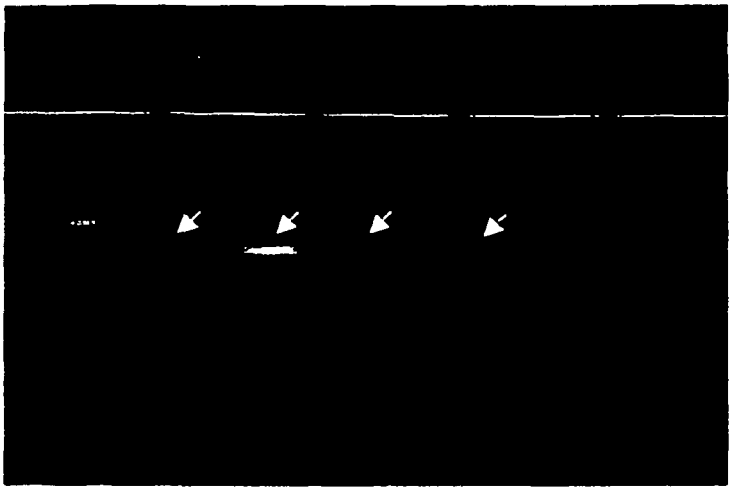


Figure 5B

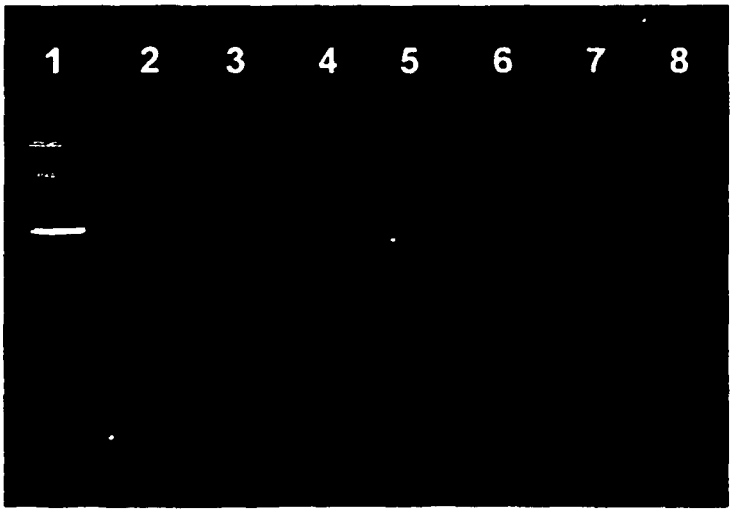


Figure 5: Agarose gel electrophoresis showing the specificity and detection limit of the PCR assay for cDNA from very virulent strain UPM94/273.

Figure 6A

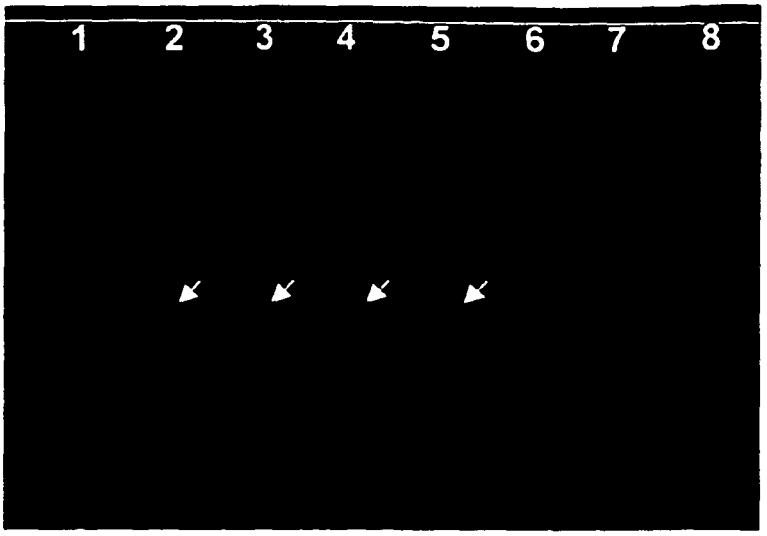


Figure 6B

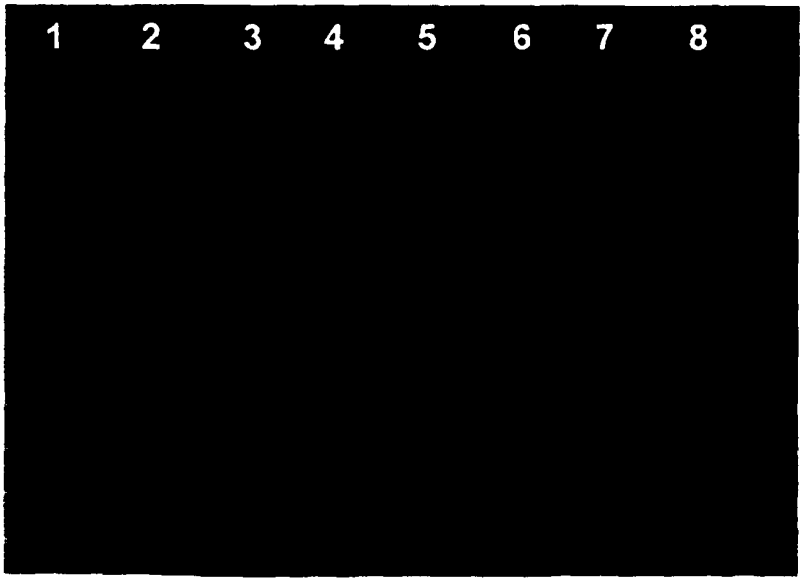


Figure 6: Agarose gel electrophoresis showing the specificity and detection limit of the PCR assay for cDNA from vaccine strain D78.

Figure 7: 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.

Figure 7A

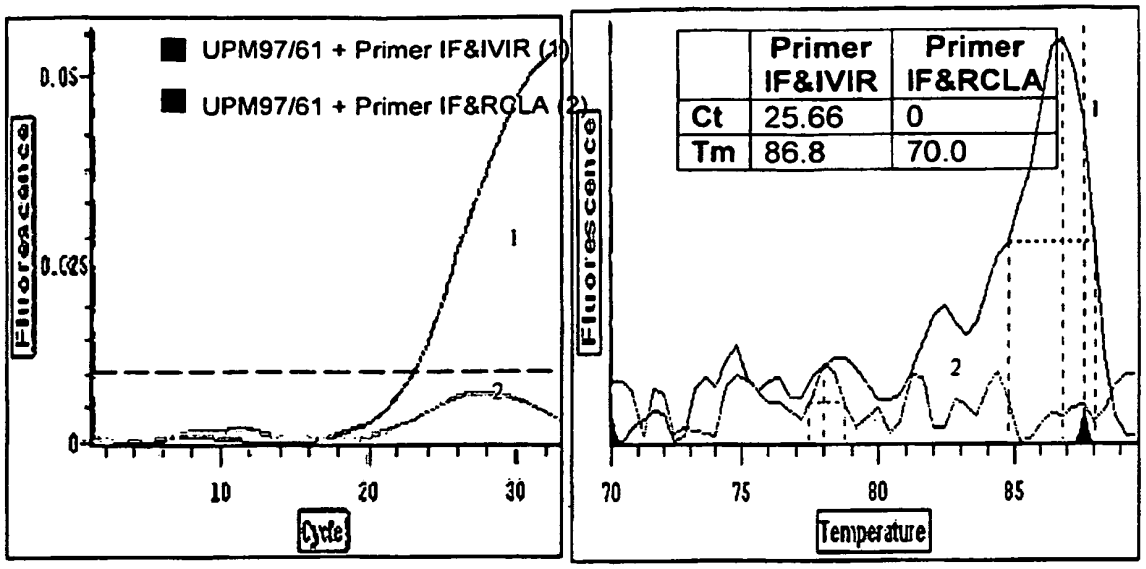
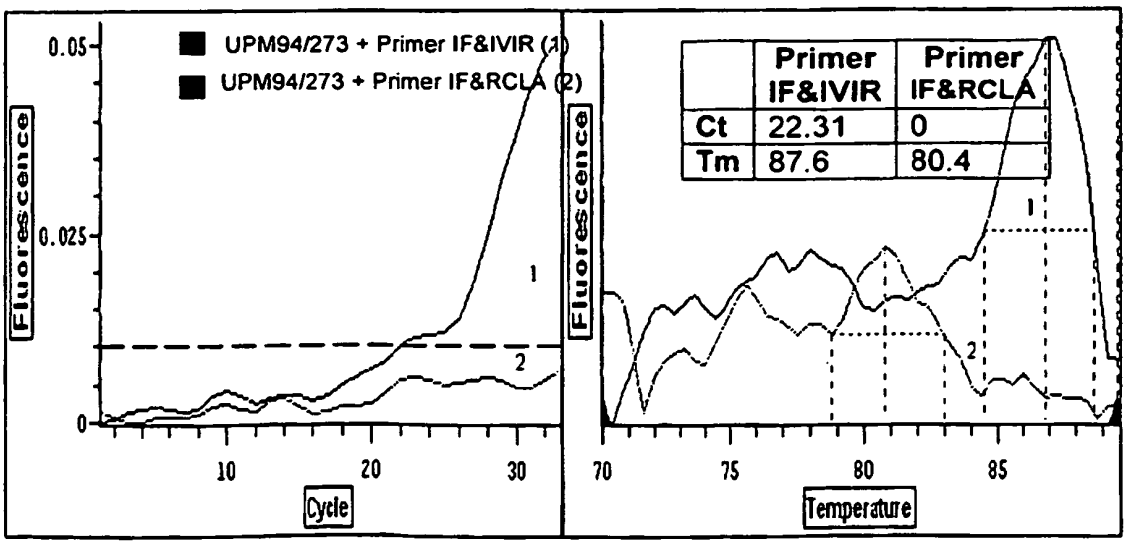


Figure 7B



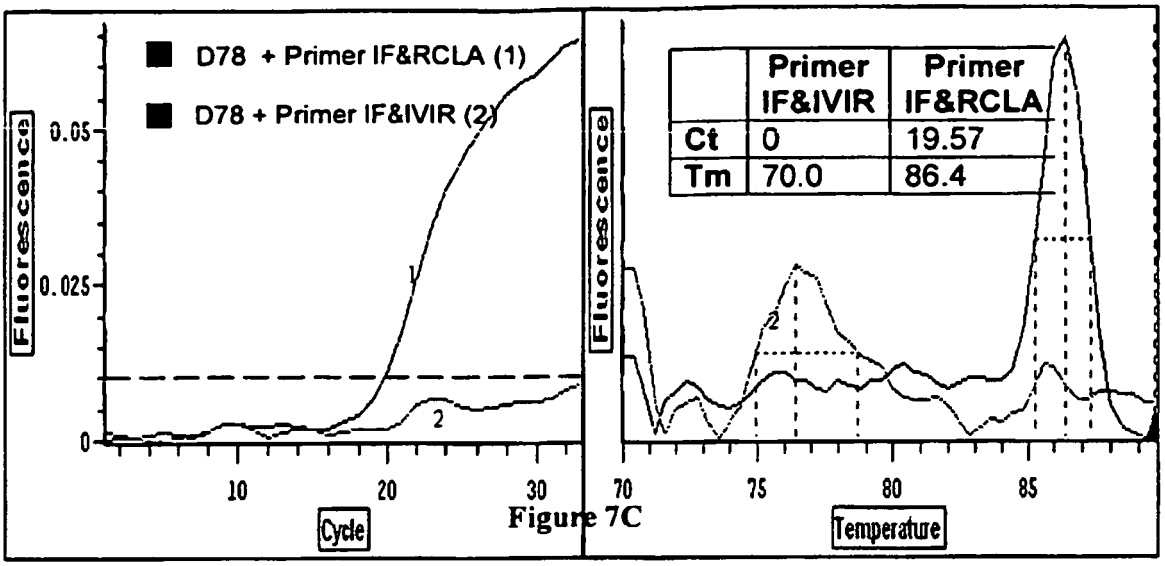
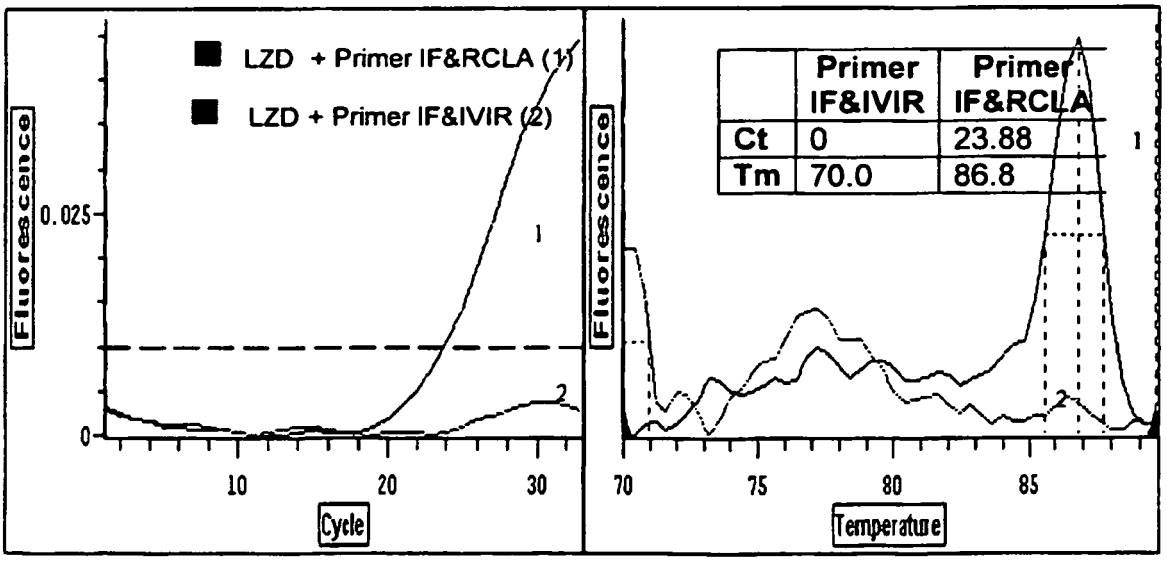


Figure 7C

Figure 7D



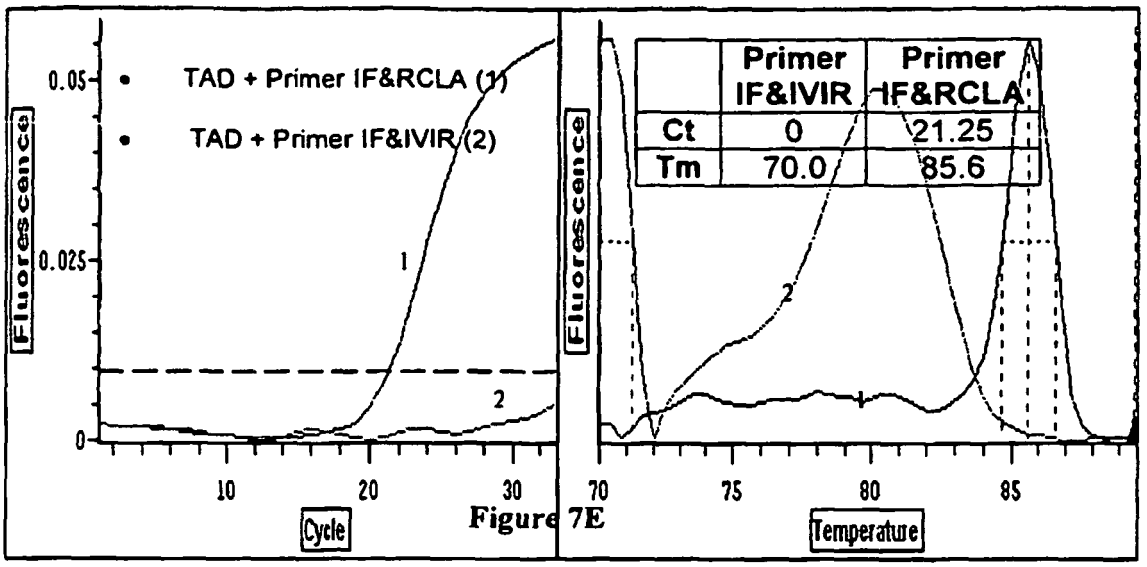


Figure 7E

Figure 7F

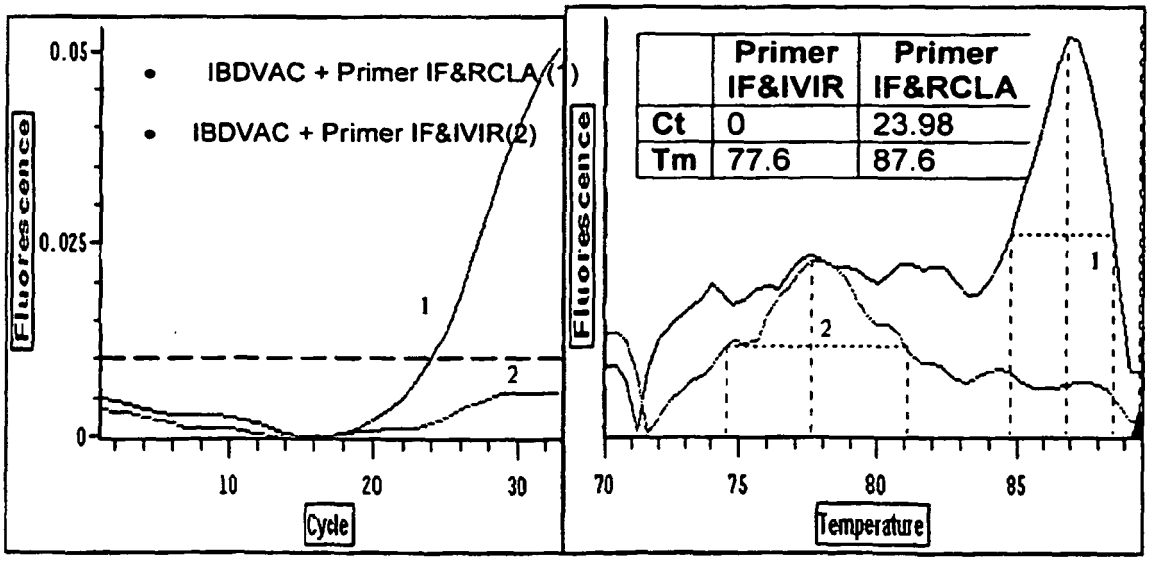


Figure 8: Agarose gel electrophoresis showing the specificity of the real-time PCR assay in detecting vaccine strains; TAD, LZD and IBDVAC using both match and mismatch primer combinations.

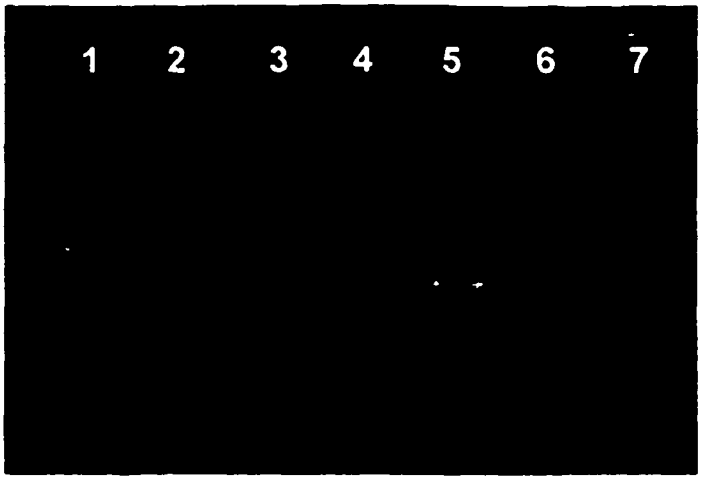
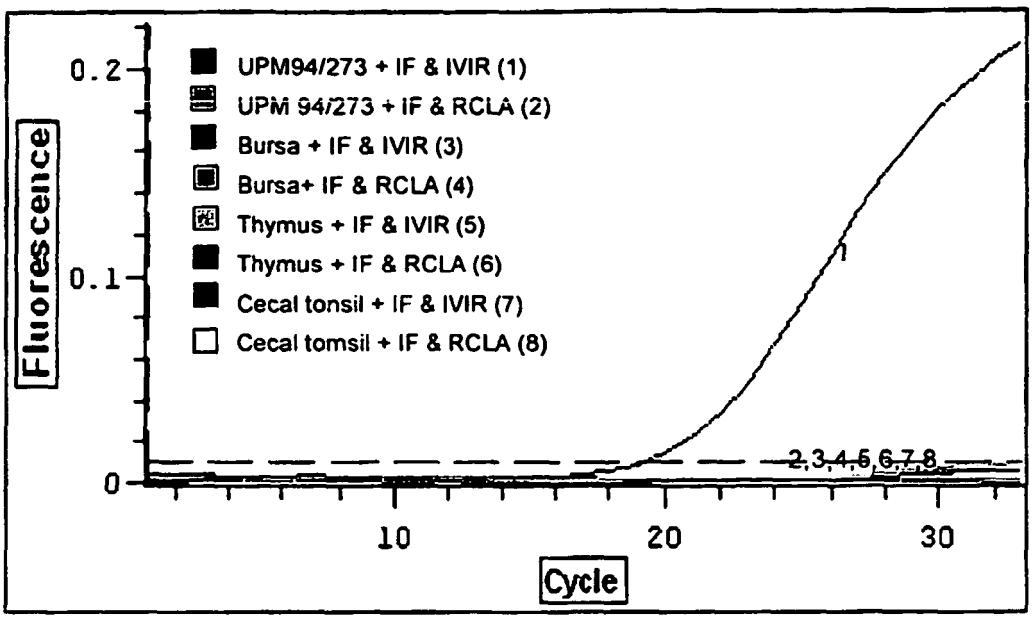


Figure 9: The specificity of real-time PCR using IF & IVIR and IF & RCLA primer combinations on control uninfected tissue samples.



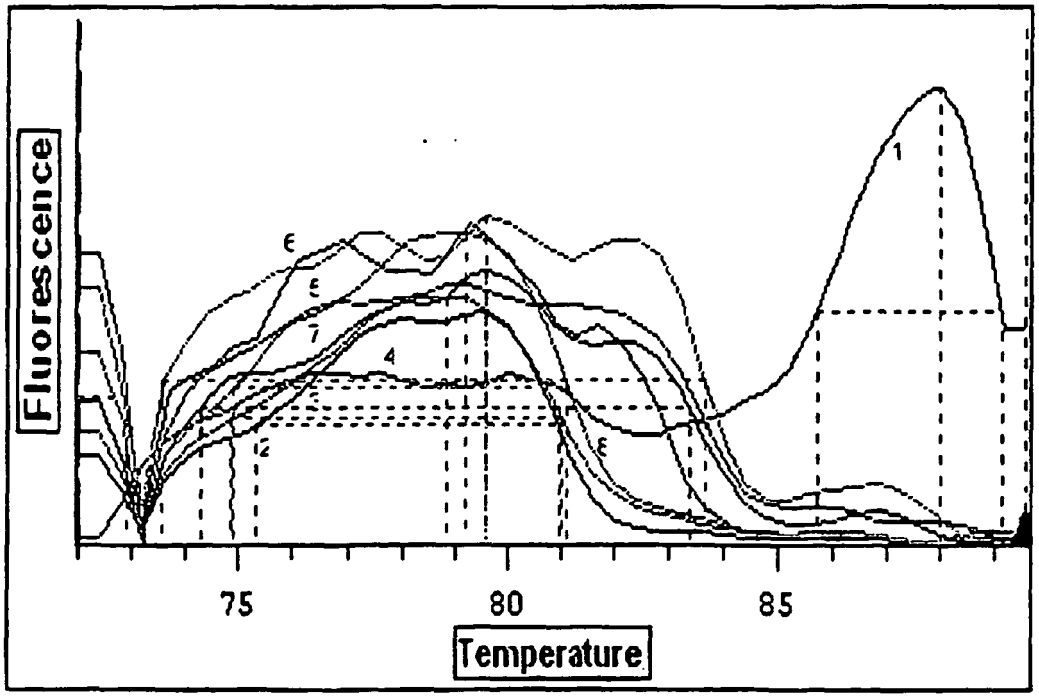


Figure 10: Nucleotide sequences (316 bp) of UPM94/273, UPM97/61, D78, LZD, TAD and IBDVAC used in this study

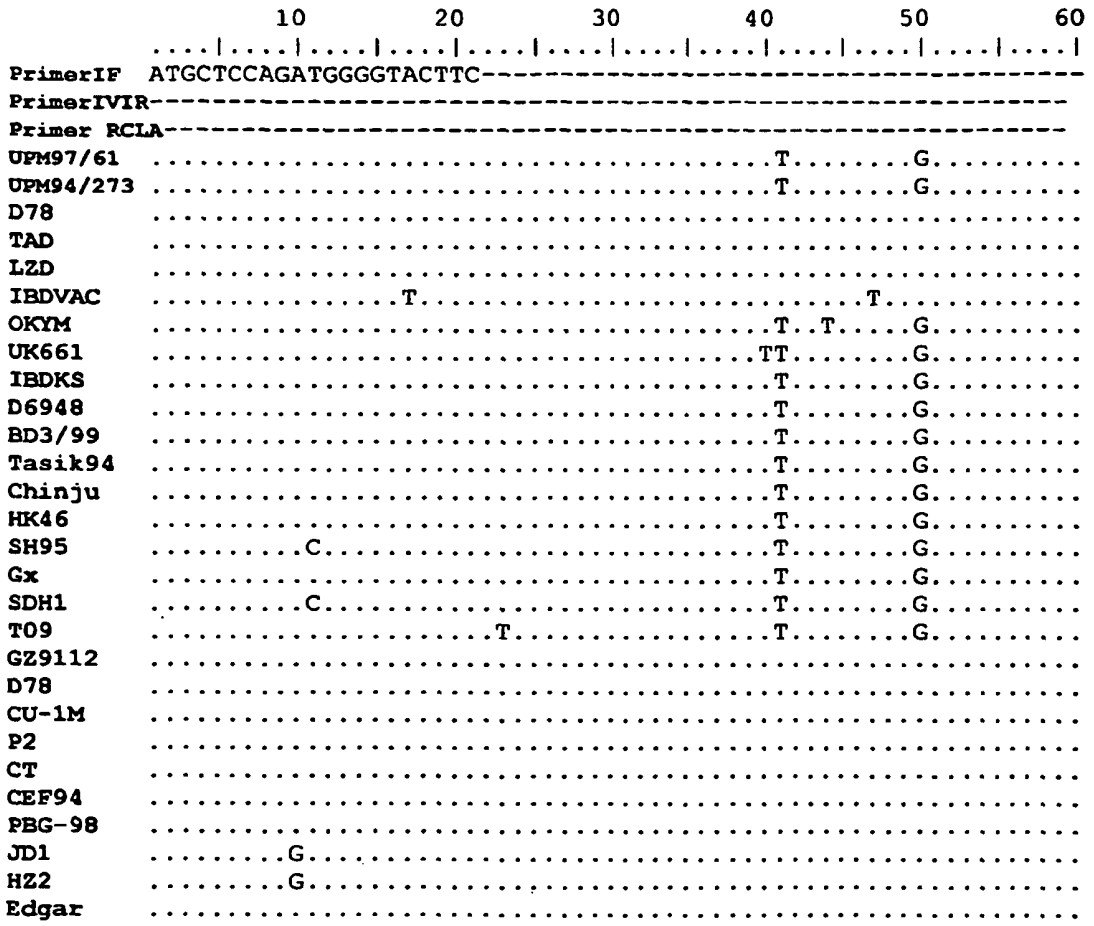


Figure 10 (continued)

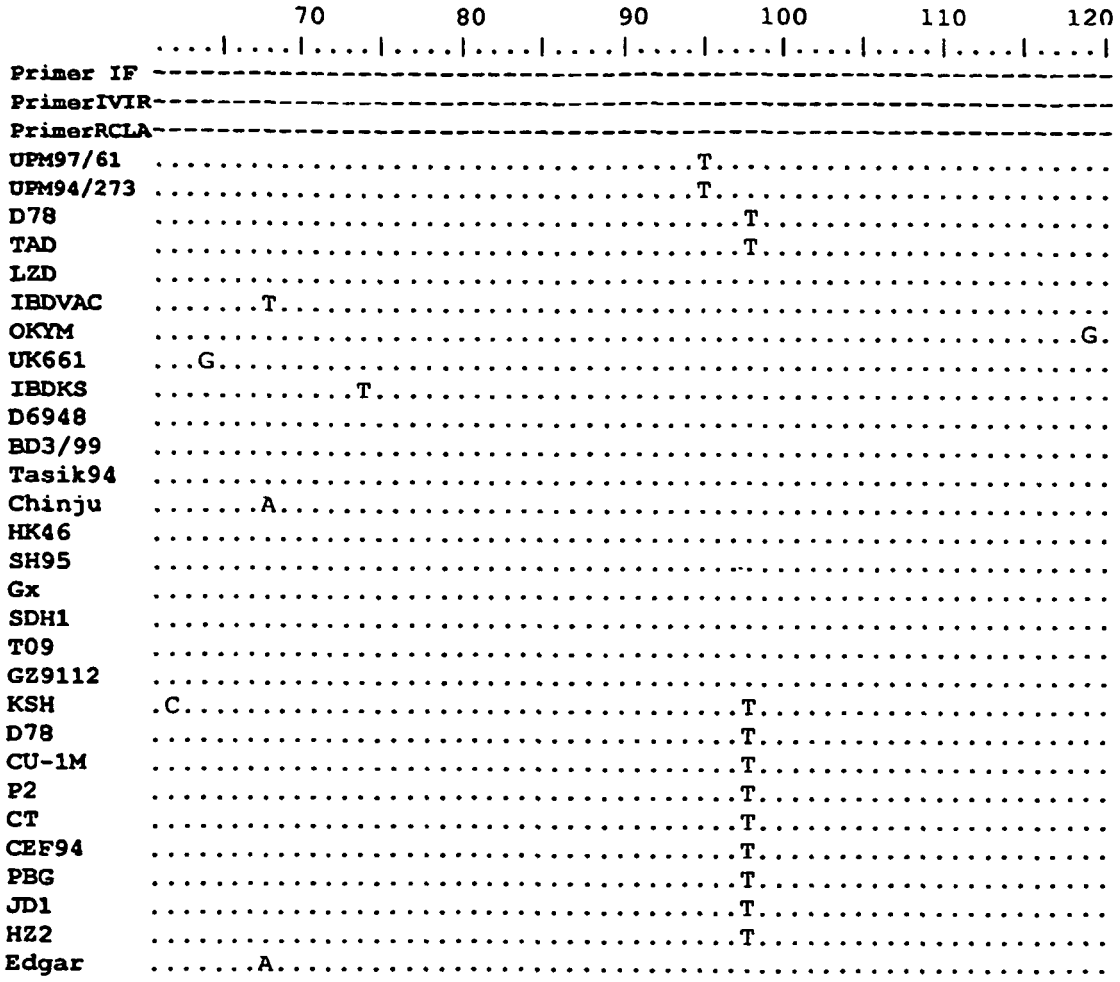


Figure 10 (continued)

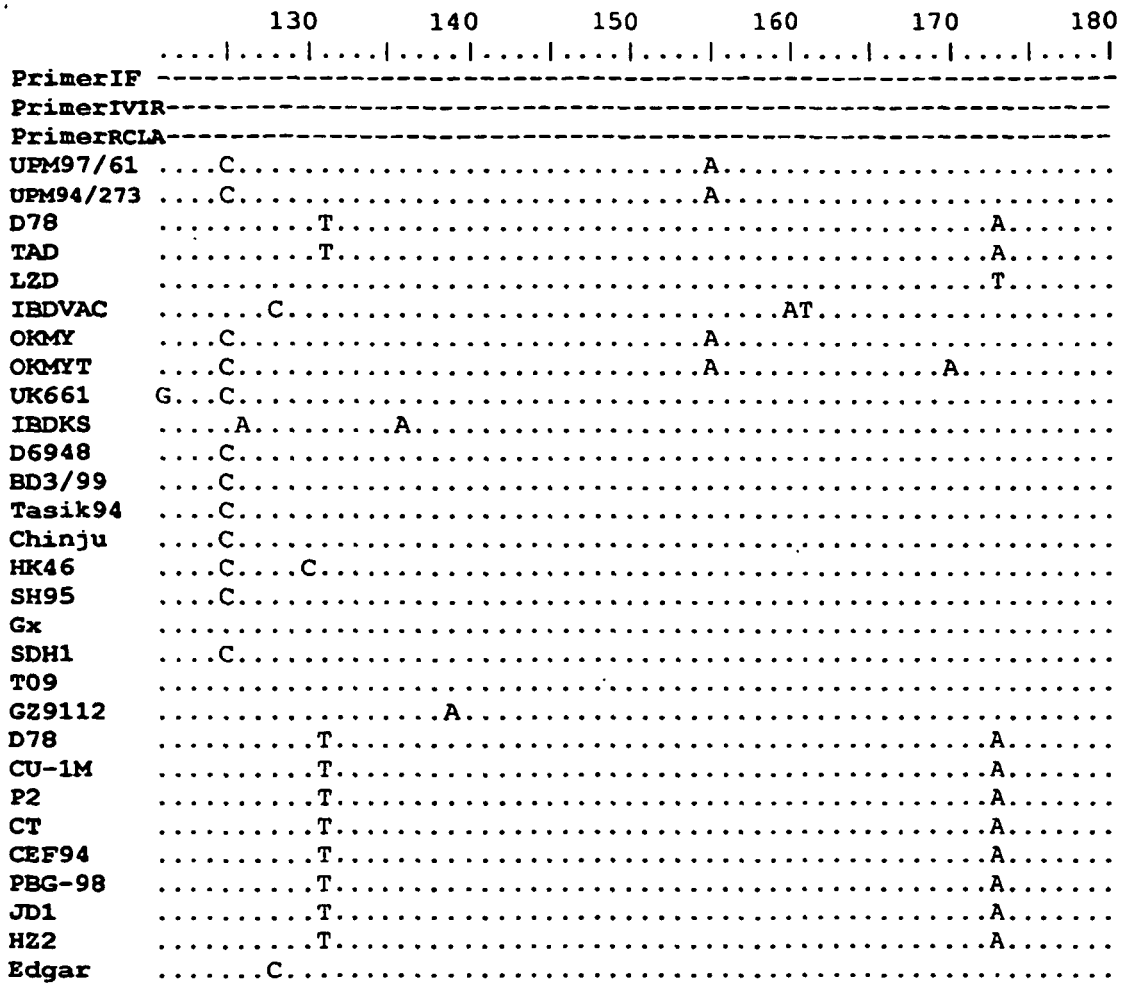


Figure10 (continued)

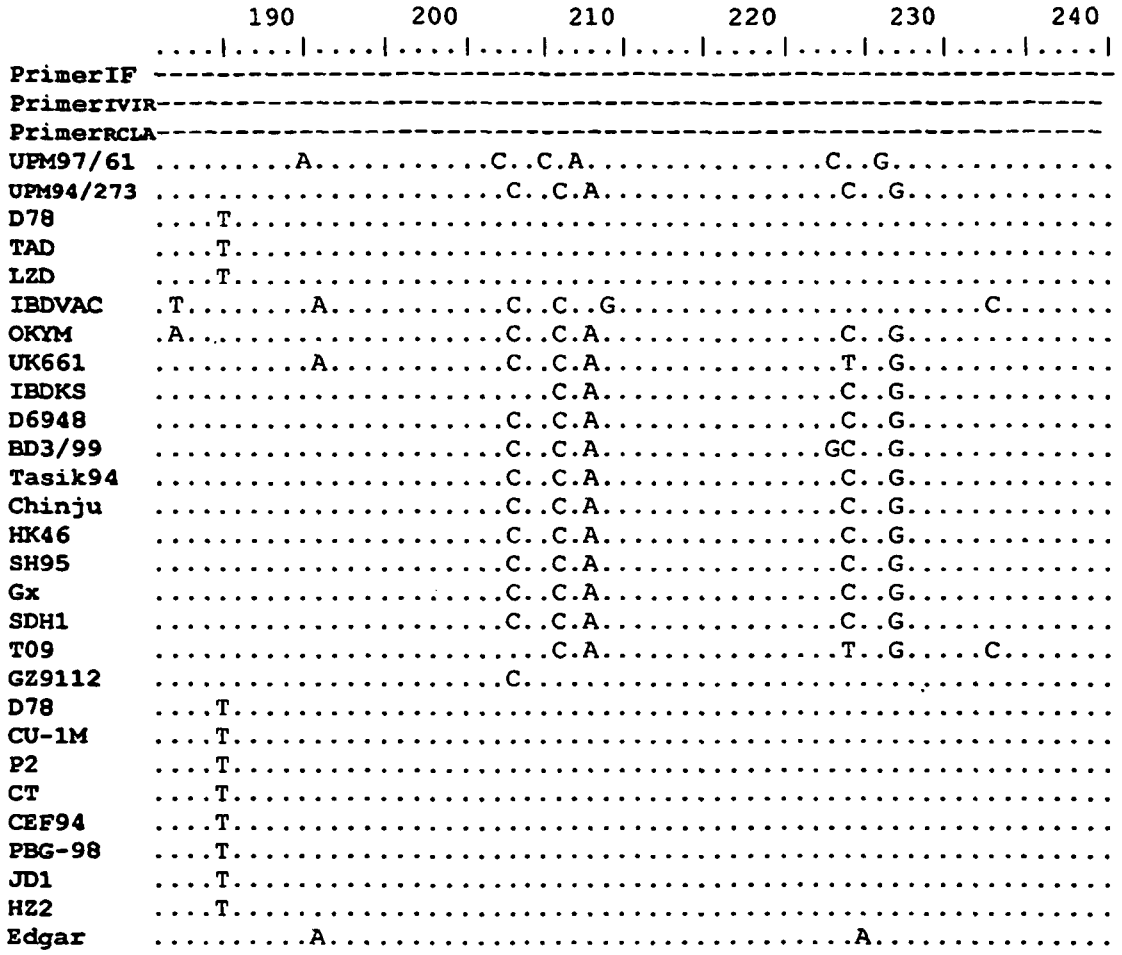


Figure 10 (continued)

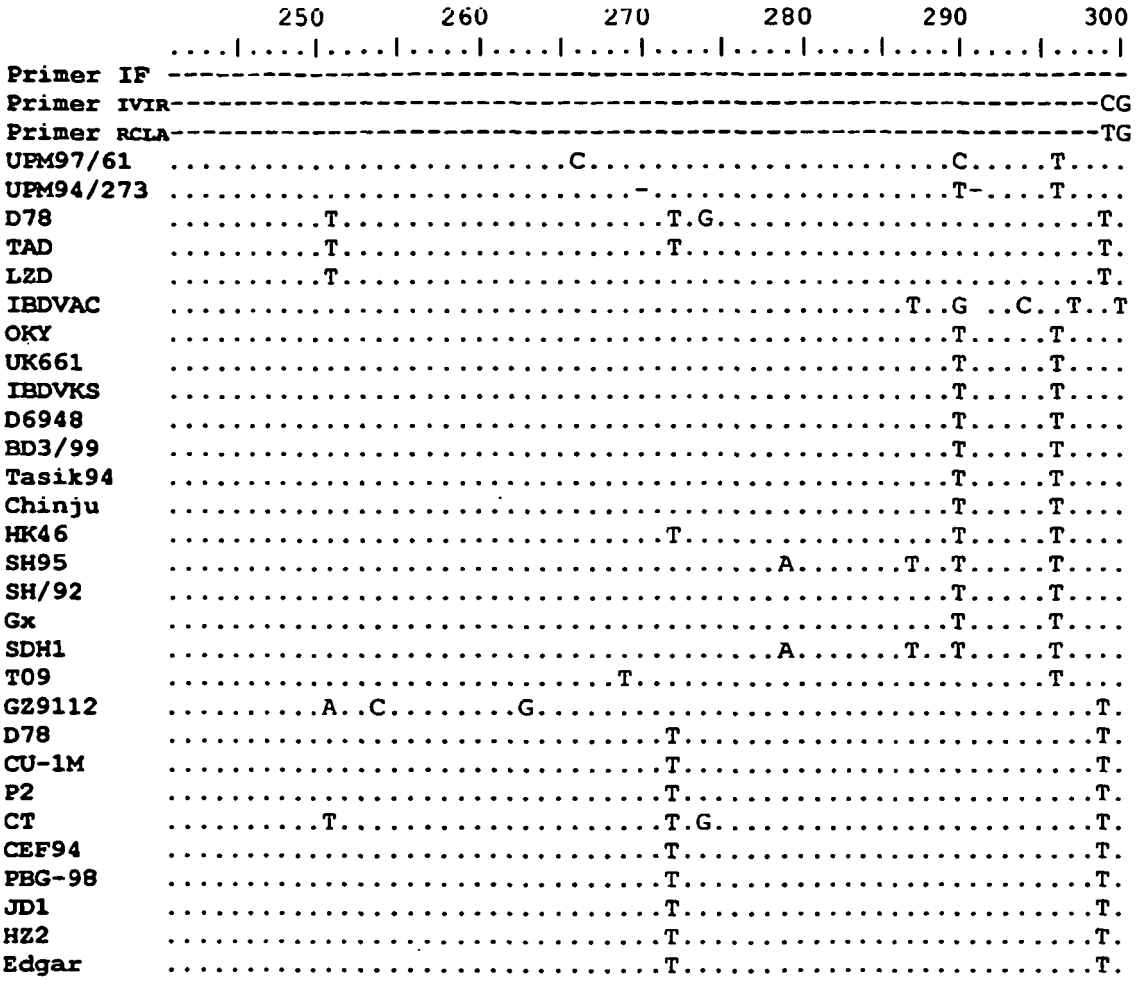


Figure 10 (continued)

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                      310
                ....|....|....|.
Primer IF       ----------------
Primer IVIR     TGAACACCGGGTCCAA
Primer RCLA     TAAACACCGGGCCCAA
UPM97/61        .G.........T....
UPM94/273       .G.........T....
D78             ................
TAD             ................
LZD             ................
IBDVAC          .......T........
OKYM            .G.........T....
UK661           .G.........T....
IBDKS           .G.........T....
D6948           .G.........T....
BD3/99          .G.........T....
Tasik94         .G.........T....
Chinju          .G.........T....
HK46            .G.........T....
SH95            .G.........T....
Gx              .G.........T....
SDH1            .G.........T....
T09             .G.........T....
GZ9112          ................
D78             ................
CU-1M           ................
P2              ................
CT              ................
CEF94           ................
PBG-98          ................
JD1             ................
HZ2             ................
Edgar           ................
```

REFERENCES CITED IN THE DESCRIPTION

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Non-patent literature cited in the description

- **Kong et al.** *Comparative immunology, Microbiology & Infectious Diseases,* 2004, vol. 27, 433-443 **[0015]**