

Rapid detection and non-subjective characterisation of infectious bronchitis virus isolates using high-resolution melt curve analysis and a mathematical model

Kylie Hewson · Amir H. Noormohammadi ·
Joanne M. Devlin · Karim Mardani ·
Jagoda Ignjatovic

Received: 14 October 2008 / Accepted: 2 March 2009 / Published online: 20 March 2009
© Springer-Verlag 2009

Abstract Infectious bronchitis virus (IBV) is a coronavirus that causes upper respiratory, renal and/or reproductive diseases with high morbidity in poultry. Classification of IBV is important for implementation of vaccination strategies to control the disease in commercial poultry. Currently, the lengthy process of sequence analysis of the IBV S1 gene is considered the gold standard for IBV strain identification, with a high nucleotide identity (e.g. $\geq 95\%$) indicating related strains. However, this gene has a high propensity to mutate and/or undergo recombination, and alone it may not be reliable for strain identification. A real-time polymerase chain reaction (RT-PCR) combined with high-resolution melt (HRM) curve analysis was developed based on the 3'UTR of IBV for rapid detection and classification of IBV from commercial poultry. HRM curves generated from 230 to 435-bp PCR products of several IBV strains were subjected to further analysis using a mathematical model also developed during this study. It was shown that a combination of HRM curve analysis and the mathematical model could reliably group 189 out of 190 comparisons of pairs of IBV strains in accordance with

their 3'UTR and S1 gene identities. The newly developed RT-PCR/HRM curve analysis model could detect and rapidly identify novel and vaccine-related IBV strains, as confirmed by S1 gene and 3'UTR nucleotide sequences. This model is a rapid, reliable, accurate and non-subjective system for detection of IBVs in poultry flocks.

Introduction

Infectious bronchitis virus (IBV) is a group 3 *Coronavirus*, a member of the family *Coronaviridae*, order *Nidovirales*, and is highly contagious and prevalent in all types of poultry flocks worldwide. IBV is the causal agent of infectious bronchitis (IB), which is characterised by respiratory and/or renal and/or reproductive disease [5]. The clinical signs can be accompanied by mortalities in broiler chickens and reduced egg shell and albumin quality in layer hens, leading to economic loss for poultry farmers. Many different IBV strains have been isolated and classified around the world [3, 10, 26] since IB was first identified in the United States in the 1930s [1]. Notably, international strains have been constantly diverging, creating a distinct and diverse sub-lineage of IB exclusive to countries such as Australia [11, 26, 28]. The types of strains identified in Australia to date have been classified as either subgroup 1, 2 or 3, [10] or unrelated field strains.

There is no treatment for IB, and thus implementation of preventative measures is the most effective way to control spread of the disease. Due to the airborne nature of IBV transmission [6], biosecurity alone is not always an effective control measure. As a consequence, vaccination protocols are widely implemented [24]. The use of live-virus vaccines, however, may involve reversion of the

K. Hewson (✉) · A. H. Noormohammadi · J. Ignjatovic
Department of Veterinary Science,
The University of Melbourne,
250 Princes Highway Werribee, Melbourne,
VIC 3030, Australia
e-mail: khewson@unimelb.edu.au

J. M. Devlin
Department of Veterinary Science,
The University of Melbourne, Parkville,
Melbourne, VIC 3010, Australia

K. Mardani
Faculty of Veterinary Medicine, The University of Urmia,
Nazloo, Urmia, West Azarbaijan, Iran

vaccine virus to virulence and subsequent disease [9]. It is therefore important to determine if disease is a result of infection with a vaccine strain (reverted to virulence), a known virulent field strain, or a previously unidentified IBV strain so that appropriate measures can be taken.

At present, the most effective method for IBV detection and characterisation involves nucleotide sequencing of the S1 gene. Homologies of $\geq 95\%$ generally indicate that strains are related [4, 10]. However, the S1 gene is one of the most variable regions of the IBV genome and may be subject to recombination events [12, 13, 17]. Although S1 gene sequencing is considered the gold standard for IBV identification, it is often not rapid enough to allow an adequate response to an IBV outbreak. Detection and characterisation of IBV strains by sequencing analysis can take weeks; however, an IBV infection only lasts 3–10 days [5]. The inability to achieve rapid identification severely restricts the implementation of appropriate measures to prevent spread and reoccurrence.

A recent study in our laboratory [20] identified a deletion in the 3' untranslated region (3'UTR) of the Australian subtype B vaccines [28] and genetically related (based on S1 gene nucleotide sequence) field isolates. It was suggested that the 3'UTR could be used to differentiate vaccine and field isolates using real-time polymerase chain reaction (RT-PCR), which is more rapid than sequence analysis. Williams et al. [30] have also discussed the presence of this hypervariable region in the 3'UTR of IBV, with conserved flanking regions, indicating that this region could be used to differentiate IBV strains.

Recently, a new technology termed high-resolution melt (HRM) curve analysis has been used to differentiate influenza A virus subtypes [18], detect various bacterial genotypes [23] and screen for mutations linked to specific diseases [14, 15, 27]. This method is reported to accurately identify single base changes in nucleotide sequences of up to 400 bp in length [25].

The main aim of this study was to evaluate the reliability of HRM curve analysis of the 3'UTR of the IBV genome for the detection, differentiation and characterisation of IBV strains.

Materials and methods

IBV strains

A number of Australian field and vaccine strains of IBV were used in this study. The IBV vaccine strains were vaccine strain A, I, VicS (Fort Dodge Australia Pty Ltd), S (Intervet Australia Pty Ltd) and B (Vaxsafe IB[®], Bioproperties Australia Pty Ltd.). Vaccines I, VicS and S have been further classified as subtype B [28] and subgroup 1 [10]

viruses. The S1 gene and 3'UTR nucleotide sequences were available in GenBank [2] for vaccine strains A and VicS only (GenBank accession numbers DQ490205 and DQ490221, respectively). The S1 gene amino acid sequences for vaccines I and S have been characterised previously [10], but their nucleotide sequences were unavailable in GenBank. Therefore, along with those of vaccine B (previously uncharacterised vaccine), the S1 gene and 3'UTR nucleotide sequences for vaccines I and S were determined as part of this study (GenBank accession numbers FJ235189, FJ235187 and FJ235188 and FJ235183, FJ235181 and FJ235182, respectively).

The field strains used were Q1/99, N1/62, Q1/73, Q1/76, V1/71, V5/90 [20], and subgroup 2 strains Q3/88, V6/92, V18/91 and N1/88 [10, 21, 26]. The nucleotide sequences for the S1 gene and 3'UTR of these strains were available in GenBank (accession numbers DQ490211, DQ490206, DQ490209, DQ490210, DQ490214, DQ490218, DQ490212, DQ490219, DQ490220, and DQ490207, respectively).

The S1 gene and 3'UTR nucleotide sequences for the previously described IBV strains N1/03 (subgroup 3) [10], H104 [20], V1/02 [22] and Q4/99 [20] were determined as part of this study and submitted to GenBank (accession numbers FJ235194, FJ235193, FJ235190 and FJ235192 and FJ235186, FJ235185, FJ235184 and FJ235180, respectively). A recent field isolate, V1/07, was isolated from the tracheae of 31-day-old broiler chicks in Victoria and its S1 gene (GenBank accession number FJ235191), and 3'UTR sequences (GenBank accession number FJ235179) were determined.

Real-time PCR and sequencing primers

The sequence and location of the primers designed and used for amplification and/or sequencing of the S1 gene and 3'UTR are provided in Table 1. All primers were synthesized by Invitrogen (Invitrogen, Victoria, Australia).

IBV RNA extraction

All IBV strains (excluding V1/07) were propagated by passaging allantoic fluids once in 10-day-old specific-pathogen-free (SPF) chicken eggs. For V1/07, the tracheal scraping was homogenised in medium 199 containing 10% foetal calf serum and 1% penicillin/streptomycin, and passaged five times as above. All vaccines were diluted to 1 labelled dose/ μl before viral RNA was extracted. One hundred microliter of diluted vaccine or allantoic fluid containing field strains was lysed in 400 μl RLT buffer (Qiagen, Victoria, Australia) with 1% β -mercaptoethanol for 2 h at 4°C. RNA was extracted using spin columns (Pacific Laboratory Products, Victoria, Australia) bedded with QiaexII suspension matrix beads (Qiagen) and eluted

Table 1 The sequences and binding sites of the primers used in this study

Primer	Primer direction	Sequence (5' – 3')	Gene / location ^a	Product size ^b (bp)	Reference
All1-F	Sense	CAGCGCCAAAACAACAGCG	3'UTR / 26930-26948	} 433	<i>This study</i> [20]
Del1-F	Sense	GAGAGGAACAATGCACAGC	3'UTR / 27016-27034		
Del1-R	Anti-sense	CATTTCCTGGCGATAGAC	3'UTR / 27362-27344		
PP1-F	Sense	GGTAAGTTACTGGTAAGAGATGTTGG	S1 / 20349-20374	} 749	<i>This study</i> <i>This study</i>
PP1-R	Anti-sense	GATACAATCCATCTGAAAAATTACC	S1 / 21097-21073		
PP2-F	Sense	TAGCATGCCAATATAACTG	S1 / 21053-21074	} 1821	<i>This study</i> <i>This study</i>
PP2-R	Anti-sense	GTTTGTATGTACTCATCTGTAACAG	S1 / 22169-22145		

^a Location refers to the primer's corresponding position on the reference IBV strain Beaudette (GenBank accession no. NC001451)

^b Product size indicates the size in bp of the two primers indicated by }

in 50 µl diethylpyrocarbonate (DEPC)-treated water. DEPC-treated water and a tracheal scraping from a healthy 31-day-old broiler chicken passaged five times through SPF eggs were used as negative controls for extraction, reverse transcription and PCR.

Reverse transcription

One micro liter of Oligo(dT)₁₅ primer (Promega, Victoria, Australia) was added to 5 µl of extracted RNA, and the mixture was heated at 75°C for 1 min, then chilled on ice for 5 min. Nineteen microliter of cDNA master mix containing 1 U RNasin[®] ribonuclease inhibitor (Promega), 1 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega), 5 µl MMLV 5× buffer, 9.5 µl DEPC-treated water and 50 µM each dATP, dTTP, dCTP, dGTP, was added to each tube, resulting in a final volume of 25 µl. The mixture was incubated at 42°C for 1 h, then 100°C for 5 min, and then subjected to RT-PCR.

Real-time PCR of the IBV 3'UTR

The All1-F primer was designed by aligning all available Australian IBV nucleocapsid gene sequences using ClustalW2 (<http://www.ebi.ac.uk>) and selecting a conserved region at the 3' end of this gene (approximately 170 bp upstream of the 3'UTR).

Each 25-µl RT-PCR reaction consisted of 5 µl 5× Green GoTaq[®] Flexi Buffer (Promega), 2 µM each primer (All1-F, Del1-F and Del1-R), 2 mM MgCl₂, 8 µM Syto-9 (Invitrogen), 50 µM of each dATP, dTTP, dCTP and dGTP, 1 U GoTaq[®] DNA polymerase (Promega) and 5 µl cDNA. RT-PCR was performed using a Rotorgene 6000 (Corbett Life Science, NSW, Australia) with an initial denaturation step of 94°C for 1 min, followed by 30 cycles of 94°C for 20 s, 57°C for 20 s, 72°C for 30 s, and a final extension of 72°C for 2 min. The quantity of template cDNA was adjusted to ensure that the copy threshold (Ct) values for all samples fell within the range of 25 ± 5. Each reaction was performed in triplicate.

PCR amplification of the IBV S1 gene

PCR amplification of the S1 gene was carried out on vaccines I, S and B, and field strains Q4/99, V1/02 and V1/07. Each 50-µl PCR reaction consisted of 5 µl 10× High Fidelity PCR Buffer (Invitrogen), 5 µl IBV cDNA, 0.4 µM each primer (PP1-F and PP2-R), 50 µM each of dATP, dTTP, dCTP and dGTP (Promega), 2 mM MgSO₄ and 1.5 U Platinum[®]Taq High Fidelity (Invitrogen). This reaction was carried out using a BioRad iCycler Thermal Cycler with an initial denaturation step of 94°C for 1 min and then 40 cycles of 94°C for 30 s, 53°C for 30 s and 68°C for 140 s, with a final extension of 68°C for 3 min.

Nucleotide sequencing and sequence analysis

Following amplification, 20 µl of each 3'UTR PCR reaction for H104, N1/03, V1/07, V1/02, Q4/99, and vaccine strains I, S and B, was subjected to electrophoresis through a 1.5% agarose gel. The appropriate bands were excised and purified using a QIAquick Gel Extraction Spin Kit (Qiagen). DNA was eluted in 50 µl EB buffer (Qiagen) and subjected to DNA sequencing (Applied Genetic Diagnostics, The University of Melbourne, Parkville, Australia), using 5 µM of each primer (All1-F and Del1-R). Nucleotide sequencing of the S1 gene for the vaccines I, S and B, and field strains Q4/99, V1/02 and V1/07 was carried out using primers PP1-F, PP1-R, PP2-F and PP2-R as described above.

Identities for the 3'UTR corresponding to nucleotides 26977–27351 of the IBV Beaudette strain (GenBank accession number NC001451), and S1 gene sequences corresponding to nucleotides 20488–22108 of the Beaudette strain were deduced using ClustalW2 (<http://www.ebi.ac.uk>).

HRM curve analysis

HRM curve analysis was carried out on the 3'UTR triplicate PCR products immediately after PCR using a Rotorgene 6000 (Corbett Life Science). In order to

determine the optimal melting condition for differentiation of IBV strains, the 3'UTR PCR products were subjected to temperature increase increments (ramp) of 0.1, 0.2 and 0.3°C between 75 and 85°C. Each strain was set as a 'genotype' (reference strain) and the average HRM genotype confidence percentages (GCPs) (value attributed to each strain being compared to the genotype, with a value of 100 indicating an exact match) for the replicates (disregarding the most outlying replicate) were tabulated.

The HRM GCPs for all related IBV strains (based on an S1 gene identity $\geq 95\%$ and a 3'UTR identity of $\geq 97\%$) were tabulated. Pairs containing comparisons to S, V1/02 or VicS were removed due to discrepancies in their melting pattern (e.g. temperature shifts) that resulted in low GCPs. The GCPs for the remaining pairs were averaged, and the standard deviation (SD) calculated. A value of $-1.645 \times SD$ (-1.645 corresponds to the lower 5% of the area under the normal distribution that has a mean of zero and a SD of one) was used to establish the GCP range.

Results

A high inter-strain correlation exists between S1 gene and 3'UTR sequence identities

In order to investigate if inter-strain variations in IBV 3'UTR sequences were reflected in the S1 gene sequences, the S1 gene and 3'UTR sequence identities were determined (Table 2) and used to calculate the Spearman's rank correlation coefficient between the two sets of figures. The initial correlation coefficient for 190 comparisons was determined to be 0.45, but after exclusion of the values for comparisons involving N1/03 and its related strain V1/07, the correlation coefficient for the remaining 154 comparisons was 0.83. Note that the N1/03 strain, a subgroup 3 virus, is presumed to be a recombinant strain [10] and was found to have an S1 gene identity of 99% and a 3'UTR identity of 100% with V1/07.

Subgroup 2 Australian IBV strains had S1 gene sequence identities of 78–99% with each other but 64–67% with other Australian strains. Subgroup 2 strains also had 3'UTR identities of 67–99% with each other and 3'UTR identities of 19–53% with other strains.

A reliable RT-PCR to detect all available strains of IBV

For analogous HRM analysis it is necessary for all strains to be amplified to comparable quantities. The oligonucleotide primer pair Del1-F/Del1-R [20] was initially used to amplify the 3'UTR of IBV strains available in our laboratory. However, the efficiency of PCR, as determined by the quantity of PCR products on an agarose gel (Fig. 1),

was low for a number of IBV strains (all subgroup 2 and three strains) using the Del1-F primer compared to the PCR performed using All1-F, despite using comparable quantities of template cDNA in both reactions. This indicated that the All1-F forward primer was more efficient than the Del1-F primer. Alignment of all available nucleocapsid-3'UTR sequences for the Australian IBV strains revealed a number of mismatches between the sequence of the oligonucleotide primer Dell-F and these IBV strains (a representation of this alignment is shown in Fig. 2). Thus a new forward primer (All1-F) was designed and used in conjunction with primer Del1-R to amplify a 230–435-bp region of the IBV genome from all available Australian strains to approximately similar efficiencies (Fig. 1).

In order to further increase the reproducibility and reliability of the HRM curve analysis (by obtaining similar amplified quantities of final PCR products before melting), finer adjustments, by diluting, were made to the cDNA templates. Therefore, a Ct parameter of 25 ± 5 cycles at a threshold of 0.1 was established. The cDNA for 15 of the 20 strains was diluted accordingly to obtain a Ct value within this range. For subsequent experiments examining unknown strains, the previously adjusted quantity of reference strain cDNA was used. Template cDNA from unknown specimens required adjustment only if a Ct value of less than 20 was generated.

HRM analysis of the 3'UTR as a reliable tool for identification of IBV strains

Visual examination of the conventional melt curves at different ramps revealed that a ramp of 0.3°C resulted in the most distinct conventional melt curves for IBV strains (Fig. 3). Ramps of 0.1 and 0.2°C produced jagged peaks that made visual comparison and further analysis of the melt profiles difficult (results not shown). Subtype B vaccines (and their related field isolates) were distinguished by a sharp double-peaked melt curve, with the first peak being shorter than the second. Vaccine B produced this conventional melt curve of shape. In contrast, vaccine strain A generated a double-peaked melt curve with a broad first peak slightly higher than the second peak, $\sim 1.5^\circ\text{C}$ later. V1/02 generated a similar two-peak conventional melt curve shape, while Q1/73 and H104 generated a triple-peaked melt curve. Subgroup 2 strains were distinguished by a single-peaked melt curve, and all remaining strains had a variation of a double-peaked melt curve shape with the first peak being higher than the second. VicS gave a conventional melt curve similar to that of the subtype B vaccines, but it varied by producing two broad peaks as opposed to two sharp peaks.

Normalised HRM curves at different ramps revealed that a ramp of 0.3°C (Fig. 4) resulted in distinct profiles for

Table 2 S1 gene and 3'UTR nucleotide sequence identities of 20 IBV strains calculated using ClustalW2

	3' UTR nucleotide identity (%)																				
	A ^a	I ^a	B ^a	S ^a	S ^a	VicS ^a	Q1/73	Q1/76	Q3/88	Q1/99	Q4/99	N1/62	N1/88	N1/03	V1/71	V5/90	V18/91	V6/92	V1/02	V1/07	H104
A ^a	100	94	94	94	94	94	91	88	46	94	98	94	46	98	93	93	53	53	99	98	91
I ^a	85	100	100	99	100	100	91	93	32	100	94	92	32	95	93	99	20	20	94	95	91
B ^a	85	99	100	99	100	100	91	93	32	100	94	92	32	95	93	99	20	20	94	95	91
S ^a	85	99	99	100	99	99	90	93	32	99	95	92	32	95	93	99	20	20	94	95	90
VicS ^a	85	99	99	99	100	100	91	93	32	100	94	92	32	95	93	99	20	20	94	95	91
Q1/73	86	91	91	91	91	100	100	86	25	91	91	90	30	92	90	90	20	20	91	92	100
Q1/76	85	99	99	99	99	99	91	100	31	93	89	87	30	90	88	93	19	19	89	90	86
Q3/88	65	67	67	67	67	67	66	67	100	32	46	45	92	45	32	32	68	73	46	45	25
Q1/99	85	99	99	99	99	99	91	99	67	100	94	92	32	95	93	99	20	20	94	95	91
Q4/99	94	90	90	90	90	90	87	90	65	90	100	94	46	98	94	94	20	20	99	98	91
N1/62	97	85	85	85	85	85	86	85	65	85	93	100	44	94	95	92	19	20	94	94	90
N1/88	65	65	65	65	65	65	64	65	78	65	65	64	100	45	44	32	67	73	46	45	30
N1/03	64	64	64	64	64	64	64	64	64	64	64	63	62	100	94	95	20	20	98	100	92
V1/71	84	88	88	88	88	88	86	88	67	87	85	85	66	64	100	93	19	20	93	94	90
V5/90	85	99	99	99	99	99	91	99	67	99	90	85	65	64	88	100	20	20	94	95	90
V18/91	64	65	65	65	65	65	64	65	78	65	64	64	95	61	65	65	100	99	53	20	20
V6/92	64	66	66	66	66	66	64	65	78	65	65	64	95	61	65	65	99	100	19	20	20
V1/02	99	84	84	84	84	84	86	84	65	84	94	98	64	63	84	84	64	64	100	98	91
V1/07	64	64	64	64	64	64	64	64	63	64	65	63	61	99	64	64	60	60	64	100	92
H104	86	91	92	91	91	91	99	91	66	91	87	86	64	64	86	91	64	64	86	64	100

^a Vaccine

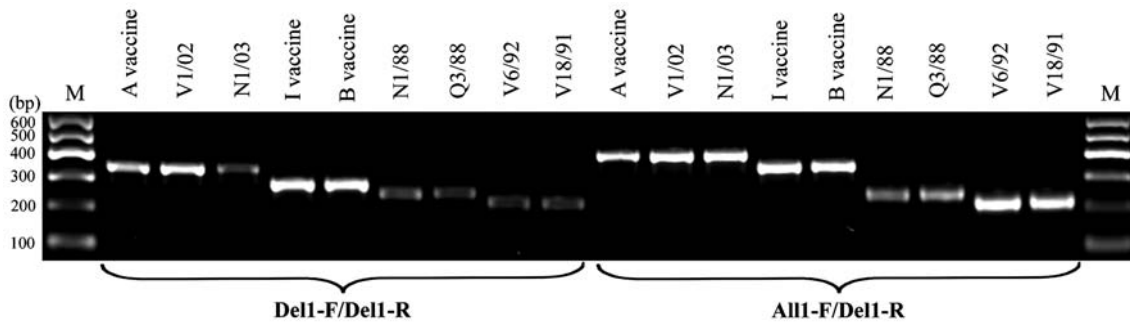


Fig. 1 Agarose gel electrophoresis of PCR products of the 3'UTR region generated using oligonucleotide primers Del1-F and Del1-R or All1-F and Del1-R from a number of IBV strains. The molecular weight marker used was Gelpilot 100-bp ladder (Qiagen)

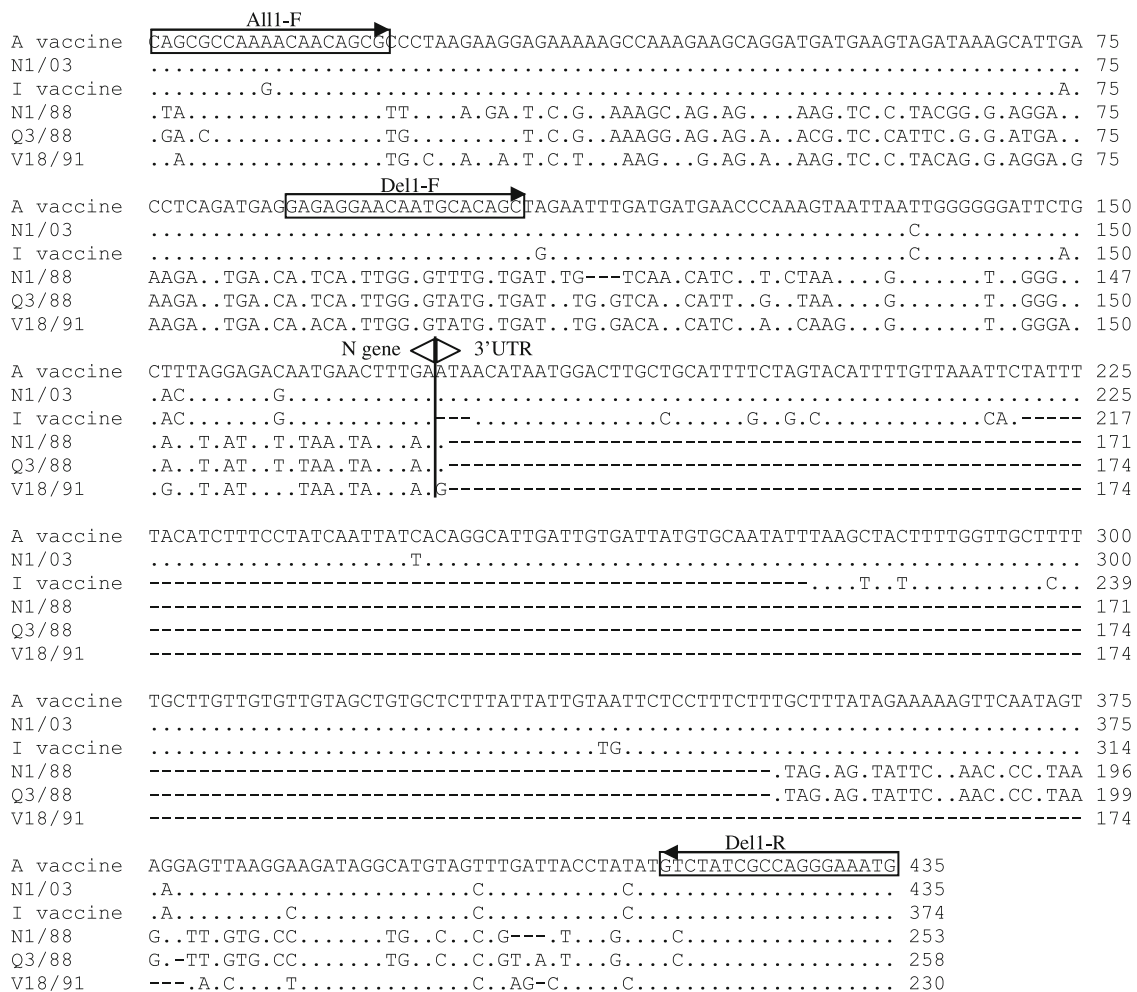


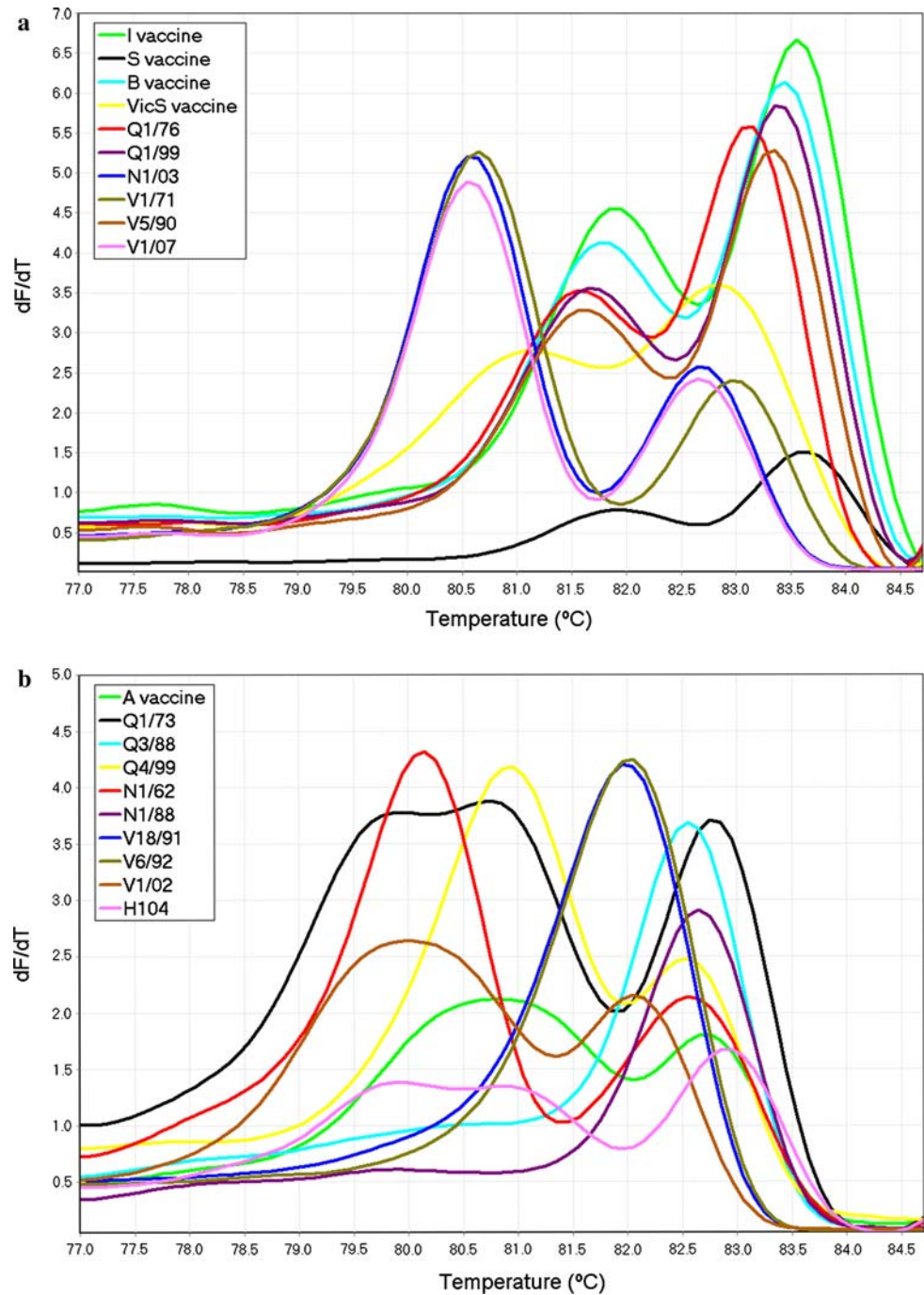
Fig. 2 Alignment of the 3'UTR for representative IBV strains used in this study. Nucleotide sequences for 3'UTR were aligned using ClustalW2 (<http://www.ebi.ac.uk>). Conserved nucleotides are shown by *dot*, while deletions are shown by *dash*. The 3' end of the

nucleocapsid gene (N gene) and the 5' end of the 3'UTR are shown by *arrowheads*. The locations of primers used in this study are indicated with *boxed arrows*

IBV strains and conveyed highest identities between known related strains (previously established by S1 gene sequence identities). Average GCPs (± 2.12) at a ramp of 0.3°C for strains in this study are shown in Table 3.

The conventional melt and normalised HRM curves were different for all strains determined to be unrelated based on their S1 gene sequence (e.g. V1/07 and N1/03 with vaccine A and A-related strains). It was also observed

Fig. 3 **a** Conventional melt curves of the 3'UTR PCR products from Australian subtype B vaccines and vaccine-related strains, subgroup 3 strains N1/03 and V1/07, and field strain V1/71.
b Conventional melt curves of the 3'UTR PCR products from vaccine strain A and related strains, subgroup 2 strains and various unrelated field strains



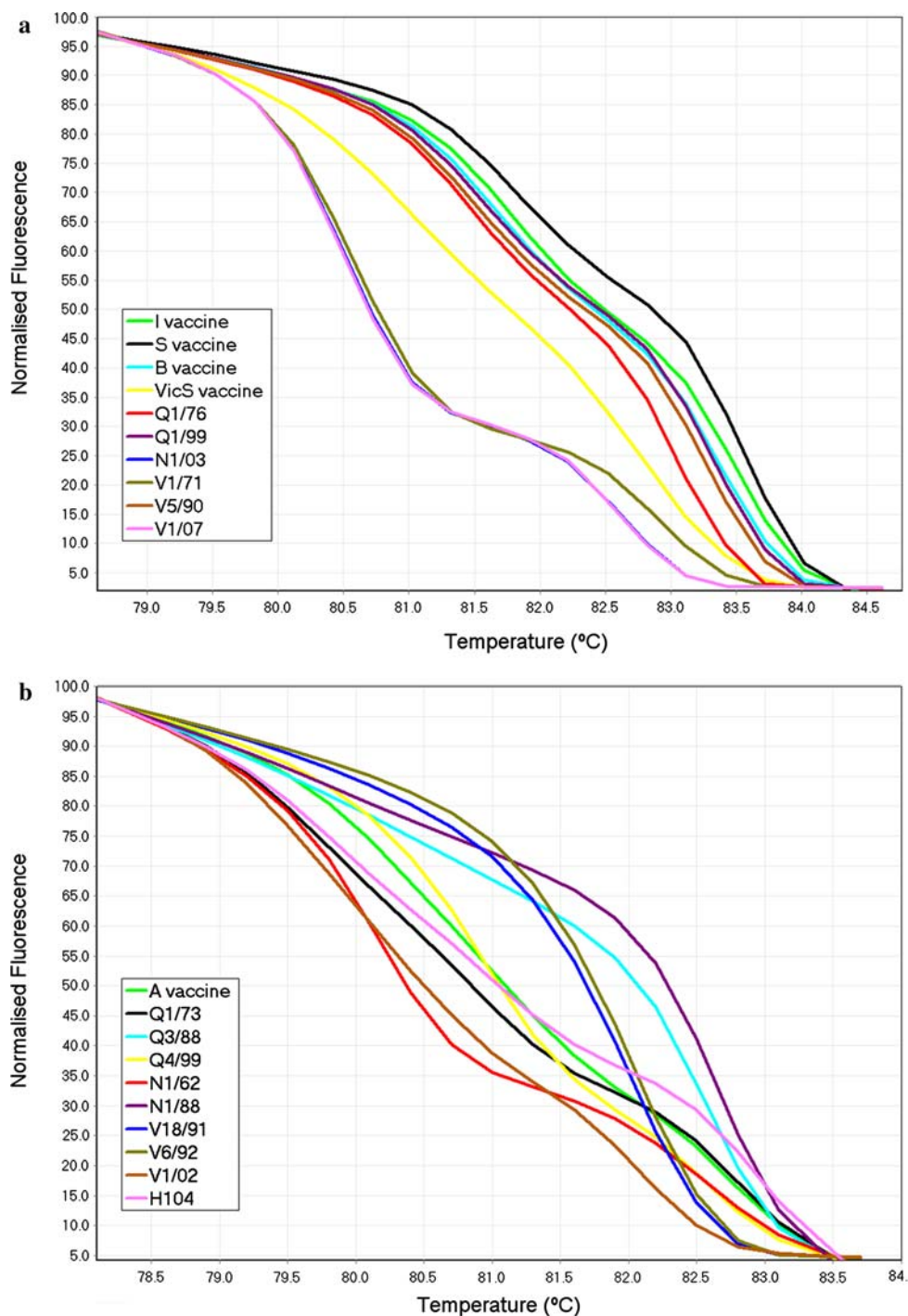
that strains with S1 gene identities of $\geq 95\%$ and 3'UTR identities $\geq 97\%$ (see Tables 2, 4) had similar conventional melt and normalised HRM curves as indicated by the relative height, number of peaks and temperature differences between peaks. For example, see the melt profiles of V1/07 and N1/03, H104 and Q1/73, and V6/92 and V18/91 (Figs. 3, 4). Field strains V5/90 and Q1/99 had profiles that resembled those of vaccines I and B. Exceptions to this observation were VicS, S and V1/02, with the latter two strains differing from subtype B vaccines and vaccine

strain A, respectively, by a melting temperature shift of their melt/HRM curve profiles only.

A mathematical system to relate or distinguish IBV strains

Using GCPs for pairs of IBV strains that had an S1 gene identity $\geq 95\%$ and 3'UTR identity of $\geq 97\%$ (Table 4), a cutoff value was generated as a mathematical model to assess the relationship of the IBV strains without visual

Fig. 4 a Normalised melt curves of the 3'UTR PCR products from Australian subtype B vaccines and vaccine-related strains, subgroup 3 strains N1/03 and V1/07, and field strain V1/71. **b** Normalised melt curves of the 3'UTR PCR products from vaccine strain A and related strains, subgroup 2 strains and various unrelated field strains



interpretation by the operator (non-subjective). VicS, S and V1/02 were not included in these calculations as their GCPs were influenced by the temperature shifts described above. The average of the GCPs was 93.09 with an SD of 7.88. A value of 12.96 (7.88×-1.645 (see “[Materials and methods](#)”))) was then calculated and subtracted from the average GCP to determine a cutoff point. Thus the GCP range for related field samples was determined to be 80.13–100.

Comparisons of each possible pair of IBV strains (excluding comparison of each strain to itself) generated 190 GCPs. Using the range of 80.13–100 calculated above, 168 out of 190 GCPs were typed in accordance with the identities of their S1 gene and 3'UTR sequences. Ten out of the 168 comparisons had 3'UTR identities $\geq 97\%$ and S1 gene identities ≥ 95 , and GCPs above 80.13 (and hence were related), while the remaining 158 had 3'UTR

Table 3 Average genotype confidence percentages (± 2.12) resulting from HRM analysis of the IBV 3'UTR amplicon of 20 IBV strains at a ramp of 0.3°C

Genotype confidence percentage for the 3'UTR		A ^a	I ^a	B ^a	S ^a	VicS ^a	Q1/73	Q1/76	Q3/88	Q1/99	Q4/99	N1/62	N1/88	N1/03	V1/71	V5/90	V18/91	V6/92	V1/02	V1/07	H104
A ^a		100	0.07	0.08	0	1.24	60.86	0.57	0.32	0.52	81.96	8.11	0.08	59.73	64.34	0.11	3.83	4.25	21.84	26.69	83.57
I ^a		100	99.75	14.92	63.80	0.03	63.79	9.61	82.31	0.03	0	0	17.51	0	0	99.19	0.70	0.55	0	0	0.43
B ^a		100	100	15.29	65.37	0.03	63.68	9.68	82.64	0.03	0	0	17.40	0.01	0.03	97.05	1.05	0.84	0	0	0.77
S ^a		100	100	5.59	0	0	2.27	0.18	4.72	0	0	0	0.55	0	0	23.89	0.01	0.01	0	0	0.02
VicS ^a		100	100	0.50	100	0.50	68.90	14.26	84.76	0.66	0	0	15.73	0.32	0.78	50.58	4.70	4.08	0	0.11	10.07
Q1/73		100	0.15	0.17	32.98	29.41	60.56	73.63	0.09	1.07	1.17	24.29	47.93	83.14	3.70	0.03	0	0	0	0	0
Q1/76		100	32.36	88.14	0.31	0	42.07	0.06	0.19	54.97	5.53	4.82	0	0.03	3.70	0	0	0	0	0	0
Q3/88		100	17.41	0.22	0	77.46	0.04	0.07	10.25	14.43	12.4	0	0.11	3.97	0	0	0	0	0	0	0
Q1/99		100	0.26	0	23.83	0	0.02	98.23	0.78	6.65	0	0	1.94	0	0	0	0	0	0	0	0
Q4/99		100	100	3.51	0.04	37.55	37.84	0.08	7.65	8.18	16.65	31.73	46.62	0	0	0	0	0	0	0	0
N1/62		100	0	73.98	69.16	0	0.07	0.07	0.07	0.07	61.06	78.25	17.51	0	0	0	0	0	0	0	0
N1/88		100	100	0	0	26.52	1.83	1.48	0	0.01	1.15	1.15	0	0	0	0	0	0	0	0	0
N1/03		100	86.31	0	0.39	0.46	53.88	96.27	30.08	45.82	0	0	0	0	0	0	0	0	0	0	0
V1/71		100	0.02	0.32	0.35	34.91	80.52	45.82	0	0	0	0	0	0	0	0	0	0	0	0	0
V5/90		100	100	0.58	0.45	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V18/91		100	99.27	0.08	1.23	6.88	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V6/92		100	100	0.10	0.39	3.19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V1/02		100	100	71.55	9.55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V1/07		100	100	23.63	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H104		100	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

^a Vaccine

Table 4 Genotype confidence percentages for strains that had a 3'UTR identity of ≥ 97 and an S1 gene identity of ≥ 95

Strain 1	Strain 2	Identity score (%)		Genotype confidence percentage
		3'UTR	S1 gene	
I Vaccine	Q1/99	99	99	82.31
I Vaccine	V5/90	99	99	99.19
I Vaccine	B vaccine	100	99	99.75
B Vaccine	V5/90	99	99	97.05
B Vaccine	Q1/99	100	99	82.64
V5/90	Q1/99	99	99	98.23
N1/03	V1/07	100	99	96.27
H104	Q1/73	100	99	83.14
V18/91	V6/92	99	99	99.27

identities $< 97\%$ and S1 gene identities < 95 , and GCPs below 80.13 (and hence were unrelated).

Out of the 22 GCPs that were not typed or differentiated in accordance to their 3'UTR and/or S1 gene identities, four involved GCPs of > 80.13 that were not in accordance with their low ($< 97\%$) 3'UTR identities and low ($< 95\%$) S1 gene identities. These included comparison of V1/71 with N1/03 and V1/07 and comparison of vaccine A with H104 and Q4/99. Nine comparisons involved GCPs of < 80.13 that were not in accordance with both their high ($\geq 95\%$) S1 gene identities and high 3'UTR ($\geq 97\%$) identities. GCPs of < 80.13 were in accordance with the low ($< 97\%$) 3'UTR identities of Q1/76 with subtype B vaccines and related field strains, N1/62 and vaccine A and of N1/88 with subgroup 2 strains V18/91 and V6/92, but not in accordance with their high S1 gene identities ($\geq 95\%$). Also, one comparison involved a GCP of > 80.13 that was in accordance with the high ($\geq 95\%$) S1 gene identity but not with the 3'UTR identity of $< 97\%$. Visual examination of the conventional melt curves for 21 of the 22 comparisons that were not in accordance with 3'UTR or S1 gene identities could type them in accordance with their 3'UTR and S1 gene identities. For example, Q1/76 generated the curve displayed only by subtype B vaccines and their related field strains (Fig. 3). The exception was strain N1/62, which could not be typed according to its S1 gene with vaccine A.

Discussion

This study describes, for the first time, a rapid and reliable strain identification technique for IBV. This study also establishes, for the first time, a mathematical model for non-subjective interpretation of the data derived from HRM curve analysis.

Using the GCPs in conjunction with the shape of the conventional melt curves, all strains under investigation in

this study were typed in accordance to their 3'UTR sequences. With the exception of N1/62, all strains were also typed in accordance to their S1 gene sequences.

The results of this investigation are consistent with previous studies exploring the relationships between Australian IBV strains [11, 20, 22, 26], with no unrelated strains being typed as similar in the current study. The correlation coefficient for the S1 gene and 3'UTR values, with the exceptions of N1/03 and V1/07, was 0.83. This represents a strong inter-strain correlation between the S1 gene and the hypervariable region in the 3'UTR, and assists in validating the use of the 3'UTR for IBV strain differentiation.

The IBV 3'UTR hypervariable region was used for HRM analysis as it appears less prone to spontaneous mutation than the S1 gene [16]. Current techniques for IBV strain identification require virus isolation from infected tissues in SPF eggs to produce high-quality RNA for efficient PCR and S1 gene sequencing. The technique developed in this study is comparatively faster as it utilises a small PCR product that can be generated directly from infected tissue (unpublished data), obviating the need for viral isolation. Indeed, the entire procedure described here (from receipt of infected tissue to HRM analysis) can be completed in < 5 h. This is much faster than conventional DNA sequencing, which takes at least 24 h (provided that the relevant equipment is readily available), excluding subsequent comparison and interpretation of the sequencing results. In terms of cost, the described method also has further advantages over sequencing as the Syto-9 dye is the only added cost to standard PCR. This does not include the initial outlay for the real-time thermocycler, although with the introduction of RT-PCR for a variety of pathogens, a real-time thermocycler is now becoming an essential piece of equipment in many diagnostic laboratories. The skill required to perform HRM and interpret the related results for strain identification is easily gained, with many diagnostic laboratory technicians already possessing the relevant skills.

Differentiating strains using the hypervariable 3'UTR has other advantages over using the S1 gene. Firstly, there is no reliable pair of primers for amplification of the S1 gene for all IBV strains. This is particularly problematic where isolates of unknown identity (and hence unknown S1 gene sequences) are examined. Secondly, the S1 gene is known to vary slightly even between passages of a single strain [8, 19] or be a site of recombination [7, 29]. Such a mutation rate in the IBV S1 gene makes HRM curve analysis of the S1 gene unreliable for strain identification.

The GCP range of 80.13–100 calculated in this study may require adjustment as more field and reference isolates are analysed with this technique. V1/02 and S vaccine could not be included in the calculation for the GCP range due to a 1-bp substitution in each that resulted in differences in their conventional melt curves. This ultimately produced GCPs that did not accurately represent their nucleotide sequence identities with their respective related strains, although both of these isolates could still be classified correctly based on the distinctive shapes of their conventional melt curves. It was observed that the GCPs for the different IBV strains fell either within this range, just outside this range (GCP of 60–80), or far below this range (GCP value <30). More than three quarters of the strain comparisons in this study fell into this last category, meaning that, in most cases, extensive analysis of data may not be necessary.

Monitoring the shape of the conventional melt/HRM curves, in addition to analysis of their GCPs, is important as it may enable more reliable typing. This was useful when rare melting variability (evident sometimes even within replicates) caused discrepancies in GCPs. For example, the GCP of 86.31 for V1/71 with N1/03 relates these strains, but observation of their melt/HRM curves could differentiate them (see Fig. 3).

The VicS vaccine used in this study produced a GCP that was inconsistent with other subtype B vaccines despite a high 3'UTR nucleotide sequence identity (99–100%) and a similar conventional melt curve shape. The additional, smaller DNA band that was amplified during PCR of the VicS vaccine strain [20] is likely to have caused this discrepancy by modifying the shape (broadening the peaks) of the curve produced. The appearance of the two DNA bands after PCR of the 3'UTR in VicS suggested mis-priming by one or both of the primers used; however, analysis of the respective area of the VicS vaccine genome did not reveal any region with similarity to either of the primers. Thus, it seems unlikely that mis-priming was responsible for amplification of a second product by any of the primers. In recent studies in our laboratory, the nucleotide sequences of the two PCR products amplified from the commercial VicS vaccine strain were determined. The smaller band was identical in sequence to the larger band, which

matched the published VicS sequence, except that it contained a 40-bp deletion in the amplified region (unpublished data). Thus, it is suggested that the commercial VicS vaccine contains two populations of viruses with different 3'UTRs, making mathematical analysis of this vaccine impossible. Irrespective of the origin of the two strains in the VicS vaccine preparation, this demonstrates a further capacity of the HRM curve analysis for detection of more than one strain in a single preparation. Additional studies are required, however, to investigate the ability of HRM to differentiate simultaneous IBV infections.

The system developed in this study represents a significant advancement in IBV detection and classification, utilising RT-PCR and melt/HRM curve analysis. In particular, this new system is faster, more cost-effective and more reliable than the currently established methods for detecting and differentiating IBV isolates and has many potential benefits for rapid control of IBV outbreaks. Further studies are necessary to assess the sensitivity of the method for quantitative analysis and its potential as a diagnostic assay for direct examination of field specimens.

Acknowledgments Funding to support this research was provided by the Australian Egg Corporation Limited (AECL). The authors thank Naomi Kirkpatrick and Denise O'Rourke for technical assistance throughout this study. The authors would also like to thank Garry Anderson for help with statistical analyses.

References

1. Beaudette FR, Hudson CB (1937) Cultivation of the virus of infectious bronchitis. *J Am Vet Med Assoc* 90:51–58
2. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL (2008) GenBank. *Nucl Acids Res* 36:D25–D30
3. Cavanagh D (2005) Coronaviruses in poultry and other birds. *Avian Pathol* 34(6):439–448
4. Cavanagh D, Davis PJ, Mockett APA (1988) Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. *Virus Res* 11(2):141–150
5. Cavanagh D, Naqi SA (2003) Infectious bronchitis. In: Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR, Swayne DE (eds) *Diseases of poultry*, 11th edn. Iowa State Press, USA, p 101–119
6. Cumming RB (1970) Studies on Australian infectious bronchitis virus. IV. Apparent farm-to-farm airborne transmission of infectious bronchitis virus. *Avian Dis* 14(1):191–195
7. Estevez C, Villegas P, El-Attrache J (2003) A recombination event, induced in Ovo, between a low passage infectious bronchitis virus field isolate and a highly embryo adapted vaccine strain. *Avian Dis* 47:1282–1290
8. Fang SG, Shen S, Tay FPL, Liu DX (2005) Selection of and recombination between minor variants lead to the adaptation of an avian coronavirus to primate cells. *Biochem Biophys Res Commun* 336(2):417–423
9. Hopkins SR, Yoder HW Jr (1986) Reversion to virulence of chicken-passaged infectious bronchitis vaccine virus. *Avian Dis* 30(1):221–223

10. Ignjatovic J, Gould G, Sapats S (2006) Isolation of a variant infectious bronchitis virus in Australia that further illustrates diversity among emerging strains. *Arch Virol* 151(8):1567–1585
11. Ignjatovic J, Sapats SI (1997) A long-term study of Australian infectious bronchitis viruses indicates a major antigenic change. *Avian Pathol* 26(3):535–553
12. Jia W, Karaca K, Parrish CR, Naqi SA (1995) A novel variant of avian infectious bronchitis virus resulting from recombination among three different strains. *Arch Virol* 140(2):259–271
13. Kottier SA, Cavanagh D, Britton P (1995) Experimental evidence of recombination in coronavirus infectious bronchitis virus. *Virology* 213(2):569–580
14. Krypuy M, Ahmed AA, Etemadmoghadam D, Hyland SJ, Group AOCS, deFazio A, Fox SB, Brenton JD, Bowtell DD, Dobrovi CA (2007) High resolution melting for mutation scanning of TP53 exons 5–8. *BMC Cancer* 7:168–181
15. Krypuy M, Newnham GM, Thomas DM, Conron M, Dobrovi A (2006) High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. *BMC Cancer* 6:295–307
16. Lai MMC (1990) Corona virus: organization, replication and expression of genome. *Annu Rev Microbiol* 44(1):303
17. Lee CW, Jackwood MW (2000) Evidence of genetic diversity generated by recombination among avian coronavirus IBV. *Arch Virol* 145(10):2135–2148
18. Lin J-H, Tseng C-P, Chen Y-J, Lin C-Y, Chang S-S, Wu H-S, Cheng J-C (2008) Rapid differentiation of influenza A virus subtypes and genetic screening for virus variants by high-resolution melting analysis. *J Clin Microbiol* 46(3):1090–1097
19. Liu S, Han Z, Chen J, Liu X, Shao Y, Kong X, Tong G, Rong J (2007) S1 gene sequence heterogeneity of a pathogenic infectious bronchitis virus strain and its embryo-passaged, attenuated derivatives. *Avian Pathol* 36(3):231–234
20. Mardani K, Browning GF, Ignjatovic J, Noormohammadi AH (2006) Rapid differentiation of current infectious bronchitis virus vaccine strains and field isolates in Australia. *Aust Vet J* 84(1–2):59–62
21. Mardani K, Noormohammadi AH, Hooper P, Ignjatovic J, Browning GF (2008) Infectious bronchitis viruses with a novel genomic organization. *J Virol* 82(4):2013–2024
22. Mardani K, Noormohammadi AH, Ignjatovic J, Browning GF (2006) Typing infectious bronchitis virus strains using reverse transcription-polymerase chain reaction and restriction fragment length polymorphism analysis to compare the 3' 7.5 kb of their genomes. *Avian Pathol* 35(1):63–69
23. Price EP, Smith H, Huygens F, Giffard PM (2007) High-resolution DNA melt curve analysis of the clustered, regularly interspaced short-palindromic-repeat locus of *Campylobacter jejuni*. *Appl Environ Microbiol* 73(10):3431–3436
24. Ratanasethakul C, Cumming RB (1983) Immune response of chickens to various routes of administration of Australian infectious bronchitis vaccine. *Aust Vet J* 60(7):214–216
25. Reed GH, Wittwer CT (2004) Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clin Chem* 50(10):1748–1754
26. Sapats SI, Ashton F, Wright PJ, Ignjatovic J (1996) Sequence analysis of the S1 glycoprotein of infectious bronchitis viruses: identification of a novel genotypic group in Australia. *J Gen Virol* 77(3):413–418
27. Vandersteen JG, Bayrak-Toydemir P, Palais RA, Wittwer CT (2007) Identifying common genetic variants by high-resolution melting. *Clin Chem* 53(7):1191–1198
28. Wadey CN, Faragher JT (1981) Australian infectious bronchitis viruses: identification of nine subtypes by a neutralisation test. *Res Vet Sci* 30:70–74
29. Wang L, Junker D, Collisson EW (1993) Evidence of natural recombination within the S1 gens of infectious bronchitis virus. *Virology* 192(2):710–716
30. Williams AK, Wang L, Sneed LW, Collisson EW (1993) Analysis of a hypervariable region in the 3' non-coding end of the infectious bronchitis virus genome. *Virus Res* 28(1):19–27