Application of high-resolution melt curve analysis for classification of infectious bronchitis viruses in field specimens

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Objective A real-time polymerase chain reaction (PCR)/high-resolution melt (HRM) curve analysis protocol was developed in our laboratory to differentiate infectious bronchitis (IB) virus reference strains. In the current study, this method was used to detect and classify IB viruses in field submissions.

Procedure Over an 11-month period samples from 40 cases of suspected IB virus were received and 17 submissions were positive for IB virus by polymerase chain reaction. HRM curve analysis classified each strain as subgroup 1, 2 or 3 strain (12 submissions) or a strain that was unable to be classified (5 submissions). The 3′ untranslated region (UTR) and partial S1 gene nucleotide sequences for the 17 IB virus strains were determined and their identity with those of the relative reference strains compared to confirm the classifications generated using the HRM curve analysis.

Results Of the 12 IB field viruses classified as subgroup 1, 2, or 3 using HRM curve analysis, the 3′UTR and S1 gene nucleotide sequences had identities ≥99% with the respective subgroup reference strain. Analysis of the 3′ UTR and S1 gene nucleotide sequences for the five IB virus strains that could not be classified indicated that four belonged to one of the subgroups, and one was a potential recombinant strain (between strains from subgroups 2 and 3). A novel recombinant strain was also detected.

Conclusion HRM curve analysis can rapidly assign the majority of IB viruses present in field submissions to known subgroups. Importantly, HRM curve analysis also identified variant genotypes that require further investigation.

Keywords coronavirus; high-resolution melt; infectious bronchitis virus; recombinant viruses; strain identification

Abbreviations bp, base pairs; GCP, genotype confidence percentage; HRM, high-resolution melt; IB, infectious bronchitis; NSW, New South Wales; PCR, polymerase chain reaction; QLD, Queensland; SPF, specific-pathogen free; UTR, untranslated region; VIC, Victoria; WA, Western Australia

A type 3 coronavirus is the causative agent of infectious bronchitis (IB), which is a disease of major significance throughout the international poultry industry. Infection in chickens causes tracheitis and/or interstitial nephritis, the extent of which is dependent on a number of factors, including the virulence of the strain. In layers, infection with IB virus has also been implicated in declines in egg quality and production rate, caused by cytopathological changes in the oviduct, which may or may not be accompanied by characteristic respiratory signs.

Recently, we demonstrated the application of an emerging technology, high-resolution melt (HRM) curve analysis following real-time polymerase chain reaction (PCR), to differentiate IB virus strains in Australia against known reference and vaccine strains. This method could successfully and objectively classify IB virus strains as subgroup 1, 2 or 3, based on differences in their 3′ untranslated region (3′UTR).

However, the ability of HRM analyses to differentiate IB virus strains in field submissions and/or detect novel and/or recombinant strains has not been studied to date. Recombination of coronaviruses has been demonstrated in the group 2 coronavirus mouse hepatitis virus. Recombination of coronaviruses that infect different species of animals has also been detected. The ability of the IB virus to generate new strains through recombination has been suggested and examined in a number of investigations and a number of recombination sites have been detected throughout the length of the IB virus genome.

The purpose of this study was to assess the capacity of HRM curve analysis to differentiate IB virus strains in field submissions, in comparison with S1 gene sequencing, as a routine method for diagnosis.

Materials and methods

IB virus strains

The viral strains used in this study included vaccine strain 1 (subgroup 1 reference strain) (Fort Dodge Australia), N1/88 (subgroup 2 reference strain) and N1/03 (subgroup 3 reference strain). The S1 gene GenBank accession numbers are FJ235187, DQ490207 and FJ235194, respectively. The 3′UTR GenBank accession numbers are FJ235181, DQ490207 and FJ235186, respectively.

Specimens from 40 cases of suspected IB virus infection, which were submitted to the University of Melbourne between 1 June 2008 and 30 April 2009, were examined by PCR to detect IB virus and positive specimens were further examined using HRM curve analysis. Table 1 shows the location of each farm, the age of the flock, the type of tissue submitted and the age at which the chickens were vaccinated against IB virus (in all cases with subgroup 1 vaccines) for each IB-virus-positive field submission. All IB virus strains identified in field submissions and not classified within a subgroup were designated 'unclassified'.
The specimens submitted were from Queensland (QLD), New South Wales (NSW), Victoria (VIC) and Western Australia (WA) and most were trachea and/or kidney tissue, but swabs and allantoic fluid (from the second passage through specific-pathogen free (SPF) eggs) were also submitted.

**Extraction of RNA**

Total extracted RNA was available in our laboratory for all reference and vaccine strains. For field submissions, 400 µL of Qiagen RLT lysis buffer (Qiagen, VIC, Australia) with 5% β-mercaptoethanol was added to a small section (approximately 5 × 5 × 5 mm) of kidney, or a tracheal scraping or swab. For cultured viruses, 100 µL of allantoic fluid was mixed with the lysis buffer. Total RNA was extracted and complementary DNA prepared from each of the field submissions as previously described.7 Diethyl pyrocarbonate-treated water was used as a negative extraction control.

**Real-time PCR and HRM curve analysis of the 3’ UTR of IB viruses**

All primers used in this study are shown in Table 2. Real-time PCR and HRM curve analysis was performed as previously described,7 using a Rotorgene 6000 (Corbett Life Science, NSW, Australia), and the conventional melt and HRM curves were used to classify each field submission as subgroup 1, 2 or 3. The genotype confidence percentage (GCP) and subsequent classification recorded at the time of initial processing (or most recent repeat analysis) were retrieved.

The 3’UTR amplicons from each field submission were separated on a 1% agarose gel, the band was excised and the DNA extracted using the

| ID   | Submission type       | Farm location | Age of chicken (days) | Age vaccinated against IB virus (days) |
|------|-----------------------|---------------|-----------------------|---------------------------------------|
| 013  | Tissue (trachea)      | VIC           | 28                    | 1 and 7                               |
| 015  | Tissue (trachea)      | NSW           | 54                    | 1 and 21                              |
| 018  | Tissue (trachea)      | NSW           | 25                    | 1 and 7                               |
| 020  | Tissue (kidney)       | QLD           | 21                    | 1                                     |
| 025  | Tissue (kidney)       | VIC           | 8                     | 1                                     |
| 026  | Tissue (kidney)       | VIC           | 12                    | 1                                     |
| 027  | Tissue (trachea)*     | VIC           | 7                     | 1                                     |
| 028  | Tissue (kidney)*      | VIC           | 18                    | 1                                     |
| 031  | Tissue (kidney)*      | VIC           | 8                     | 1                                     |
| 032  | Swabs (trachea)       | NSW           | 7                     | 1                                     |
| 038a | Allantoic fluid (trachea, passage 2) | WA | 7 | 1 |
| 038b | Allantoic fluid (unknown, passage 2) | WA | 21 | 1 |
| 040  | Tissue (trachea)      | NSW           | 40                    | 1                                     |
| 041  | Tissue (kidney)       | QLD           | 14                    | 1                                     |
| 043  | Tissue (trachea)      | NSW           | 31                    | 1 and 19                              |
| 044  | Tissue (kidney)       | VIC           | 11                    | 1 (VicS)                              |
| 050  | Tissue (trachea)      | NSW           | 47                    | 1                                     |

*Tissue specimen chosen for analysis because both trachea and kidney specimens tested positive for IB viruses with similar HRM curves and GCPs.

*Subgroup 1 vaccines I, S and B were used.

GCP, genotype confidence percentage; HRM, high-resolution melt; NSW, New South Wales; QLD, Queensland; VIC, Victoria; WA, Western Australia.

**Table 1. Field submissions from broiler chickens determined to be positive for infectious bronchitis (IB) virus**

| Primer | Target/location* | Sequence (5’–3’) | Citation no. |
|--------|------------------|------------------|--------------|
| Poly-F1| S1 gene/20070-20090 | GATTGTGCATGGTGAGCAATG | 15 |
| PP2-R  | S1 gene/22169-22145 | GTTTGTATGTACTCATCTGTAACAG | 7 |
| All1-F | 3’UTR/26930-26948 | CAGCGCCAAAACACGGCG | 7 |
| Del1-R | 3’UTR/27362-27344 | CATTTCCTGCGGATAGAC | 24 |

*Location of the primer on the reference IB virus strain Beaudette (GenBank accession number NC001451).

UTR, untranslated region.
Qiaquick Gel Extraction Spin Kit (Qiagen) and sequenced (Applied Genetic Diagnostics, University of Melbourne, Parkville, VIC, Australia) using 5 mmol/L of each PCR primer (All1-F and Del1-R). The resulting sequences were aligned with subgroup reference strains using ClustalW2 (www.ebi.ac.uk/clustalw) and the highest identity with the reference sequence was recorded.

Amplification and sequencing of the S1 gene from each field submission
All field submissions were subjected to PCR using primers Poly-F1 and PP2-R, as previously described, to amplify a product of approximately 2100 base pairs (bp). The amplified PCR products were separated on a 1.2% agarose gel, the band was excised and sequenced as described above, using 5 mmol/L each of primer Poly-F1 and PP2-R. The sequences obtained were aligned using ClustalW2 and the nucleotide identity to reference strains recorded.

Results

Identification of IB viruses in field submissions using HRM curve analysis
Of the 40 field submissions received for IB virus detection, 17 were found to contain virus by PCR–HRM curve analysis. All these samples were from broiler flocks. In cases where both tracheal and kidney tissues had been submitted and determined to be positive for IB virus, the results of the HRM curve analysis from both organs were identical (data not shown) and only one tissue sample was used for subsequent analyses.

Two IB viruses in submissions from QLD (020 and 041), six in submissions from VIC (013, 025, 026, 027, 028 and 031), and one each from NSW (032) and WA (038a) were classified as subgroup 1 (vaccine or vaccine-related strains). Two IB viruses (018 and 040), both from NSW, were classified as subgroup 3. These classifications were based on both their conventional melt curve shape and the derivation of a GCP > 80.13 with the relevant reference strain from the normalised HRM curves. Three IB viruses detected in submissions from NSW (015, 043 and 050) and one each from VIC (044) and WA (038b) were deemed ‘unclassified’ as they had GCP values <80.13 with each of the subgroup reference strains. The conventional and normalised melt curves for all 17 IB virus field strains are shown in Figure 1.

The unclassified field strains 015 and 038b produced conventional melt curves that were distinct from the subgroup reference strains. Both 043 and 050 produced melt curve profiles that were similar to the subgroup 3 reference strain. However, a slight variation in the shape of the curves resulted in GCP values below the cut-off with the subgroup 3 reference strain (data not shown).

Submission 044 was a kidney tissue submitted from an 11-day-old SPF chicken, vaccinated at 1 day old with a subtype 1 vaccine (VicS). The

| Table 3. Classification of field submissions into subgroups of infectious bronchitis virus |
|-----------------|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| ID   | HRM classification (subgroup) | GCP with subgroup reference | S1 gene length used to generate identities | Nucleotide sequence identity with subgroup reference (%) |
|      |                                |                               |                              | S1 gene | 3′UTR |
| 013  | 1                              | 95.66                         | 1717                         | 99      | 100  |
| 015  | Unclassified                   | NA                           | 1723                         | Subgroup 2 (93) | Subgroup 3 (98) |
| 018  | 3                              | 84.95                         | 1705                         | 99      | 99   |
| 020  | 1                              | 97.41                         | 1138                         | 99      | 99   |
| 025  | 1                              | 93.32                         | 1684                         | 99      | 99   |
| 026  | 1                              | 94.14                         | 1717                         | 99      | 99   |
| 027  | 1                              | 92.43                         | 1425                         | 100     | 99   |
| 028  | 1                              | 87.94                         | 1666                         | 99      | 99   |
| 031  | 1                              | 95.86                         | 1294                         | 99      | 99   |
| 032  | 1                              | 89.99                         | 1717                         | 99      | 99   |
| 038a | 1                              | 86.19                         | 1249                         | 99      | 99   |
| 038b | Unclassified                   | NA                           | 1225                         | Subgroup 1 (99) | Subgroup 1 (99) |
| 040  | 1                              | 94.30                         | 925                          | 99      | 99   |
| 041  | 1                              | 82.80                         | –                            | 99      | 99   |
| 043  | Unclassified                   | NA                           | 800                          | Subgroup 3 (99) | Subgroup 3 (99) |
| 044  | Unclassified                   | NA                           | 1717                         | Subgroup 1 (99) | Subgroup 1 (99) |
| 050  | Unclassified                   | NA                           | –                            | Subgroup 3 (99) | Subgroup 3 (99) |

aVaccine I used as the genotype/reference. bHRM profile did not match any of the class reference strains. cReference strain N1/03 used as the genotype/reference. dHighest GCP below cut-off. eMaximum length of sequence obtained from S1 gene sequencing that was used to generate the nucleotide sequence identities (full amplicon approximately 1720 bp). fComplete S1 gene sequence obtained. gAmplification/sequencing not successful. hReference strain N1/88 used as the genotype/reference.

bp, base pairs; GCP, genotype confidence percentage; HRM, high-resolution melt; NA, not available; UTR, untranslated region.
conventional melt curve for strain 044 was similar in shape to that produced by subgroup 1 strains, but a melt curve shift to a higher temperature resulted in a GCP value below the cut-off with the subgroup 1 reference strain.

3’UTR sequences and HRM curve analysis classification

The 3’UTR of the genome was sequenced for all 17 field submissions that were positive for IB virus. These sequences were aligned with the subgroup reference strains and the highest identity with the reference strains for each of the 17 IB virus field strains was determined (Table 3). The 3’UTR nucleotide sequences for all 10 IB field viruses classified as subgroup 1 by HRM had identities of 99% and 100% with the Australian vaccine strains I and S, respectively. The 3’UTR of the unclassified field IB virus 038b contained a 40-bp deletion in its 3’UTR (data not shown), but the remaining sequence had 99% identity with vaccine strains I and S. There was a G to A base substitution in the 3’UTR of IB field virus 044 compared with subgroup 1 strains (data not shown).

IB field viruses 018 and 040 (both classified as subgroup 1) had 3’UTR nucleotide sequence identity scores of 99%, whereas the unclassified strain 015 had an identity score of 98% with the subgroup 3 reference strain N1/03 (Table 3). The unclassified IB field viruses 043 and 050 both had 3’UTR nucleotide sequence identity scores of 99% with subgroup 3 reference strain N1/03 (Table 3), with both containing the same C to T base substitution at position 353 of the amplicon (data not shown), which explained the variations in the melt curves.

S1 gene nucleotide sequences for classified field viruses

The complete S1 gene sequence (approximately 1720 bp excluding the flanking sequences for the polymerase and S2 genes) was determined for submissions 013, 015, 018, 026, 028 and 044. Only partial S1 gene sequences could be obtained for IB field viruses 025, 027, 031, 032, 038a, 038b and 040 because of the small amount of template amplified (relative to the viruses for which the complete S1 gene was successfully sequenced). The length of the partial S1 gene sequences ranged from 800 bp (043) to 1684 bp (027). The S1 gene nucleotide sequence iden-
The IB virus field strain 015 had the highest S1 gene nucleotide sequence identity score (93%) with the subgroup 2 reference strain N1/88, and nucleotide sequence identities \( \approx 67\% \) with all other strains. This contrasted with its 3′UTR nucleotide sequence identity score of 98% with the subgroup 3 reference strain N1/03, suggesting that this IB field virus is likely to be a recombinant virus.

IB field viruses 013, 025, 026, 027, 028, 031, 032 and 038a had S1 gene nucleotide sequence identities \( \geq 99\% \) with the subgroup 1 reference strain vaccine I, correlating with their 3′UTR nucleotide sequence identity scores with this strain and the HRM curve analyses. IB field viruses 038b and 044 also had S1 gene nucleotide sequence identities \( \geq 99\% \) with the subgroup 1 reference strain vaccine I correlating with their 3′UTR nucleotide sequence identity scores with this strain.

IB field viruses 018 and 040 had nucleotide sequence identities of 99% with the subgroup 3 reference strain N1/03, correlating with their 3′UTR nucleotide sequence identity scores with this strain and the HRM curve analyses. The S1 gene nucleotide sequence identity of IB field virus 043 was 99% with the subgroup 3 reference strain N1/03, correlating with its 3′UTR nucleotide sequence identity with this strain.

**Discussion**

HRM curve analyses of field submissions containing IB viruses identified strains of the virus that required further investigation, and enabled the detection of an Australian recombinant IB virus strain. The classification of field submissions as either subgroup 1 or 3 using HRM curve analysis correlated with both S1 gene and 3′UTR nucleotide sequence comparisons. No IB field viruses were classified by HRM curve analysis as being related to the subgroup 2 reference strain N1/88.

IB field virus 015 had contrasting nucleotide sequence identity matches for its 3′UTR and S1 gene sequences, and also produced a distinct melt curve profile. Its 3′UTR sequence suggested a close relationship with the subgroup 3 reference strain N1/03, whereas its S1 gene was most similar to the subgroup 2 reference strain N1/88. Strains belonging to subgroup 2 are distinct from strains belonging to subgroup 3. This suggests that IB field virus 015 was a recombinant of two viruses from different subgroups. Recombination involving the S1 gene alone is not sufficient or reliable for IB virus classification. Sequencing alone of the S1 gene of field strains 015 and 038b would have resulted in incorrect characterisation of these two IB field viruses.

Further analysis of field strain 015 is necessary to establish the origin of this strain and to determine its virulence for chickens. It would also be of interest to assess whether the current Australian vaccines protect against this recombinant strain.

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