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Research Note—

Detection of Infectious Bronchitis Virus by Real-Time Reverse Transcriptase–Polymerase Chain Reaction and Identification of a Quasispecies in the Beaudette Strain

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SUMMARY. In this report, we describe a real-time reverse transcriptase–polymerase chain reaction (RRT-PCR) diagnostic test for infectious bronchitis virus (IBV) with the use of fluorescence resonance energy transfer (FRET) technology. Two primers that amplify a 383-base pair product between nucleotide positions 703 and 1086 relative to the start codon for the S1 gene of the Massachusetts 41 virus were designed and used to amplify the Beaudette, Massachusetts 41, Florida 18288, Connecticut, Iowa 97, Arkansas DPI, CA/NE95/99, DE/072/92, and GA/0470/98 strains of IBV. The primers were specific and did not amplify New Castle disease virus, *Mycoplasma* spp., or infectious laryngotracheitis virus. For RRT-PCR by FRET, an anchor probe conjugated to fluorescein and a detection probe conjugated to a red fluorophore were designed to anneal to a hypervariable region within the 383-base pair product. The level of sensitivity was 1×10^4 RNA molecules used as starting template. After amplification, a melting curve analysis was conducted to specifically identify IBV types. Because of sequence differences in the annealing position of the detection probe, the Arkansas, Connecticut, Beaudette, and Massachusetts 41 strains could be differentiated. No fluorescence was observed for the DE/072/92 and GA/0470/98 viruses with the anchor and detection probes. When the Beaudette strain was examined, two melting peaks were observed at 44 C and 51 C, indicating a quasispecies in that laboratory strain of IBV. Routine typing of vaccine strains of IBV was possible with this technology, but high standard deviations associated with the melting curve analysis of the FRET probes described herein made it difficult to use this test reliably for routine typing of IBV field isolates.

RESUMEN. *Nota de Investigación*—Detección del virus de la bronquitis infecciosa aviar mediante la técnica de reacción en cadena por la polimerasa y trascripción reversa en tiempo real e identificación de una cuasi-especie en la cepa Beaudette.

En este reporte se describe una prueba diagnóstica basada en la técnica de reacción en cadena por la polimerasa y trascripción reversa en tiempo real (de las siglas en Inglés RRT-PCR) para la detección del virus de bronquitis aviar, en la cual se utiliza la tecnología de transferencia de energía resonante de fluorescencia (de las siglas en Inglés FRET). Se diseñaron dos iniciadores de reacción que amplifican un producto de 383 pares de bases a partir de los nucleótidos ubicados entre las posiciones 703 y 1086 después del codon inicial del gen que codifica por la proteína S1 del virus de la cepa Massachussets 41, los cuales fueron utilizados para amplificar productos a partir de las cepas Beaudette, Massachussets 41, Florida 18288, Connecticut, Iowa 97, Arkansas DPI, CA/NE95/99, DE/072/92 y GA/0470/98 del virus de bronquitis. Estos iniciadores son específicos y no amplifican productos cuando son usados en reacciones con cepas del virus de Newcastle, *Mycoplasma* spp o el virus de laringotraqueítis aviar. Para la prueba de RRT-PCR mediada por la técnica FRET, se utilizó una sonda de anclado conjugada a fluoresceína y una sonda de detección conjugada a un fluoroforo rojo, las cuales fueron diseñadas para interactuar con la región hipervariable que se encuentra en el producto de 383 pares de bases que amplifican los iniciadores mencionados. El nivel de sensibilidad fue de 1×10^4 moléculas de ARN utilizadas como plantilla o molde inicial. Después de la reacción de amplificación se realizó un análisis de la curva de disociación con el fin de identificar los diferentes tipos de virus de bronquitis. Debido a que la sonda de detección se une al producto amplificado en posiciones diferentes en las diferentes cepas del virus de bronquitis, fue posible diferenciar las cepas

Arkansas, Connecticut, Beaudette y Massachussets 41 mediante esta técnica. No se detectó fluorescencia cuando se utilizaron las cepas DE/072/92 y GA/0470/98 y las sondas mencionadas. Cuando se examinó la cepa Beaudette, se detectaron dos picos de disociación a 44 y 51 grados Celsius, lo que indica la presencia de una cuasi-especie en esta cepa de laboratorio del virus de bronquitis. La tipificación rutinaria de las cepas vacunales del virus de bronquitis es factible mediante el uso de esta técnica, pero los altos valores de desviación estándar observados en los análisis de las curvas de disociación con las sondas usadas en la técnica de FRET descritas en este estudio hacen difícil el uso de esta técnica para la tipificación rutinaria de aislados de campo.

Key words: infectious bronchitis virus, real-time reverse transcriptase–polymerase chain reaction, diagnosis, quasispecies

Abbreviations: DEPC = diethylpyrocarbonate; EID_{50} = 50% embryo infectious dose; FITC = fluorescein isothiocyanate; FRET = fluorescence resonance energy transfer; IBV = infectious bronchitis virus; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; RT = reverse transcriptase; RRT = real-time reverse transcriptase; Tm = melting temperature

Infectious bronchitis virus (IBV) is a coronavirus that causes a highly contagious upper respiratory tract disease in chickens. The disease is worldwide in distribution and is extremely difficult to control because multiple serotypes and variants of the virus occur that are not cross protective. Live attenuated vaccines for the major serotypes of IBV can be used to control most outbreaks, but it is first necessary to isolate and definitively identify the serotype of the virus responsible for the outbreak. The disease caused by IBV can appear similar to infectious laryngotracheitis, avian influenza, and viscerotropic velogenic Newcastle disease, which are high priority diseases. This is particularly true when secondary pathogens like *Escherichia coli* are involved. Thus, it is important to be able to diagnose IBV rapidly so one can determine if it is or is not the cause of an upper respiratory disease outbreak.

Traditionally, the virus-neutralization test conducted in embryonating eggs was used to isolate and identify different serotypes of IBV. Today, the reverse transcriptase (RT)–polymerase chain reaction (PCR) is routinely used (4,5,7). The rapid RT-PCR/restriction fragment length polymorphism (RFLP) identification test has been used to identify and characterize traditional and variant types of IBV from commercial poultry and research laboratories all over the world (2,4,7). The RT-PCR/RFLP test is based on amplification and restriction enzyme digestion of the S1 portion of the spike glycoprotein gene. The restriction fragment patterns for the S1 gene are observed after electrophoresis on a 2% agarose gel and compared with patterns of known serotypes and variants of the virus.

In this report, we describe a real-time RT (RRT)-PCR diagnostic test for IBV by the LightCycler™ PCR amplification and detection system (Roche Diagnostics Corp., Indianapolis, IN). To identify different IBV types, a set of probes complementary to a hypervariable sequence region of the S1 gene were designed to utilize the fluorescence resonance energy transfer (FRET) technology (1). The FRET technology uses an anchor probe that is conjugated to fluorescein and a detection probe conjugated to a red fluorophore (LightCycler™ Red 640). Fluorescein on the anchor probe emits a green fluorescent light when it is excited by the ultraviolet light source in the LightCycler™. When that probe comes into close proximity to the serotype-specific probe, by hybridizing to the PCR product, energy from the green fluorescent light is transferred to and excites the red dye, which then emits light at 640 nm (Fig. 1). After each round of amplification, the probes are allowed to anneal to the product and the reaction is monitored for red dye emission at 640 nm. After RT-PCR amplification, the probes are allowed to anneal to the amplified product, and a melting curve analysis is conducted to determine the number of mismatches between the detection probe and the template. In the melting curve analysis, fluorescence is monitored while the temperature of the hybridization reaction is slowly increased. The melting temperature (Tm) of the detection probe is determined by a rapid decrease in fluorescence. The anchor probe, which is much longer than the detection probe, remains attached to the template because it has a higher Tm. Because the number of mismatches between the detection probe and the template will affect the temperature at which the probe disassociates from the template, that temperature can be used to distinguish different IBV types. A perfect match will dissociate at the highest temperature, a related virus where the probe has some mismatches will detach at a lower temperature, and a different serotype with the most mismatches will separate at the lowest temperature.

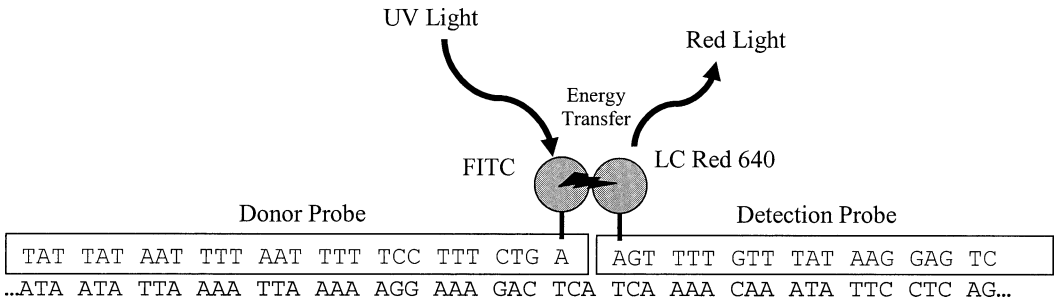

Fig. 1. FRET technology between an anchor probe conjugated with fluorescein isothiocyanate (FITC) and a detection probe conjugated with LC Red 640. Emission of light at 520 nm from ultraviolet light striking FITC is transferred (represented by the lightening bolt) to the LC Red 640 when the anchor and detection probes are in close proximity because of specific annealing to the amplified viral template (lower line of sequence data based on Massachusetts 41 sequence). The resulting emission of red light at 640 nm is monitored after each amplification cycle and increases with the amount of amplified template in the reaction.

Advantages of the new LightCycler™ system are that running agarose gels is no longer necessary, and identification is almost immediate. By this system, we identified and confirmed by cloning and sequencing a quasispecies in the Beaudette laboratory strain of IBV.

MATERIALS AND METHODS

Viruses. We selected several common strains of IBV (Table 1) to demonstrate the feasibility of using RRT-PCR for detection and, possibly, diagnosis of the virus. The serotypes of these standard strains of IBV have been verified in our laboratory by RT-PCR/RFLP and routine serology in embryonating eggs (3).

RRT-PCR. We designed primers to the first third of the S1 gene, which contains three hypervariable regions. It is necessary to amplify the hypervariable regions because they contain serotype-specific sequences that will be identified during the actual PCR amplification process with fluorescent dye–labeled probes. The primers were designed to conserved regions outside the hypervariable regions with the Oligo v4.05 computer program (National Biosciences, Inc., Plymouth, MN). Primers were designated IBVLC5′ and IBVLC3′, synthesized at Research Genetics, Inc. (Huntsville, AL), and tested for their ability to amplify each of the viruses listed above.

The viral RNA from 200 µl of allantoic fluid from IBV-inoculated 10-day-old embryonating eggs (virus titer = approximately $1 \times 10^{4.5}$ 50% embryo infectious dose [EID_{50}]/ml) was extracted with the High Pure RNA isolation kit (Roche Diagnostics Corporation). The purified RNA was resuspended in 35 µl of diethylpyrocarbonate (DEPC)-treated water. The RRT-PCR amplification was conducted with the LightCycler-RNA amplification kit SYBR Green I (Roche Diagnostics Corp.). In that kit, amplification is monitored with SYBR Green I dye, which emits a fluorescent signal at 530 nm when it binds to the double-stranded DNA and is exposed to ultraviolet light. The 20-µl RRT-PCR reaction mixture contained 4 µl of reaction mixture (included in the kit), 1 µl of each primer (approximately 125 ng), 1.6 µl of $MgCl_2$ (included in the kit), 0.4 µl of enzyme mixture (included in the kit), 4 µl of IBV RNA (from 300 ng to 800 ng per reaction), and 8 µl of DEPC-treated water.

The RRT-PCR amplification was done in a LightCycler™ (Roche Diagnostics Corp.). The RT step was incubated at 55 C for 10 min, then the reaction mixture was heated to 95 C for 30 sec. Next, 45 cycles of 95 C for 0 sec, 52 C for 10 sec, and 72 C for 13 sec were conducted and SYBR Green I fluorescence was monitored after each cycle.

Sensitivity. A Massachusetts 41 S1 gene RT-PCR amplified with previously reported primers (4) was cloned into the TOPO XL vector (Invitrogen,

Table 1. Viruses used in this study.

Virus strain	Serotype	Source
Arkansas DPI	Arkansas	Dr. J. Gelb Jr.[A]
Connecticut	Connecticut	Dr. J. Gelb, Jr.[A]
Beaudette	Massachusetts	ATCC VR-22[B]
Massachusetts 41	Massachusetts	ATCC VR-21[B]
Florida 18288	Florida	Dr. P. Villegas[C]
Iowa 97	Iowa	Dr. P. Villegas[C]
CA/NE95/99	California	PDRC[D]
DE/072/92	Delaware	Dr. J. Gelb, Jr.[A]
GA/0470/98	Georgia	PDRC[D]

[A]University of Delaware, Newark, DE.
[B]American Type Culture Collection, Rockville, MD.
[C]University of Georgia, Athens, GA.
[D]Poultry Diagnostic and Research Laboratory, University of Georgia, Athens, GA.

Carlsbad, CA). Runoff RNA transcripts were produced by the RiboMAX™ RNA production system (Promega, Madison, WI) according to the manufacturer's recommendations. The amount of RNA produced was determined with an Eppendorf BioPhotometer spectrophotometer (Hamburg, Germany). To determine the sensitivity of the test, serial 10-fold dilutions of the RNA product were made and used as template in the RRT-PCR reaction described above.

RRT-PCR probes. An anchor probe and a detection probe to distinguish the different types of IBV were designed with the Oligo v4.05 computer program (National Biosciences, Inc.) and synthesized at TIB Molbiol LLC (Adelphia, NJ). After each round of amplification, the probes were annealed to the product and the amount of amplified DNA was monitored real time by measuring for red dye emission at 640 nm. In addition, the probes were tested for their ability to detect different serotypes of IBV by running a melting curve analysis after amplification. After the RRT-PCR amplification, the reaction mixture was heated to 95 C for 1 sec and the two probes were allowed to anneal to the PCR product. The mixture was cooled to 30 C, then slowly heated at a rate of 0.1 C/sec to 92 C. Fluorescence at 640 nm was monitored in a stepwise fashion after each 0.1 C change in temperature.

Cloning and sequencing. The RRT-PCR product from the Beaudette strain of IBV was cloned with the TOPO TA cloning kit (Invitrogen) according to the manufacturer's recommendations. Clones containing the RRT-PCR product were identified by size on a 1% agarose gel. The cloned inserts were amplified with the LightCycler-RNA amplification kit SYBR Green I and the LightCycler™ (Roche Diagnostics Corp.) as above but without the RT step. In addition, a melting curve analysis with the anchor and detection probes described above was conducted for each amplified clone. Finally, the amplified products from the clones were sequenced with the ABI Prism DNA sequencing kit (Applied Biosystems, Foster City, CA) with the IBVLC5′ and IBVLC3′ primers and an ABI Prism 310 genetic analyzer (Applied Biosystems). The nucleotide sequence data were analyzed with the MacDNASIS Pro v3.5 software (Hatachi Software Engineering Co., San Bruno, CA).

Testing samples from experimentally inoculated birds. To examine the ability of this new test to detect IBV, we tested samples taken from experimentally inoculated chickens. Briefly, chickens were inoculated intraocularly with 1×10^4 EID_{50} of the Arkansas virus at 4 wk of age, and tracheal swabs were collected at 5 days postchallenge. Tracheal swab material was inoculated into 10-day-old embryonating eggs as previously described (3), and the allantoic fluid was harvested 48 hr postinoculation and tested by the LightCycler™ method described above.

Statistical analysis. The least significant difference of the means was statistically calculated with the Tukey–Kramer test for all pairs and JMP Statistical Discovery Software (SAS Institute, Inc., Cary, NC).

RESULTS

RRT-PCR. We designed two primers to amplify a 383-base pair product between nucleotide positions 703 and 1086 relative to the start codon for the S1 gene of the Massachusetts 41 virus (GenBank accession no. X04722). The sequence of the 5′primer is 5′-ACTGGCAATTTTTCAGA-3′ and is designated IBVLC5′. The sequence of the 3′ primer is 5′-ACAGATTGCTTGCAACCAC-3′ and is designated IBVLC3′. The primers were used to amplify the Beaudette, Massachusetts 41, Florida 18288, Connecticut, Iowa 97, Arkansas DPI, CA/NE95/99, DE/072/92, and GA/0470/98 strains of IBV (Fig. 2). The level of sensitivity with RNA runoff transcripts of the Massachusetts 41 S1 gene was approximately 1×10^4 RNA molecules (0.01 pg RNA). The primers were tested for specificity against other upper respiratory tract pathogens, including New Castle disease virus, *Mycoplasma* spp., and infectious laryngotracheitis virus, and no amplification products were observed (data not shown). Different slopes of the amplification curves in Fig. 2 are due to the amount of RNA template used and the efficiency of the reaction in each tube.

Melting curve analysis. We designed an anchor and a detection probe based on the Massachusetts 41 sequence of the S1 gene (Fig. 1). A region of variability was selected for the detection probe based on comparison with sequences for the other viruses used in this study. The sequence of the detection probe and the mismatches with the Connecticut, Arkansas, and DE/072/92 viruses are shown in Fig. 3.

To determine if the anchor and detection probes could indeed be used to type IBV, the Connecticut, Arkansas, and Massachusetts 41 viruses were amplified with IBVLC5′ and IBVLC3′ primers, then a detection probe melting curve analysis was conducted on the RT-PCR–amplified products. On the basis of the melting curve analysis, a clearly different rate of change in fluorescence was observed for the Arkansas, Connecticut, and Massachusetts 41 strains (Fig. 4). However, the average detection probe Tms, based on a minimum of four tests for each virus examined in this study (Table 2), were statistically not significantly different for Massachusetts 41, Connecticut, and Florida strains.

No fluorescence was observed for the DE/072/92 and GA/0470/98 viruses with the anchor and detection probes. We tried to lower the initial temperature for the melting curve analysis to room temperature, but still no fluorescence was detected (data not shown).

The Beaudette strain of IBV was examined four different times with allantoic fluid from two

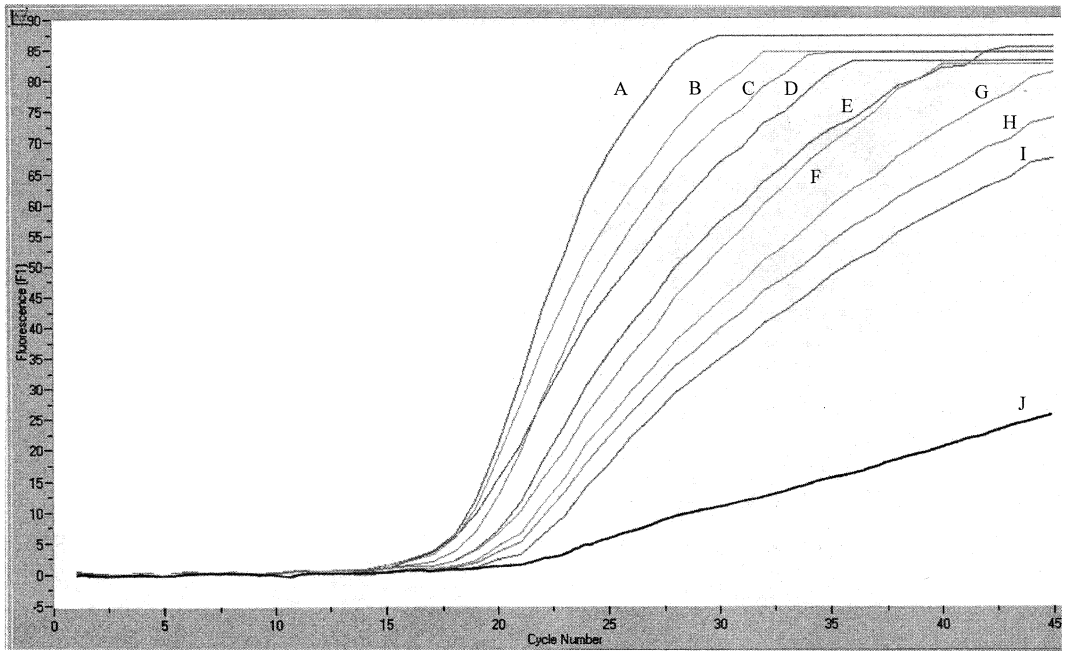

Fig. 2. A representative graph of RRT-PCR amplification of (A) Massachusetts 41, (B) Beaudette, (C) Arkansas, (D) Connecticut, (E) GA/0470/98, (F) CA/NE95/99, (G) DE/072/92, (H) Florida 18288, (I) Iowa 97, and (J) negative control, with primers IBVLC5′ and IBVLC3′. The y-axis is normalized as the percentage of the difference between minimum and maximum fluorescence values measured at 530 nm for each tube with the formula F1 = (F measured − F minimum)/(F maximum − F minimum) × 100. The x-axis is the cycle number.

different passages of the virus, and each time, two melting peaks were observed at 44 C and 51 C (Fig. 5). Sequence data obtained for nine clones of the amplified product from the Beaudette strain of IBV showed a base change from T to A at position 14 from the 5′ end of the detection probe in five of the nine clones. No differences were observed in the region of the anchor probe.

Detection in experimentally inoculated birds. When chickens were inoculated with the Arkansas strain of IBV, all of the samples (10/10) taken at 5 days postchallenge were positive for that serotype of the virus, and virus was not detected (0/10) in the negative control birds (data not shown) with the Lightcycler™ and FRET anchor and detection probes.

DISCUSSION

In this study, we used RRT-PCR to detect several common strains of IBV. The test takes 38 min (35 min for RT-PCR reaction and 3 min for melting curve analysis) to run and, with the FRET probes, costs approximately $19.00 per test. The RRT-PCR reaction with SYBR Green I alone costs approximately $8.00. We found that the RRT-PCR test could be used to detect 1×10^4 RNA molecules (0.01 pg RNA). It is difficult to compare the sensitivity of this test with other RT-PCR tests for IBV because of differences in the efficiency of viral RNA extraction and RT-PCR reactions. However, the test reported herein appears to be adequately sensitive for detection of laboratory strains and bird isolates of IBV propagated in 10-day-old embryonating eggs.

We found that detection and differentiation of the common IBV types (Massachusetts 41, Arkansas, and Connecticut) were reliable when the appropriate

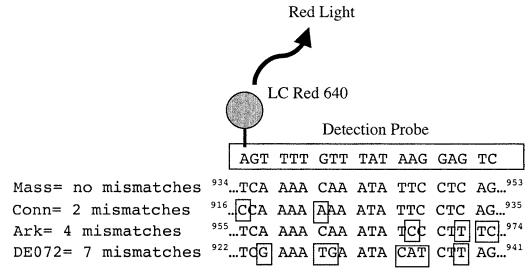

Fig. 3. The Massachusetts 41 detection probe sequence showing mismatches with the Connecticut, Arkansas, and DE/072/92 strains of IBV. Nucleotide positions are relative to the start codon for the S1 gene of each virus.

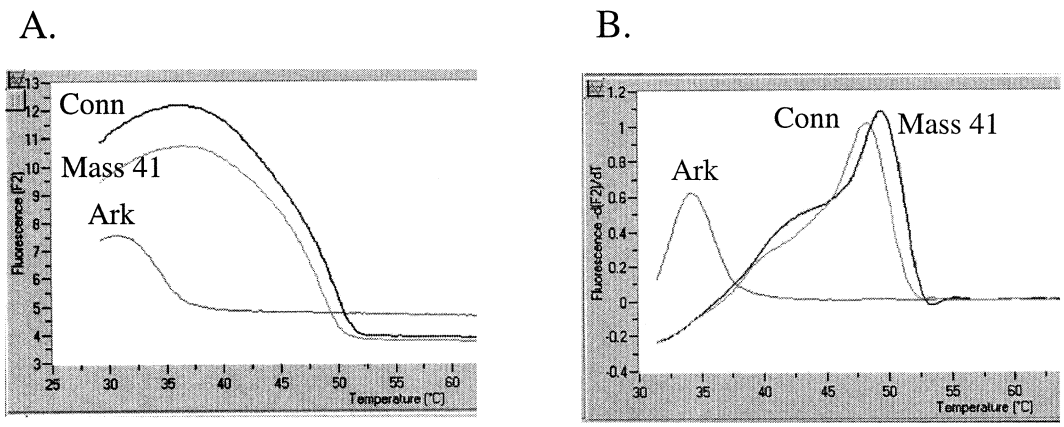

Fig. 4. Melting curve analysis of the anchor and detection probes with the amplified product for the viruses indicated. (A) The y-axis is normalized as the percentage of the difference between minimum and maximum fluorescence values measured at 640 nm for each tube with the formula F2 = (F measured − F minimum)/(F maximum − F minimum) × 100. (B) To more precisely determine the Tm of the probe, the first derivative of the run data (y-axis = $-dF/dT$) was calculated and represents the rate of change in fluorescence measured at 640 nm.

controls were used. However, the relatively high standard deviation calculated for the detection probe Tm for other types of IBV made it difficult to distinguish among them even when the appropriate controls were used. It appears that the number and type of nucleotide mismatches, as well as the relative position of the mismatches and the surrounding sequence, affect the Tm of the detection probe. This made it difficult to distinguish among IBV types with a similar number of G/C mismatches.

No fluorescence was detected when the FRET anchor and detection probes were used with RRT-PCR product from the DE/072/92 and GA/0470/GA98 strains. Because those strains were amplified with the IBVLC5′ and IBVLC3′ primers, we assume that the detection probe, which had seven mismatches with the amplified product of those strains, was not hybridizing. Lowering the initial temperature of the melting curve analysis did not help.

Table 2. Average[A] detection probe Tm for each of the IBV strains examined in this study.

IBV type	Tm ± SD[B]	Nucleotide mismatches[C]
Massachusetts 41	50.5 C ± 1.4[a]	0
Florida 18288	49.4 C ± 2.0[a]	1 (A/T)
Connecticut	49.0 C ± 1.5[a]	2 (1 A/T, 1 G/C)
Iowa 97	32.0 C ± 1.0[b]	3 (1 A/T, 2 G/C)
Ark DPI	34.7 C ± 1.6[c]	4 (2 A/T, 2 G/C)
CA/NE95/CA99	33.2 C ± 1.1[d]	5 (3 A/T, 2 G/C)
DE/072/92	No fluorescence	7 (4 A/T, 3 G/C)
GA/0470/GA98	No fluorescence	7 (4 A/T, 3 G/C)

[A]Each data point represents a minimum of four replicates.

[B]Different lowercase superscripts signify significant differences ($P < 0.05$).

[C]Number and type of nucleotide mismatches between the detection probe and the IBV type examined.

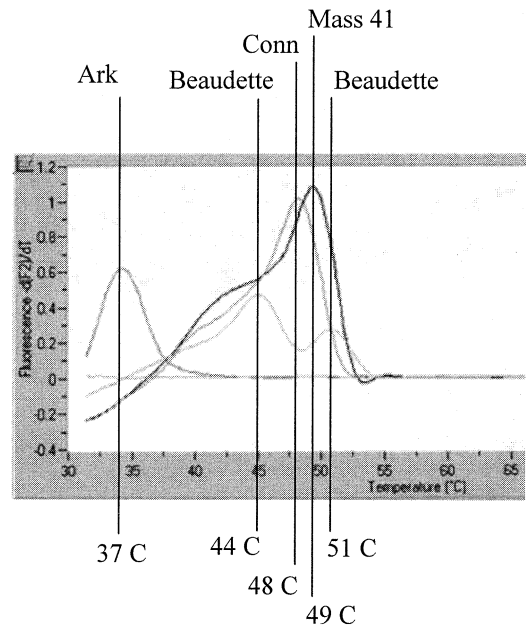

Fig. 5. Melting curve analysis (calculated first derivative of the run data, $-dF/dT$) of the anchor and detection probes with the amplified product for the viruses indicated and the average melting temperature indicated for each peak.

Because of the high standard deviations associated with the melting curve analysis, we could not reliably develop this test for routine diagnosis of IBV types. We have developed the test for use in the laboratory, however, and found it to be extremely reliable and accurate at identifying IBVs after experimental inoculation of chickens or in vaccine/challenge studies. In this study, we were able to detect and identify the Arkansas strain of IBV in all of the birds experimentally exposed to that virus. The ability to detect and identify IBV type with a test that takes about 1 hr is extremely useful when testing a large number of samples like those generated in *in vivo* experiments.

Each time the Beaudette strain of IBV was examined, melting peaks were observed at 44 C and 51 C, indicating that two populations or quasispecies of that virus occur in the same sample. In the region of the detection probe, the sequence of the Beaudette strain is reported to be the same as that of Massachusetts 41 (GenBank accession no. AJ311362), and a Tm of 51 C ± 1.4 calculated for the Massachusetts 41 strain is consistent with that sequence. On the basis of the area under the two peaks for the Beaudette strain, 59% of the population has a sequence different from the reported sequence. This percentage is consistent with the sequence data generated from cloned RRT-PCR product of the Beaudette strain, which showed a T to A nucleotide change at position 14 from the 5′ end of the detection probe in 55% (5/9) of the clones examined. Considering that the Connecticut strain with two mismatches only dropped the Tm of the detection probe to 49 C, that one A/T nucleotide mismatch for the Beaudette strain dropped the Tm of the detection probe to 44 C was unexpected. However, and as previously mentioned, the number, type, and position of nucleotide mismatches can significantly affect the Tm of the detection probe.

While conducting RRT-PCR on different IBV strains, we fortuitously identified the presence of a quasispecies in the sample of the Beaudette strain from our laboratory. Quasispecies have been reported for coronavirus (6,8,9). Those studies have shown that coronaviral RNAs exist as a diverse population, which is important for viral evolution and persistence of the virus. This diversity can contribute to the emergence of new virus types in the field and continued pathogenicity of the virus for the host (8). Our data confirm the existence of quasispecies in IBV, but the significance of two major populations of virus in a highly attenuated laboratory strain of IBV is not known.

REFERENCES

1. Bernard, P. S., R. S. Ajioka, J. P. Kushner, and C. T. Wittwer. Homogeneous multiplex genotyping of hemochromatosis mutations with fluorescent hybridization probes. Am. J. Pathol. 153:1055–1061. 1998.

2. Callison, S. A., M. W. Jackwood, and D. A. Hilt. Molecular characterization of infectious bronchitis virus isolates foreign to the United States and comparison with United States isolates. Avian Dis. 45:492–499. 2001.

3. Gelb, J., Jr., and M. W. Jackwood. Infectious bronchitis. In: A laboratory manual for the isolation and identification of avian pathogens, 4th ed. D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 169–174. 1998.

4. Jackwood, M. W., N. M. Yousef, and D. A. Hilt. Further development and use of a molecular serotype identification test for infectious bronchitis virus. Avian Dis. 41:105–110. 1997.

5. Keeler, K. L., K. L. Reed, W. A. Nix, and J. J. Gelb. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S1) gene. Avian Dis. 42:275–284. 1998.

6. Kiss, I., S. Kecskemeti, J. Tanyi, B. Klingeborn and S. Belak. Preliminary studies on feline coronavirus distribution in naturally and experimentally infected cats. Res. Vet. Sci. 68:237–242. 2000.

7. Kwon, H. M., M. W. Jackwood, and J. Gelb, Jr.Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis 37:194–202. 1993.

8. Nix, W. A., D. S. Troeber, B. F. Kingham, C. L. J. Keeler, and J. J. Gelb. Emergence of subtype strains of the Arkansas serotype of infectious bronchitis virus in Delmarva broiler chickens. Avian Dis. 44:568–581. 2000.

9. Rowe, C. L., S. C. Baker, M. J. Nathan, J. Y. Sgro, A. C. Palmenberg, and J. O. Fleming. Quasispecies development by high frequency RNA recombination during MHV persistence. Adv. Exp. Med. Biol. 440:759–765. 1998.

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