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## Molecular Detection and Serotyping of Infectious Bronchitis Virus from FTA<sup>®</sup> Filter Paper

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**SUMMARY.** We investigated the feasibility of using Flinders Technology Associates (FTA<sup>®</sup>) filter cards for the storage of allantoic fluid containing an infectious bronchitis virus (IBV), such as Arkansas-DPI, Connecticut, and Massachusetts, and for their identification by reverse transcriptase (RT)-polymerase chain reaction (PCR) and characterization by restriction fragment length polymorphism (RFLP) or nucleotide sequencing. FTA<sup>®</sup> paper is a cotton-based cellulose membrane containing lyophilized chemicals that lyses many types of bacteria and viruses. IBV was inactivated upon contact with the FTA<sup>®</sup>, as shown by the inability of the virus to be propagated in embryonating chicken eggs. RT-PCR of the S1 gene showed that viral RNA in allantoic fluid remained stable after storage on FTA<sup>®</sup> filter cards and that the stability was time and temperature sensitive for the large (1700 base pair [bp]) but not the small (383 bp) PCR products. Analysis of the amplified products showed that molecular characterization is feasible in allantoic fluid stored on FTA<sup>®</sup> under nonfavorable environmental conditions (41 C) for at least 15 days. The use of FTA<sup>®</sup> cards for the collection, transport, and storage of IBV-containing samples is safe, inexpensive, and adequate for molecular diagnosis. We propose that specimens coming from overseas on FTA<sup>®</sup> cards would be first analyzed by RT-PCR with primers yielding a 1700-bp product followed by RFLP of the positive cases. Negative cases would be analyzed with primers yielding a 383-bp product (to exclude detrimental effect of the storage conditions) followed by nucleotide sequencing of the positive cases.

**RESUMEN.** Identificación del virus de bronquitis infecciosa por medio de la prueba de transcriptasa reversa-reacción en cadena por la polimerasa en papel de filtro FTA<sup>®</sup>.

Se investigó la posibilidad de utilizar tarjetas de papel de filtro FTA<sup>®</sup> para almacenar líquido alantoide de las cepas Arkansas-DPI, Connecticut, y Massachusetts del virus de bronquitis infecciosa aviar. El objetivo fue el de identificar la presencia de estas cepas por medio de las pruebas de transcriptasa reversa-reacción en cadena por la polimerasa (de las siglas en Inglés RT-PCR), la caracterización por medio de la prueba del polimorfismo de la longitud de los fragmentos de restricción (de las siglas en Inglés RFLP), o mediante la secuencia de nucleótidos. El papel de filtro FTA<sup>®</sup> es una membrana de algodón y celulosa que contiene químicos liofilizados que lisan muchos tipos de bacterias y virus. El virus de bronquitis se inactivó al entrar en contacto con el papel FTA<sup>®</sup>, como se demostró por la incapacidad del virus de propagarse en embriones de pollo. La prueba RT-PCR del gen S1 demostró que el ARN viral permaneció estable al almacenamiento en el papel FTA<sup>®</sup> y esa estabilidad fue sensible a la temperatura y al tiempo de almacenamiento para los oligonucleótidos de mayor tamaño (1700 pares de bases) pero no para los pequeños (383 pares de bases). El análisis de los productos amplificados mostró que es posible la caracterización molecular del virus en muestras almacenadas en el papel FTA<sup>®</sup>, aún bajo condiciones ambientales desfavorables (41 C) por un mínimo de 15 días. El uso del papel de filtro FTA<sup>®</sup> para la toma, transporte y almacenamiento de muestras infectadas con el virus de bronquitis es un procedimiento seguro, barato y adecuado para el diagnóstico molecular. Proponemos que las muestras que vengan del extranjero en papel FTA<sup>®</sup> sean analizadas primero por la prueba de RT-PCR utilizando iniciadores que produzcan nucleótidos de 1700 pares de bases, seguido de análisis por medio de la prueba de RFLP de los casos positivos. Los casos negativos se pueden analizar utilizando iniciadores que produzcan nucleótidos de 383 pares de bases (para descartar efectos dañinos debido a las condiciones de almacenamiento), seguido por la secuenciación de los casos positivos.

Key words: infectious bronchitis virus, RT-PCR, FTA<sup>®</sup> card, RNA, diagnosis

Abbreviations: AF = allantoic fluid; FTA<sup>®</sup> = Flinders Technology Associates; IBV = infectious bronchitis virus; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; RT = reverse transcriptase

Infectious bronchitis virus (IBV) is a coronavirus that produces upper respiratory tract disease in chickens resulting in significant economic losses to the poultry industry. The disease can occur worldwide, even in vaccinated flocks, because of poor or no cross-protection among serotypes or to IBV variants. The identification of IBV is primarily performed by the hemagglutination (HA) test, hemagglutination-inhibition (HI) test, and the virus-neutralization (VNS) test conducted in embryonated eggs (8). More recently, the reverse transcriptase (RT)-polymerase chain reaction (PCR) along with restriction fragment length polymorphism (RFLP) has been used for the identification and molecular characterization of IBV variants (4,10,13). In our diagnostic center, the original RT-PCR/RFLP (10,13) has been modified to improve the turnaround time

and the simplicity of the test without affecting the specificity or sensitivity.

Collection and transport of infectious agents through the mail, especially from foreign countries to reference laboratories in the United States, require chemical inactivation of the specimens and compliances with strict federal, state, and civil regulations (19). These procedures are expensive and time consuming and logistically complex. As an alternative, we propose using the FTA<sup>®</sup> filter paper (Whatman, Newton, MA) for the collection and transport of biological samples. FTA<sup>®</sup> is a cotton-based cellulose paper impregnated with anionic detergent and buffer that provides chelating and free radical-trapping properties (3). Most cell types are lysed on contact with FTA<sup>®</sup>, including tumor cells (7), white blood cells (6), and bacteria (14). Viruses are also inactivated (1,2,9,12,18,20), leaving the nucleic acids suitable for molecular

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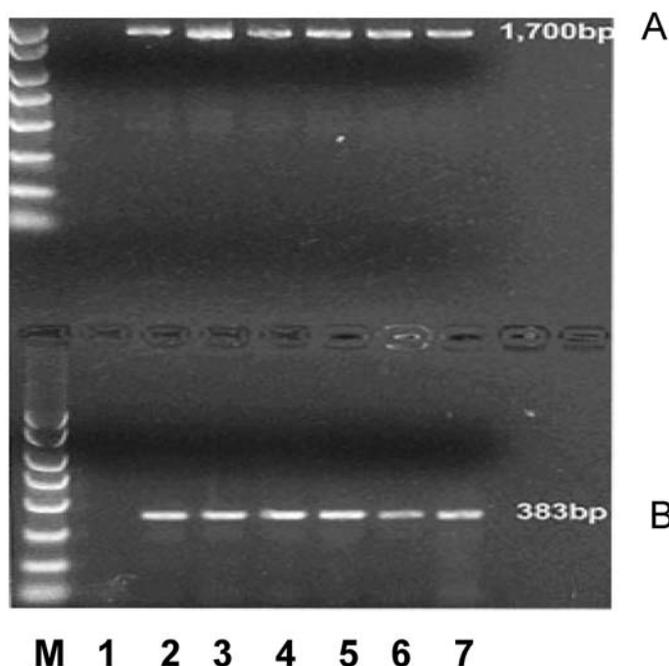


Fig. 1. Electrophoresis of FTA®/RT-PCR products on a 2% agarose gel stained with ethidium bromide. (A) Primers S1 5' oligo/3' degen yielding a 1700 bp product. (B) Primers IBV LC5'/LC3' yielding a 383 bp product. M = 2000-50 bp DNA marker; 1 = phosphate-buffered saline (PBS); 2 = 1 µl of IBV AF; 3 = 5 µl; 4 = 10 µl; 5 = 25 µl; 6 = 50 µl; and 7 = Trizol® LS RNA extraction.

identification. Identification of viruses in specimens stored on filter paper have been reported for hepatitis C (1), measles (12), HIV (2), bovine viral diarrhea (20), and chicken infectious bursal disease virus (18) by RT-PCR and orthopoxviruses (9) and turkey hemorrhagic enteritis virus (18) by PCR. A great body of information is available on the stability and molecular analysis of DNA in specimens stored on filter paper including FTA® (21), but little is known about the stability of viral RNA on FTA® cards. The detection of viral RNA in specimens stored on filters depends on many factors, including the type of filter (nitrocellulose, nylon, or FTA®), temperature and length of storage, viral source (blood, tissues, and cell cultures), type of nucleic acid (ssRNA or dsRNA), and the sensitivity of the appropriated test. Here we report, for the first time, the use of the FTA® filter as a medium to inactivate and store IBV, a single-stranded enveloped RNA virus. The specific objective of this work was to study the feasibility of performing RT-PCR, RFLP, or sequencing analysis on IBV in allantoic fluid (AF) stored on FTA® cards.

## MATERIALS AND METHODS

**Viruses.** We selected a few common strains of IBV (Arkansas-DPI, Connecticut, Massachusetts) from our depository to test the feasibility of performing molecular characterization from samples stored on FTA® cards. Clinical samples were obtained as AF after propagating the virus in embryonated eggs as part of our routine diagnostic services.

**Sample collection.** Aliquots of AF were spotted on the active area of FTA® paper and allowed to dry at ambient temperature for at least 30 min before further processing. Also, tracheal swabs (Transport Swabs, Fisher HealthCare, Houston, TX) from SPF chickens vaccinated at 3 wk of age with Newcastle B1B1 type and Massachusetts serotype of IBV (Fort Dodge Animal Health, Fort Dodge, IA) were directly applied to the FTA® cards 72 hr after vaccination.

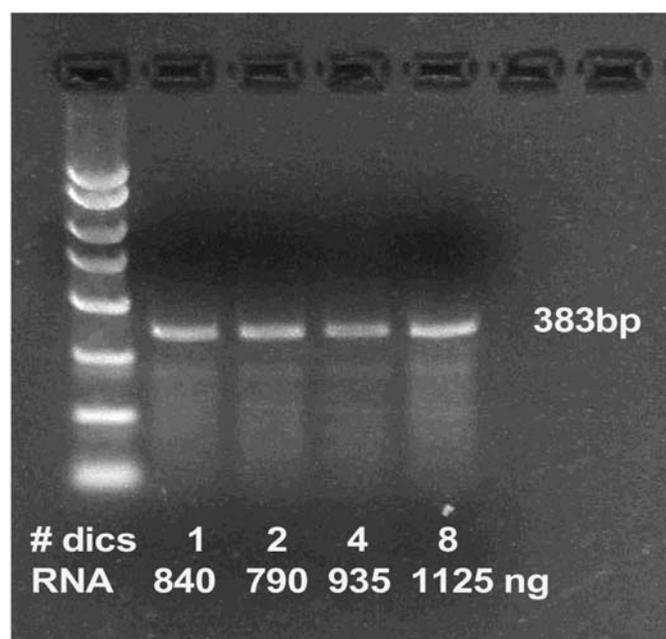


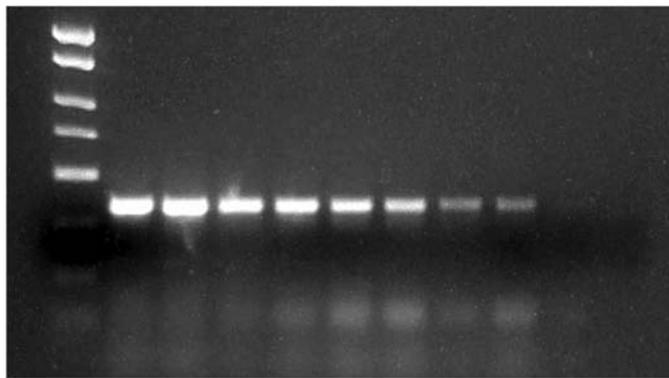
Fig. 2. Electrophoresis of FTA®/RT-PCR products on a 2% agarose gel. RNA was extracted from one, two, four, and eight 2-mm FTA®/sample discs. DNA marker is 2000-50 bp. IBV amplicon is 383 bp.

### Sensitivity, specificity, and stability of the FTA® system.

The sensitivity test was performed by spotting 1, 5, 10, 25, and 50 µl of IBV in AF ( $10^4$  EID<sub>50</sub>/ml) on FTA® cards processed 24 hr later. Also, 15 replicates of 5 µl of AF were spotted on FTA® cards, and the RNA was extracted from one, two, four, and eight 2-mm discs punched from the card. The specificity test was performed from FTA® cards spotted with 5 µl of live respiratory pathogens (*Mycoplasma gallisepticum*, *Mycoplasma synoviae*, Newcastle disease virus, adenovirus, and infectious laryngotracheitis virus), as well as other RNA viruses (avian leukosis virus subgroup J and infectious bursal disease virus) and processed 24 hr later. PCR or RT-PCR using specific primers for these organisms was performed according to Moscoso *et al.* (16,17, and unpublished data). The stability test was performed from FTA® cards spotted with 5 µl of five different AF samples containing IBV (IBV positive by virus isolation and the traditional RT-PCR), which were stored at 4 C, room temperature (RT), and 41 C for 1, 4, 9, and 15 days before processing. Some samples were tested after 15 days of storage.

**Virus inactivation on FTA® cards.** Five microliters of AF containing live IBV (Massachusetts strain) were applied to the FTA® cards, dried at RT for 1 hr, and processed for virus isolation. Two-millimeter discs of FTA® cards containing the virus were aseptically removed and soaked in triptose phosphate broth (TPB) for 30 min. A 200-µl aliquot of TPB was inoculated in 9- to 11-day-embryonated eggs in an attempt to propagate the virus following standard procedures (15). AF harvested at 48 hr postinoculation was passaged (0.2 ml) in embryonated eggs two additional times. Embryonic lesions, death, and a positive neuraminidase test would be evidence of virus isolation.

**RNA extraction from FTA® paper.** Two-millimeter discs from the spotted area of the filters were cut by a sterile hole-puncher and placed in 1.5-ml Eppendorf tubes (Brinkmann Instruments, Westbury, NY). The punch was cleaned and sterilized with 70% ethanol (according to Whatman technical bulletin) between cuts from different samples to prevent cross-contamination. Each disc was placed in 200 µl of 10 mM of Tris-HCL and 0.1 mM of EDTA, with a pH value of 8.0, vortexed, and incubated for 10 min at RT. The High Pure Viral RNA Kit (Roche, Mannheim, Germany) was then used to extract the RNA from the FTA® paper, following the manufacturer's instructions. RNA was also extracted from AF using our standard procedure with Trizol® LS Reagent (Life Technologies, Grand Island, NY). Briefly, 250 µl of AF



M 1 2 3 4 5 6 7 8 9 10

Fig. 3. Electrophoresis of FTA<sup>®</sup>/RT-PCR products from various amounts of RNA. M = 2000-50 bp DNA marker; 1 = 1600 ng; 2 = 1000 ng; 3 = 800 ng; 4 = 400 ng; 5 = 200 ng; 6 = 100 ng; 7 = 50 ng; 8 = 25 ng; 9 = 10 ng; and 10 = 5 ng. IBV amplicon is 383 bp.

were mixed with 750  $\mu$ l of Trizol<sup>®</sup> LS and 200  $\mu$ l of chloroform, vortexed, and spun for 5 min at 14,000 rpm. The RNA in the aqueous phase was precipitated with 500  $\mu$ l of isopropanol and then washed with 75% ethanol and suspended in 40–50  $\mu$ l of dep (0.1% diethyl pyrocarbonate) H<sub>2</sub>O. RNA concentration and purity were estimated by spectrophotometer readings at A<sub>260nm</sub> and A<sub>280nm</sub>.

**RT-PCR.** The standard diagnostic RT-PCR for IBV that uses the Titan One Tube RT-PCR System Kit (Roche) was also used for the FTA<sup>®</sup> system. The PCR mixture (50  $\mu$ l) contained 26  $\mu$ l of dep H<sub>2</sub>O, 8  $\mu$ l of 5x buffer, 4  $\mu$ l of 25 mM MgCl<sub>2</sub>, 2  $\mu$ l of 100 mM DTT, 1  $\mu$ l (5 U) of enzyme mix, 1  $\mu$ l of 10 mM DNTP mix (Amersham Biosciences, Piscataway, NJ), 1  $\mu$ l of each primer (50  $\mu$ M; Qiagen, Germantown, MD), and 5  $\mu$ l of template solution containing between 100 ng and 1100 ng of RNA. A set of primers were used to amplify the S1 gene that codes for the spike glycoprotein to yield a PCR product of 1700 bp (5). The primer sequences are: S1 Oligo-5' = 5'-TGA AAC TGA ACA AAA GAC-3' and S1 Degen-3' = 5'-CCA TAA GTA ACA TAA GGR CRA-3'. A second set of primers comprising a portion of the S1 gene between nucleotide positions 703 and 1086 (11) were also used. The primer sequences are: IBV-LC 5' = 5'-ACT GGC AAT TTT TTC AGA-3'. IBV-LC-3' = 5'-ACA GAT TGC TTG CAA CCA C-3. A one-step RT-

PCR program was performed in a Thermal Hybaid cycler (Franklin, MA), which consisted of heating at 50 C for 1 hr for the RT step, an initial denaturation at 95 C for 5 min, and 35 cycles of 94 C for 30 sec, 55 C for 30 sec, and 68 C for 1 min. A final extension was performed at 68 C for 7 min.

**Agarose gel electrophoresis.** PCR products were electrophoresed on a 2% agarose gel in TAE buffer (40 mM of Tris and 2 mM of EDTA, with a pH value of 8.0) containing 0.5  $\mu$ g/ml of ethidium bromide for 30 min at 70 V and visualized under an ultraviolet light transilluminator.

**Restriction fragment length polymorphism.** The S1 gene amplification products (15  $\mu$ l) were digested for at least 1 hr with 10 U of restriction endonucleases *Bst*YI, *Hae*III, and *Xcm*I, as recommended by the manufacturer (New England Biolabs, Beverly, MA), and the RFLP pattern was determined on a 2% agarose gel.

**Nucleotide sequencing and sequence analysis.** The amplified PCR products of the IBV-LC5' / IBV-LC3' primers were sequenced at the University of Georgia, Molecular Genetics Instrumentation Facility, Athens, GA, using the ABI Prism DNA sequencing kit and an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA). The sequenced data were analyzed with the nucleotide-nucleotide Blast data base (National Center for Biotechnology Information).

**Phenol treated allantoic fluid on FTA<sup>®</sup>.** Ten phenol inactivated AF (1:1 v/v solution of saturated phenol—pH 4.3) were spotted on FTA<sup>®</sup> cards (5  $\mu$ l) and analyzed by the standard RT-PCR method and by the FTA<sup>®</sup> RT-PCR system after 24–96 hr of storage at room temperature.

**Analysis of field samples by the FTA<sup>®</sup> system.** Fifty-two clinical samples that were positive for virus isolation were analyzed by the standard diagnostic RT-PCR and RFLP of the S1 product. The same AF samples were spotted on FTA<sup>®</sup> (5  $\mu$ l), dried at RT, and also analyzed by RT-PCR (S1 primers) 24–96 hr later. Twenty of the FTA<sup>®</sup>-stored AF were randomly chosen for RFLP analysis.

## RESULTS

**Virus inactivation on FTA<sup>®</sup> cards.** IBV in AF stored on FTA<sup>®</sup> cards for 1–5 days were unable to grow in chicken embryos after three egg passages, as demonstrated by the absence of embryonic lesions and negative neuraminidase test (data not shown).

**Sensitivity of the FTA<sup>®</sup>/RT-PCR system.** Fig. 1 shows the RT-PCR amplified product from an AF-containing virus 24 hr after

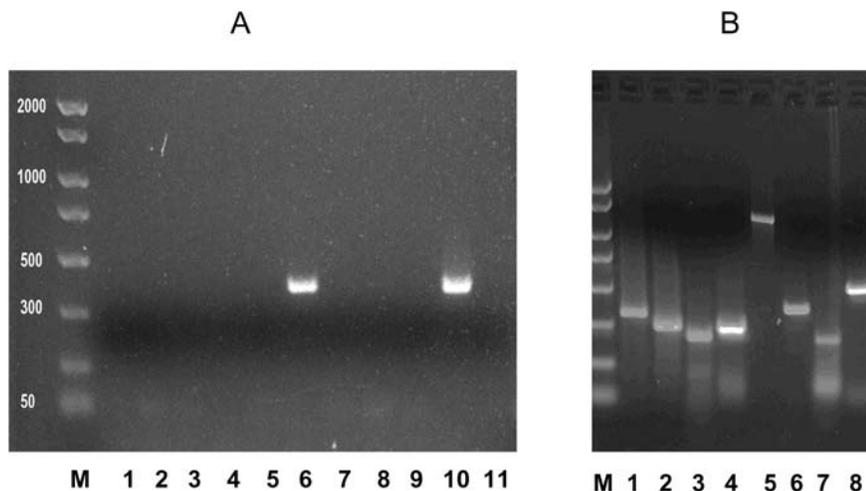


Fig. 4. Electrophoresis of RT-PCR products from FTA<sup>®</sup>/IBV system (A) and FTA<sup>®</sup>/various agents system (B). M = 2000-50 bp DNA marker; 1 = *Mycoplasma synoviae* H, 370 bp; 2 = *M. gallisepticum* ts-11, 300 bp; 3 = Newcastle disease virus, 255 bp; 4 = infectious laryngotracheitis, 296 bp; 5 = adenovirus, 1219 bp; 6 = IBV, 383 bp; 7 = infectious bursal disease virus, 248 bp; 8 = avian leukosis virus subgroup J, 546 bp; 9 = PBS; 10 = IBV standard Trizol<sup>®</sup> LS extraction, 383 bp; and 11 = RT-PCR mix only.

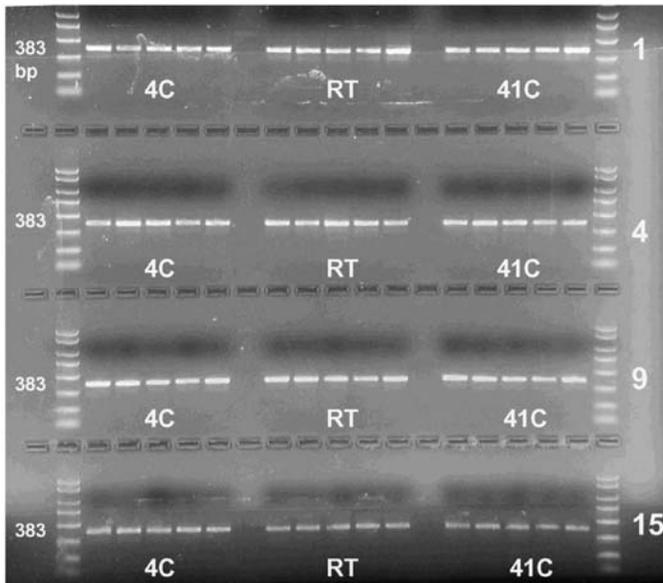


Fig. 5. Electrophoresis of RT-PCR products from five different AF containing IBV, which were stored on FTA® paper at 4 C, RT, and 41 C for 1, 4, 9, or 15 days. DNA marker is 2000-50 bp. Amplicon is 383 bp.

being spotted on FTA® in 1-, 5-, 10-, 25-, and 50- $\mu$ l volumes. No significant visual differences were seen in the intensity of the bands when a standard 2-mm disc was punched from each volume for RNA extraction (RNA = 190 ng – 950 ng/reaction). Also, no differences were seen between the two sets of primers (A = S1 5' oligo/3' Deg; B = S1 LC5'/LC3') at any volume tested. Moreover, their signals were similar to the one obtained with the standard Trizol® extraction method. These properties would allow analyzing the large PCR products (Fig. 1A) by RFLP and the small PCR products (Fig. 1B) by sequencing. To further assess the sensitivity of the FTA®/RT-PCR system, RNA was extracted from one, two, four, or eight 2-mm discs, and 5  $\mu$ l of each extraction (RNA concentration ranged from 158 ng/ $\mu$ l to 225 ng/ $\mu$ l) was subjected to RT-PCR. No significant visual differences were observed in the intensity of the amplicon bands

among the number of discs tested (Fig. 2). Furthermore, RNA extracted from a single disc can be diluted to levels between 25 ng and 100 ng and still be detectable by RT-PCR (Fig. 3).

**Specificity of the FTA®/RT-PCR system.** Fig. 4 shows specific amplification of IBV with the IBV/FTA® system and no cross-amplification with other pathogens, including mycoplasma, and other RNA and DNA viruses (Fig. 4A). However, amplification with primers specific for each infectious agent was seen in all cases (Fig. 4B).

**RNA stability on the FTA®.** Fig. 5 shows the agarose gel of IBV amplicons (383 bp) after performing RT-PCR on samples stored at 4 C, RT, or 41 C for 1, 4, 9, or 15 days. RNA extracted from the filter was readily detected at all times and temperatures. Maximum RNA stability was observed in samples stored at 4 C followed by those stored at room temperature, as judged by the brightness of the bands. A slight decrease in the visual intensity of the bands was observed after 15 days of storage at 41 C. The IBV S1 gene oligonucleotide consisting of 1700 bp was more difficult to amplify when the temperature and storage on FTA® was higher (data not shown) than the smaller IBV amplicon (383 bp), but it was still possible to perform molecular characterization using FTA® cards stored for less than 16 days at RT or 20 days at 4 C. IBV was detected in a few cases after 36 days of FTA® storage at RT when using the IBV-LC primer set (383 bp) (data not shown).

**Restriction fragment length polymorphism.** Fig. 6 shows the RFLP pattern for an Arkansas-DPI virus S1 gene amplified from allantoic fluid stored on FTA® cards at RT for 1, 4, 9, or 15 days. Although the visual intensity of the bands decreases slightly with time, the digested (1700 bp) pattern was consistent with the presence of the ARK-DPI profile. Sequence analysis of the FTA®/RT-PCR products (383 bp) from the same samples confirmed the above conclusion (data not shown).

**Phenol treated AF on FTA®.** Fig. 7 shows that the phenol-inactivated sample analyzed by the FTA® system resulted in amplicons similar to the standard method of RT-PCR amplification and of the expected size.

**Analysis of field samples by the FTA® system.** Fifty-two clinical samples positive by virus isolation were also positive by the standard diagnostic RT-PCR test and the FTA®/RT-PCR system

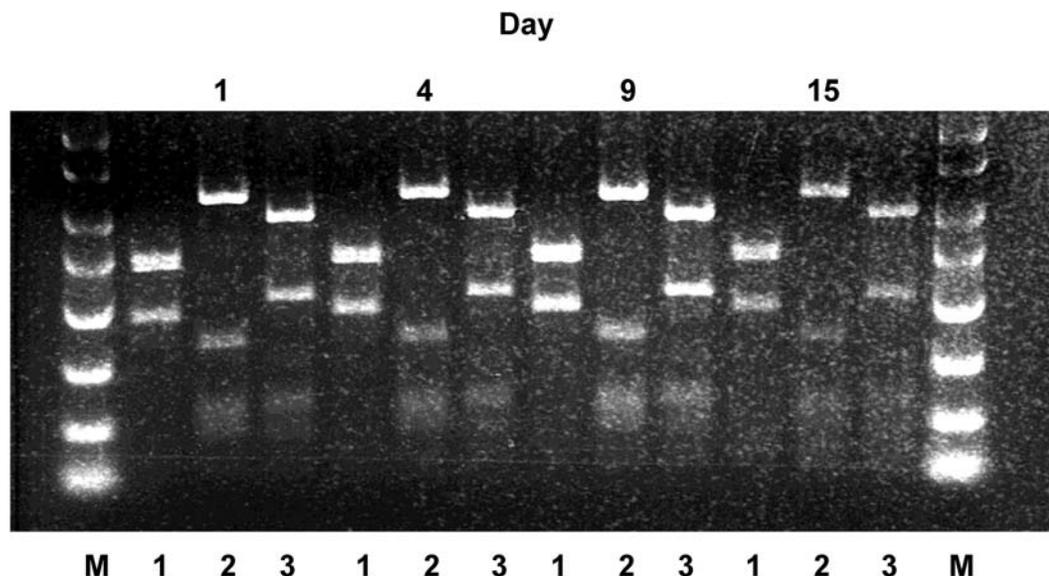


Fig. 6. Electrophoresis pattern of digested FTA®/RT-PCR products (1700 bp) from an AF containing Ark-DPI virus stored at RT for several days. M = 2000-50 bp DNA marker; 1 = restriction enzyme *BstYI*; 2 = *HaeIII*; and 3 = *XcmI*.

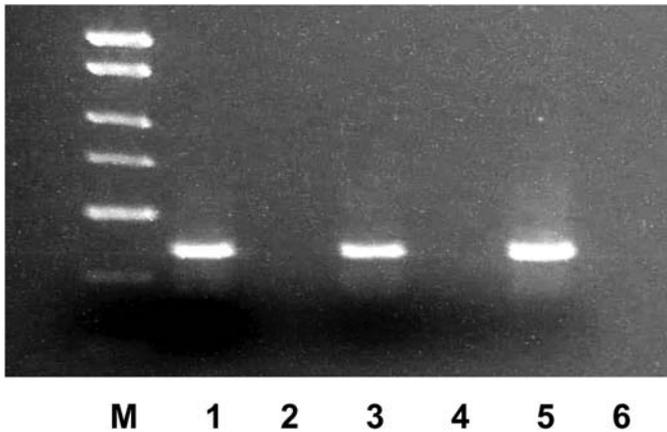


Fig. 7. Electrophoresis of RT-PCR products from phenol-treated and -untreated AF. M = 2000-50 bp DNA marker; 1 = phenol-treated AF on FTA<sup>®</sup>; 2 = phenol-treated PBS on FTA<sup>®</sup>; 3 = untreated AF on FTA<sup>®</sup>; 4 = untreated PBS on FTA<sup>®</sup>; 5 = phenol-treated AF non-FTA<sup>®</sup>, standard Trizol<sup>®</sup> LS extraction; 6 = phenol-treated PBS non-FTA<sup>®</sup>. IBV amplicon is 383 bp.

(data not shown). RFLP analyses of S1 products from both RT-PCR methods were comparable. Fig. 8 shows representative RFLP patterns from the FTA<sup>®</sup> system.

## DISCUSSION

Two main issues are involved in the use of FTA<sup>®</sup> paper as a collection and transport medium of biological specimens: inactivation of infectious agents and their possible identification by molecular techniques. We have reported here that live IBV was rendered noninfectious after being in contact with the FTA<sup>®</sup> paper for a short period of time. Similar inactivation has been seen for avian mycoplasma (16) and some avian RNA (17) and DNA viruses (18). The FTA<sup>®</sup>/RT-PCR system for IBV detection was sensitive because only 1  $\mu$ l of a positive AF sample, representing 10 EID<sub>50</sub>, was needed for the test. This would suggest that the presence of a small number of virus particles in a specimen, such as tracheal swabs from infected birds dotted on FTA<sup>®</sup> paper, could be used as a source for IBV detection and identification. This would constitute a significant improvement in the collection of samples in the field

and their transport, especially from remote areas of the world to centralized laboratories. The IBV/FTA<sup>®</sup> system had high specificity because no cross-amplification was observed with other respiratory pathogens.

The detection of IBV in AF stored on FTA<sup>®</sup> cards was possible by RT-PCR, indicating that the RNA was stable for at least 15 days. However, large PCR products were significantly affected by the time and temperature of storage (weak signal after 20 days at room temperature), whereas small products were not (strong signal after 36 days at room temperature). A possible explanation is that RNA denatures over time and is faster at higher temperatures, creating nicks in the strand. Therefore, the probability of getting a nick in the strand between primers that amplify a 1700 bp fragment is much higher than in fragment of only 383 bp in length. Notwithstanding, FTA<sup>®</sup>/RT-PCR performed with either primer set within 15 days of storage at temperatures equal or below 41 C showed 100% agreement with HA/HI/VNS tests of field samples. Furthermore, we were able to characterize the IBV by RFLP and sequencing in selected cases, demonstrating the feasibility of performing molecular analysis of amplicons from FTA<sup>®</sup>-stored specimens. Although we showed that phenol inactivated samples may be stored on FTA<sup>®</sup> paper and also be analyzed molecularly, the advantages of FTA<sup>®</sup> inactivation are superior: nontoxic, simple and rapid processing, and ambient storage conditions. Our data show that FTA<sup>®</sup> cards can be used for collecting and transporting AF-containing IBV in a way that is simple, inexpensive, safe, and suitable for molecular analysis.

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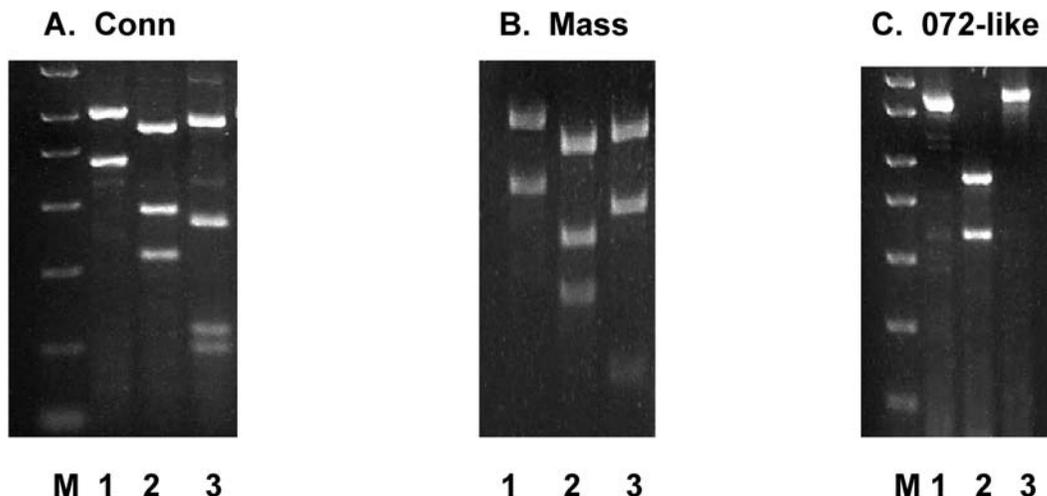


Fig. 8. RFLP pattern of FTA<sup>®</sup>/RT-PCR products (1700 bp) from AF containing Connecticut (A), Massachusetts (B), or 072-like (C) strains of IBV. M = 2000-50 bp DNA marker; 1 = restriction enzyme *BstYI*; 2 = *HaeIII*; and 3 = *XcmI*.

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