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Molecular Detection and Serotyping of Infectious Bronchitis Virus from FTA® Filter Paper

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SUMMARY. We investigated the feasibility of using Flinders Technology Associates (FTA®) 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® 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®, 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® 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® under unfavorable environmental conditions (41 C) for at least 15 days. The use of FTA® 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® 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®. Se investigó la posibilidad de utilizar tarjetas de papel de filtro FTA® 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® 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®, 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 el papel FTA® 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®, aún bajo condiciones ambientales desfavorables (41 C) por un mínimo de 15 días. El uso del papel de filtro FTA® 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® 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®, card, RNA, diagnosis

Abbreviations: AF = allantoic fluid; FTA® = 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® filter paper (Whatman, Newton, MA) for the collection and transport of biological samples. FTA® 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®, 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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RT-PCR for IBV on FTA® paper

M 1 2 3 4 5 6 7

![Image](https://bioone.org/journals/Avian-Diseases)

**Fig. 1.** Electrophoresis of FTA®/RT-PCR products on a 2% agarose gel stained with ethidium bromide. (A) Primers S1 5'-oligo/3' degenerate 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.

**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.

**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 (10⁴ EID₅₀/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 FTA® discs. DNA marker is 2000-50 bp. IBV amplicon is 383 bp.
were mixed with 750 μl of Trizol® LS and 200 μl of chloroform, vortexed, and spun for 5 min at 14,000 rpm. The RNA in the aqueous phase was precipitated with 500 μl of isopropanol and then washed with 75% ethanol and suspended in 40–50 μl of deionized pyrocarbonate H$_2$O. RNA concentration and purity were estimated by spectrophotometer readings at A-260nm and A-280nm.

**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® system. The PCR mixture (50 μl) contained 26 μl of deionized H$_2$O, 8 μl of 5x buffer, 4 μl of 25 mM MgCl$_2$, 2 μl of 100 mM DTT, 1 μl (5 U) of enzyme mix, 1 μl of 10 mM DNTP mix (Amersham Biosciences, Piscataway, NJ), 1 μl of each primer (50 μM; Qiagen, Germantown, MD), and 5 μl 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).

**RESULTS**

**Virus inactivation on FTA® cards.** IBV in AF stored on FTA® 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®/RT-PCR system.** Fig. 1 shows the RT-PCR amplified product from an AF-containing virus 24 hr after inactivation on FTA® cards.
being spotted on FTA in 1-, 5-, 10-, 25-, and 50-μl 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 μl of each extraction (RNA concentration ranged from 158 ng/μl to 225 ng/μl) 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.
DISCUSSION

Two main issues are involved in the use of FTA® 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®/C210 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®/RT/PCR system for IBV detection was sensitive because only 1 l of a positive AF sample, representing 10 EID₅₀, 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®/C210 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® system had high specificity because no cross-amplification was observed with other respiratory pathogens.

The detection of IBV in AF stored on FTA® 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®/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®-stored specimens. Although we showed that phenol inactivated samples may be stored on FTA® paper and also be analyzed molecularly, the advantages of FTA® inactivation are superior: nontoxic, simple and rapid processing, and ambient storage conditions. Our data show that FTA® cards can be used for collecting and transporting AF-containing IBV in a way that is simple, inexpensive, safe, and suitable for molecular analysis.

REFERENCES


