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Full Length Research Paper

Stability of the foot-and-mouth disease virus stored in cellulose-based FTA cards at different temperatures

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Abstract

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Foot-and-mouth disease (FMD) is a highly contagious viral infection affecting cloven-hoofed animals. In FMD-endemic regions, proper storage and transportation of clinical samples are critical for preserving virological and epidemiological data. This study evaluated the suitability of Advantec® filter paper, a novel cellulose-based medium, for storing foot-and-mouth disease virus (FMDV) RNA for subsequent amplification and sequencing. FMDV RNA stability was tested under four temperatures (-40°C to 25°C) and storage durations (2–12 weeks). Stability was assessed using RT-PCR and VP1 coding region sequencing, with nucleotide alignment performed to determine genomic integrity. Results showed that FMDV RNA remained stable for up to 10 weeks at room temperature (25°C) and 4°C, and over 12 weeks at -20°C and -40°C. Furthermore, nucleotide sequences extracted from filter paper were 100% identical to those from epithelial tissues stored at -80°C. These findings suggest that Advantec® filter paper is a viable alternative to conventional FTA cards, effectively preserving FMDV RNA for up to 10 weeks at moderate temperatures. This highlights its potential as a cost-effective transport medium for field-based virus surveillance and diagnostics, particularly in resource-limited settings.

Keywords: FTA cards, Advantec® filter paper, FMD virus, viral RNA isolation, storage temperature.

Introduction

Foot-and-mouth disease (FMD) is an extremely contagious viral disease affecting a variety of cloven-hoofed animals, including cattle, pigs, sheep, and several wildlife species. The causative agent is the foot-and-mouth disease virus (FMDV), classified within the *Aphthovirus* genus of the Picornaviridae family. This virus has seven distinct serotypes, which add complexity to vaccination efforts, as each serotype requires specific immunization (Pirbright Institute, 2023; EuFMD, 2023 WOAH, 2023). FMD's impact in endemic regions is substantial, leading to reduced livestock productivity and posing significant barriers to international trade due to stringent health and safety regulations (Knight-Jones et al., 2019). Given the rapid spread of FMD and its serious economic implications, effective management relies heavily on swift and accurate laboratory diagnosis, including

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precise serotyping of the virus during outbreaks. This approach supports targeted vaccination strategies essential for controlling and potentially eradicating the disease in affected areas (Knight-Jones & Rushton, 2013).

In resource-challenged laboratories, effective laboratory diagnosis of FMD is often hindered by inefficient preservation of samples due to logistical challenges including those linked to inadequate cold-chain transport and storage facilities (EuFMD, 2023; Fowler et al., 2014). These factors collectively undermine the capacity to detect and characterize the FMDV. Consequently, there is a vested interest in formulating and assessing approaches that can effectively conserve and retain FMDV samples to ensure the complete retrievability of the virus during analysis at reference laboratories. Diverse research endeavours have explored the feasibility of various materials, including the use of filter papers also known as Fast Technology for Analysis of nucleic acids (FTA) cards (Smith and Burgoyne, 2004) which are used for preserving biological specimens (FMD Reference Laboratories, 2023; Krambrich et al., 2022; Madhanmohan et al., 2013). Particularly noteworthy is the prospect of utilizing filter papers to conserve FMDV from epithelial tissue samples, facilitating subsequent nucleic acid amplification and sequencing, especially in regions where the disease prevails. FTA® cards stabilize nucleic acids at ambient temperatures by lysing cells and preserving genetic material, making them an ideal option for fieldwork where refrigeration is unavailable. The commonly used commercial FTA® produced by Whatman is impregnated with chemicals that can destroy and prevent the growth of bacteria to protect the nucleic acid in the sample for extended periods (GE Healthcare, 2019; Reeve et al., 2018). However, there are certain limitations in using most of the existing FTA such as degradation of nucleic acids, incomplete lysis of certain pathogens as well as compatibility issues with downstream applications. To address these limitations, Advantec® FTA cards have emerged as an alternative, offering improved performance in sample preservation and molecular processing. Unlike the widely used Whatman FTA® cards, the suitability of Advantec® filter paper for nucleic acid storage and its potential as an alternative storage medium have not been extensively tested.

The main goal of this study was to assess the stability of FMDV stored on Advantec® filter papers under various storage conditions. Specifically, the research aimed to evaluate RNA stability at different temperatures, providing guidance on the optimal storage conditions for users employing these filter papers for sample preservation.

Materials and Methods

Study area and laboratory conditions

This study took place in the Microbiology Laboratories at the College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, following approval from the university's Research Ethics Committee. During the laboratory procedures, average ambient daytime temperatures ranged from 29 to 31°C, with humidity levels between 60% and 85%.

Test material

The test filter paper under examination was Advantec®, produced by Toyo Roshi Kaisha Limited in Tokyo, Japan. It has been described by the manufacturer to be made from 100% cotton linter cellulose, pH tolerant up to 12, and thermostable up to 120°C with an estimated ash content of 0.1%. This filter paper is classified as a qualitative analysis medium (Grade No. 131) and has a smooth, white surface. It is noteworthy that Advantec® filter paper is susceptible to the effects of dimethyl sulfoxide (DMSO). As a precautionary measure, it is advised to avoid direct exposure to sunlight, ultraviolet radiation, excessive moisture, elevated temperatures beyond the thermostable limit, high humidity, open-air storage, and contact with potent oxidizing agents while utilizing it.

Epithelial suspension preparation

This virus genetic material was extracted from archived epithelial tissue specimens that had been gathered during a suspected FMD outbreak in Tanzania. Subsequently, it was verified as being of FMDV serotype O. A 1-gram segment of epithelial tissue sample was aseptically extracted from the virus transport medium employing sterile forceps. Subsequently, this tissue was carefully sectioned into smaller fragments using sterile scissors and then relocated into a mortar. To formulate a virus suspension of 10%, a total of 10mL of phosphate buffer saline was introduced, and the amalgamation was thoroughly pulverized by utilizing a pestle within a specialized biological safety cabinet.

Loading of virus onto filter papers

Twelve filter papers were divided into four sections using a sterile pair of scissors. These papers were subsequently immersed in a viral suspension before being air-dried at room conditions for one hour. The dried papers were then placed in four separate envelopes, each envelope with 12 pieces and then stored at room temperature (25°C), 4°C, -20°C, and -40°C for a maximum of twelve weeks.

Elution of virus and RNA extraction

At 2, 4, 6, 8, 10, and 12 weeks, two pieces of the filter papers from each envelope stored at the different temperatures were withdrawn for virus extraction. For each extraction the filter papers were cut into four millimetres square fragments using sterile scissors and transferred into micro centrifuge tubes. 1000 µL of nuclease-free water was added to each tube, and the mixture was incubated for one hour at 50°C with periodic vortex mixing every 30 minutes to obtain viral suspensions. Viral RNAs were extracted from the viral suspensions using the Quick-RNA® Miniprep Kit

(Epigenetics, USA), following the manufacturer's instructions. The RNA extracts were directly quantified spectrophotometrically on Nanodrop (NanoDrop™ Thermofischer scientific), and then stored at -80°C before polymerase chain reaction (PCR) analyses.

Pan-serotypic detection of FMD virus by RT-PCR

Preparation of reaction mixtures

Real time polymerase chain reaction (RT-PCR) amplification of the extracts was performed to detect and quantify FMD RNA employing forward and reverse primers as delineated in Table 1. Within the RT-PCR reaction, both negative and positive controls were incorporated. The negative control was constituted of RNase-free water, while the positive control encompassed epithelial tissue samples that had been verified as FMDV serotype O and had been stored at -80°C. The amplification procedure to produce a master mix was executed utilizing the QIAGEN® one-step RT-PCR Kit (Hilden, Germany). The master mix was distributed into individual PCR tubes, and 2.5 µL of RNA was added to each tube, bringing the total volume to 25 µL.

PCR amplification

Amplifications were done in the Thermal Cycler 9700® PCR System manufactured by Applied Biosystems, California, USA. The cycling conditions were set and carried out according to the PCR system manufacturer's guidelines. The PCR amplification products were then subjected to analysis through electrophoresis on a 1.5% agarose gel, which had been stained with E-Z Vision staining dye. Bands of 328 base pairs were observed, and the gels were photographed under UV light.

Amplification and sequencing of VP1 region of FMDV eluted from novel filter paper

To assess the stability of FMDV RNA stored on filter paper under different temperatures and durations, the VP1 coding region of FMDV was amplified and sequenced after being eluted from the filter paper and initially confirmed as positive using pan-serotypic RT-PCR. The VP1 region was chosen for RT-PCR and sequencing due to its high variability, role in serotype differentiation, antigenic importance, and established use in molecular epidemiology. Firstly, we performed a one-step RT-PCR on the elution wash obtained from filter paper samples that had tested positive in the pan-serotypic RT-PCR. This RT-PCR aimed at detecting the VP1 region of FMDV using specific primers, namely O-1C244F, O-1C272F, and EUR-2B52R (Table 1). RNase-free water was used as negative control, while epithelial tissue sample that had been confirmed to contain FMDV serotype O and were stored at -80°C was used as positive control. The QIAGEN® one-step RT-PCR Kit was used for the PCR. PCR products were analysed by electrophoresis on a 1.5% agarose gel that had been stained with E-Z Vision staining dye.

Purification of RT-PCR products

After the PCR process, the resulting products underwent several steps of purification following the guidelines of the Illustra GFX™ PCR DNA and Gel Band Purification Kit (Buckinghamshire, UK).

Cycle sequencing

The sequencing of eluted purified DNA was carried out employing the BigDye® Terminator v 3.1 Cycle Sequencing Kit (ThermoFisher Scientific, California, USA). Primers used for the cycle sequencing are described in Table 2.

Data analysis

The gel electrophoresis output of RT-PCR products from samples that were stored at varying temperatures for 12 weeks is displayed in a photographic format. Meanwhile, the information derived from sequencing the VP1 region of FMD virus RNA was collected and processed with DNASTAR Laser gene 10-core suite software. Following this, nucleotide sequence alignment and analysis were performed using MEGA 7.0.21 software, and the findings were presented in tabular form.

Results

Pan-serotypic detection of FMD virus by RT-PCR

RT-PCR products were observed following the storage of FMDV samples on filter papers under various temperature conditions, including room temperature (25°C), 4°C, -20°C, and -40°C, for a duration of up to 12 weeks (Figure 1).

The negative control showed no amplification, while the positive control successfully amplified. Notably, the FMDV genome was detectable for a duration of up to 10 weeks when stored at both room temperature and 4°C, where after 10 weeks of storage under these conditions, there were no more amplifications (Table 3). In contrast, samples stored at -20°C and -40°C continued to yield positive results by RT-PCR even after 12 weeks of storage.

Table 1: Primers information for pan-serotypic viral detection using RT-PCR

Designation	*Sequence (5' to 3')*	*Gene*	*Position*
O-1C244F	GCAGCAAAACACATGTCAAACACCTT	VP3	244-269
O-1C272F	TBGCRGGNCTYGCCCAGTACTAC	VP3	272-294
EUR-2B52R	GACATGTCCTCCTGCATCTGGTTGAT	2B	52-77

Table 2: Information on primers used for cycle sequencing

Designation	*Sequence (5' to 3')*	*Gene*	*Position*
NK72R	GAAGGGCCCAGGGTTGGACTC	2A/2B	34-48; 1-6
O-1D296aF	ATAACACCACTAATCCAAC	VP1	296-314
O-1C244F	GCAGCAAAACACATGTCAAACACCTT	VP3	244-269
O-1C272F	TBGCRGGNCTYGCCCAGTACTAC	VP3	272-294
EUR-2B52R	GACATGTCCTCCTGCATCTGGTTGAT	2B	52-77

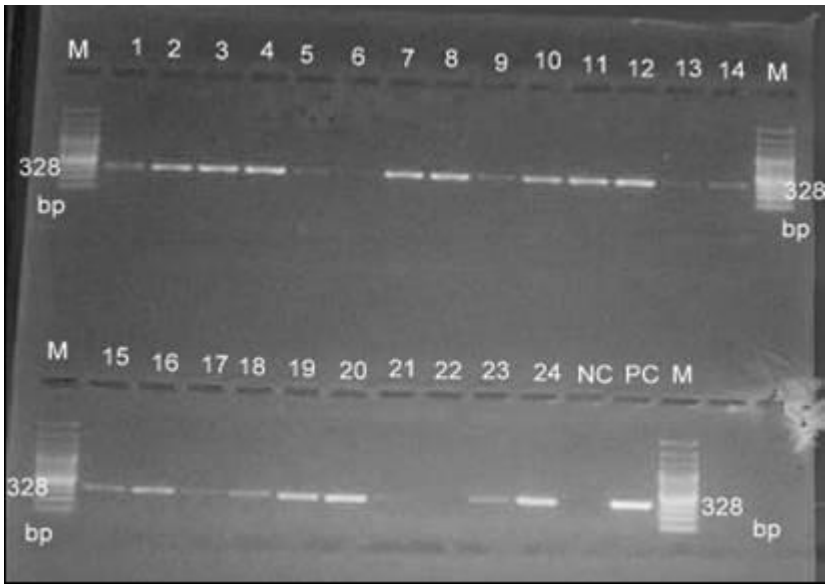

Figure 1: Gel electrophoresis of RT-PCR products targeting 5' UTR of FMDV genome for FMDV samples stored for either 2, 4, 6, 8, 10, or 12 weeks at different temperatures. Lanes 1, 5, 9, 13, 17, and 21 are filter papers stored at room temperature. Lanes 2, 6, 10, 14, 18, and 22 are filter papers stored at 4^{o}C. Lanes 3, 7, 11, 15, 19, and 23 are filter papers stored at -20^{o}C. Lanes 4, 8, 12, 16, 20, and 24 are filter papers stored at -40^{o}C. NC: negative control, PC: positive control, pb: base pair, M: 100bp DNA ladder. 328 bp amplicon indicates successful detection of FMDV RNA.

Table 3: Summary of gel electrophoresis results for RT-PCR products targeting 5' UTR of FMDV genome for FMDV samples stored for up to 12 weeks at different temperatures

Temperature	*Weeks post smearing*					
(°C)	2	4	6	8	10	12
Room temp.	+	+	+	+	+	-
4	+	+	+	+	+	-
-20	+	+	+	+	+	+
-40	+	+	+	+	+	+

+ positive signal (328 bp amplicon); - negative signal (no amplicon)

Sequences of the VP1 region of FMDV from control and filter paper

A sequence comparison, focusing on a 638-nucleotide segment within the VP1 portion of the FMDV genome, remarkably exhibited a consistent 100% similarity across all storage conditions as illustrated in Table 4.

Discussion

The current study has demonstrated the effectiveness of the tested filter paper, Advantec®, in storing FMD virus samples for up to 10 weeks at both 4°C and room temperature. Moreover, the use of the filter paper allowed for extended sample storage when maintained at lower temperatures, such as -20°C and -40°C. The practice of using filter papers for storing biological samples for diagnostic purposes has been widely adopted in both medical and veterinary fields, as evidenced by various studies (Köster et al., 2021; Shalaby et al., 2020; Cardona-Ospina et al., 2019; Picard-Meyer et al., 2007; Michaud et al., 2007; Wambura 2006). For instance, Wambura (2006) reported that Newcastle disease virus (NDV) stored on Whatman® filter paper remained detectable by RT-PCR whether stored at room temperature or 37°C for approximately 21 weeks. Picard-Meyer et al. (2007) assessed the use of FTA® Gene Guard System cards for the shipment, storage, and detection of rabies viruses using a simplified hemi-nested reverse transcriptase polymerase chain reaction (HnRT-PCR). They found that viral RNA remained stable on the cards at room temperature for an average of 6 weeks. Additionally, Michaud et al. (2007) detected nucleic acid from African swine fever and peste des petits ruminants even after 3 months of storage on Whatman FTA® cards at 32°C, and DNA viruses could be detected at least 9 months after storage at 37°C. These studies collectively suggest that filter papers have potential in viral diagnostics. Köster et al., (2021) investigated the suitability of filter cards for long-term storage of faecal samples of animal and human origin positive to the diarrhoea-causing protozoan parasites, *Giardia duodenalis* and *Cryptosporidium hominis*. They demonstrated that filter cards impregnated with faecal matrices containing these pathogens were fully compatible with downstream molecular methods for up to six months at room temperature.

In addition to detecting the virus following various storage conditions, the comparison of the 638-nucleotide sequences of VP1 region between viral RNA eluted from the filter paper and the corresponding epithelial tissue sample kept at -80°C revealed no nucleotide sequence variations. These results strongly indicate that the FMDV genome stored on Advantec® filter paper remained fully intact under the tested temperature and duration conditions. Further confirming the role of filter papers as an alternative to the cold chain for storing FMDV samples. FTA cards have successfully been used to recover intact viral genomes and facilitate sequencing, even after various storage conditions (Fowler et al., 2014). Keck et al., (2022) demonstrated that the viral material applied to FTA cards was adequately preserved, enabling detailed molecular analysis through sequencing. They successfully retrieved infectious FMDV from certain cards via chemical transfection, verifying that the full-length RNA sequences remained intact and suitable for in-depth genetic study.

The absence of the 328 bp amplicon on Advantec® filter paper after 10 weeks at room temperature in this study suggests degradation of FMDV RNA. This degradation is primarily attributed to active RNases, hydrolytic processes enhanced by ambient humidity, and the lack of protective chemical treatments characteristic of Advantec® filter paper (Mathay et al., 2012). However, storing RNA at -20°C minimizes these factors, effectively preserving RNA integrity for extended periods.

These cards, in comparison with other sample storage methods, are advantageous as they do not require a cold

Table 4 Nucleotide sequences (A and B*) of RT-PCR products targeting the VP1 coding region.

Sequence	Nucleotides
A	>>GACACAAACCACTTCTCCGGGCGAGTCGGCTGACCCTGTGACTGCCACCGTGGAAAAC
	TACGGCGGCGTGACTCAGAACCAGAGACGTCAACACACGGACGTTTCGTTCATTCTGGAC
	AGATTTGTGAAGGTCACACCCCAAGACCAGGTCAACGTCTTGGACTTGATGCAGATTCCT
	GCCCACACACTGGTGGGCGCGCTCCTACGCGCGTCCACCTACTACTTTGCTGACTTGGA
	GGTAGCAGTGAAGCATGAGGGCAACCTCACTTGGGTCCCGAACGGAGCACCCGAAGCCG
	CATTGGACAACACCACCAATCCAACAGCATACTACAAGGCACCTCTCACCCGTCTTGCAC
	TGCCTTACACGGCCCCACACCGTGTGCTGGCAACTGTGTACAACGGAAACTGCAAGTACG
	GCAGCGCCCCAGTGGCCAACGTGAGGGGCGACCTTCAGGTGCTGGCCCAGAAGGCTGC
	AAGAGCGCTGCCCACCTCCTTCAACTACGGTGCCATCAAAGCGACCCGGGTGACAGAAC
	TGCTTTACCGCATGAAGAGGGCCGAGACTTACTGTCCCCGACCCCTCTTGGCCATCCACC
	CGAGTGACGCTAGACACAAACAAAAGATTTGTGGCACCTGTCAAACAACTCCTG
B	>>GACACAAACCACTTCTCCGGGCGAGTCGGCTGACCCTGTGACTGCCACCGTGGAAAAC
	TACGGCGGCGTGACTCAGAACCAGAGACGTCAACACACGGACGTTTCGTTCATTCTGGAC
	AGATTTGTGAAGGTCACACCCCAAGACCAGGTCAACGTCTTGGACTTGATGCAGATTCCT
	GCCCACACACTGGTGGGCGCGCTCCTACGCGCGTCCACCTACTACTTTGCTGACTTGGA
	GGTAGCAGTGAAGCATGAGGGCAACCTCACTTGGGTCCCGAACGGAGCACCCGAAGCCG
	CATTGGACAACACCACCAATCCAACAGCATACTACAAGGCACCTCTCACCCGTCTTGCAC
	TGCCTTACACGGCCCCACACCGTGTGCTGGCAACTGTGTACAACGGAAACTGCAAGTACG
	GCAGCGCCCCAGTGGCCAACGTGAGGGGCGACCTTCAGGTGCTGGCCCAGAAGGCTGC
	AAGAGCGCTGCCCACCTCCTTCAACTACGGTGCCATCAAAGCGACCCGGGTGACAGAAC
	TGCTTTACCGCATGAAGAGGGCCGAGACTTACTGTCCCCGACCCCTCTTGGCCATCCACC
	CGAGTGACGCTAGACACAAACAAAAGATTTGTGGCACCTGTCAAACAACTCCTG

*Sequence A: recovered from the positive control, Sequence B: recovered from the filter paper at different temperatures of storage.

chain, a feature particularly valuable in remote areas. For FMDV, nucleic acids stored on FTA cards and similar filter papers have shown minimal degradation, proving suitable for diagnostic purposes such as RT-qPCR and sequencing (Krambrich et al., 2022; Madhanmohan et al., 2013). Although our study did not compare Advantec® to other FTA cards in terms of cost, availability, and ease of use; we are convinced that the current findings place the

former among useful and efficient storage cards for diagnostic purposes.

In summary, based on the current findings, the Advantec® filter papers can be used as an alternative to other types of FTA cards available in the market and can store FMDV during transport or in the laboratory environment at a temperature range of 25 - 31°C for up to 10 weeks without genome degradation. These findings also support the use of Advantec® filter paper for storing FMDV samples, especially in regions with tropical climates and limited access to cold chain facilities. Follow up studies and field tests are required to test other FMDV serotypes or other viruses, find out whether the FMD virus remains infective after the storage period, as well as how the Advantec® filter paper compare with other FTA cards in terms of environmental factors such as moisture and sunlight.

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Conflict of interest

The authors declare no conflict of interest.

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