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A new solid matrix for preservation of viral nucleic acid from clinical specimens at ambient temperature

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Abstract

Stabilizing paper matrix methods for retaining nucleic acid from inactivated clinical specimens offer a solution for molecular diagnostics when specimens may be stored or shipped at ambient temperature. We developed cellulose disks (UNEXP) saturated with a total nucleic acid extraction buffer (UNEX) modified from a previously developed lysis buffer for multiple enteric pathogens. Infectivity of hepatitis A virus, adenovirus and poliovirus was destroyed after 2–3 h incubation at room temperature on the UNEXP disks. Norovirus RNA could be detected in UNEXP-eluted nucleic acids by reverse transcription-quantitative PCR (RT-qPCR) from 54 stool samples after 2 weeks storage at room temperature on disks; a subset of seven samples were positive after 3 months storage. Genotyping was successful in 76% of 54 samples tested including six of seven samples stored on the UNEXP disks for up to one month. Comparison of UNEXP with the FTA elute card in a subset of 10 samples demonstrated similar detection and genotyping rates after two weeks of storage at room temperature.

UNEXP disks could be useful for epidemiologic investigations of disease outbreaks in resource-limited areas by simplifying specimen transport to regional diagnostic laboratories or shipment to international centers without the need to ship samples on dry ice.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Keywords

Norovirus; Nucleic acid preservation; Safe transport; Virus inactivation

1. Introduction

Numerous studies over several decades have described preservation and storage of clinical specimens on a paper matrix, such as the blood spot card for use in neonatal genetics to drug testing reviewed in Enderle et al. (2016). In addition these matrices have been used for disease surveillance particularly in resource limited environments for a range of pathogens from parasitic enteropathogens to HIV (Natarajan et al., 2018; Solomon et al., 2002). Although complete inactivation of infectivity is not assured with these cards, the cost is very low. The Flinders Technology Associates (FTA) card, a chemically treated paper matrix designed to lyse cells, fix and store nucleic acids and inactivate pathogens, has been more commonly used for infectious pathogens, particularly viruses. FTA cards are available in a variety of types and formats including the FTA classic (frequently called the FTA card) and a newer FTA elute card. FTA cards have been used successfully for identification of pathogens of animal origin (Abdelwhab et al., 2011; Cortes et al., 2009; Inoue et al., 2007; Linhares et al., 2012; Madhanmohan et al., 2015; Muthukrishnan et al., 2008; Perozo et al., 2006; Purvis et al., 2006). More recent studies have focused on detection of viral nucleic acid of human pathogens from FTA cards using varying elution methods and storage conditions (Naslund et al., 2011; Sakai et al., 2015; Tam et al., 2015). These newer approaches that alter the procedures for card processing have provided some successful use of the FTA card for viral pathogen detection and identification. Other approaches to the detection of viruses include the RNASound™card for West Nile virus (WNV) and influenza virus (Foss et al., 2016; Lau and Hurt, 2016). WNV was detected in dead birds equally well with RNA Sound and FTA cards, although RNA sound only preserves for one week.

Data on the use of FTA cards for the detection of human RNA viruses are limited, especially for single-stranded RNA viruses. A 6.1% recovery rate was reported for enterovirus RNA from FTA classic cards (Li et al., 2012). Variable recovery for enteropathogens including *E. coli*, *Salmonella typhimurium*, *Giardia*, and norovirus GI and GII, was obtained with the FTA elute card (Lalani et al., 2015). More recently, norovirus was detected in 43% or fewer of samples on FTA elute cards as compared to frozen stools, although overall detection of all pathogens on the card was 73% (Lalani et al., 2018). FTA cards have been used successfully to transport cell culture lysate containing RNA viruses including measles and poliovirus for molecular analysis (Bankamp et al., 2013; Montmayeur et al., 2017). DNA viruses, such as human papilloma virus, have been detected and typed with 94% agreement between the standard method and an FTA card when clinical samples were tested (Gustavsson et al., 2009). Thus, the paper matrix has been used for storage, detection and genotyping of pathogens with a variety of approaches published, but with a limited number of studies for the detection of RNA viruses directly from clinical samples.

In the present study, we modified a universal nucleic acid extraction (UNEX) buffer that has been described for the lysis of viruses, bacteria, and parasites in complex matrices (Hill et

al., 2015). We used the modified UNEX buffer (UNEXP buffer) to saturate cellulose filter disks (UNEXP disks) and evaluated their ability to inactivate several non-enveloped viruses, determined the quantitative recovery of viral nucleic acid (NA, including RNA and DNA), and assessed the ability to detect and genotype norovirus eluted from UNEXP disks stored for 2 weeks at room temperature.

2. Materials and methods

2.1. Viruses and cell culture

Several infectious non-enveloped viruses (Hepatitis A virus (HAV), human adenovirus 2 (HAdV-2), poliovirus) were used to determine if UNEXP disk would be able to destroy their infectivity. HAdV-2 was cultured in A549 cells to obtain a titer of 1×10^9 PFU/ml (Cromeans et al., 2010). HAV (strain HM175-24A) was cultured and assayed in FrhK-4 cells to obtain a titer of 1×10^7 PFU/ml (Cromeans et al., 1989). Experimental infectivity assays were performed with a $TCID_{50}$ assay including 3 replicates of each ten-fold dilution and titers were calculated as described previously (Reed and Muench, 1938). Nucleic acids were extracted from virus stocks with the UNEX buffer as described (Hill et al., 2015). Viral nucleic acid extracts from inocula or disks were analyzed using real-time PCR (TaqMan® assays) for HAdV and HAV respectively (Jothikumar et al., 2005a; Jothikumar et al., 2005b).

An isolate of Sabin 1 poliovirus with a $CCID_{50}$ 1×10^8/ml was used to study inactivation of poliovirus on the UNEXP disk and was cultured in RD cells for determination of inactivation (Rezapkin et al., 1994). All poliovirus experimentation was performed in a Class II biological safety cabinet in CDC poliovirus laboratories.

2.2. UNEXP disks: preparation, addition of samples, and elution of nucleic acid

UNEX buffer was prepared as described for viruses (Hill et al., 2015) or purchased from Microbiologics (St. Cloud, MN) made by the same formula. Cellulose Whatman™ 3 MM Chromatography paper (Fisher Scientific St. Louis, MO.) was used as the solid matrix to make disks by manually punching 16-mm disks. Prior to adding UNEX buffer to the disks, 4% Polyethylene glycol (Sigma, molecular biology grade) was added to prepare UNEXP. Sixty microliters of UNEXP was slowly dispensed dropwise to all areas of the disk (placed on clean aluminum foil). The disks were allowed to air dry completely for 2–3 h at ambient temperature in a Class II biological safety cabinet and were stored in sealed clean plastic bags until used. Prepared UNEXP disks were stored up to several months before use.

For molecular evaluation, UNEXP disks placed on clean aluminum foil were loaded drop-wise with 60 μl of virus sample, either cell culture virus serially diluted in PBS or 10% clarified stool specimens (undiluted or serially diluted). Sample storage on UNEXP disks was studied at multiple time points: 0 h, 3 days, 7 days and 14 days for HAV and HAdV2 experiments, and 0 h, 2 week, 1 month, and 3 months for norovirus experiments. For each time point, each sample was loaded on the disks in duplicate. UNEXP disks then were air-dried in a biological safety cabinet for 3 h. Nucleic acid from the baseline sample disks (0 h) was eluted immediately whereas UNEXP disks for all later time points were stored in

Ziploc® bags or in 1.7 ml microfuge tubes without desiccant at room temperature (~22 °C) until elution of NA. Clean gloves were used for handling each individual stool-inoculated disk in all procedures.

For elution of NA at the indicated times, individual disks not already in tubes were transferred to a 1.7-mL microcentrifuge tube and washed twice with 1 ml nuclease free water by re-pipetting vigorously four times. NA was eluted with 600 μl nuclease free TE buffer [DNA suspension buffer] (Teknova, Hollister, CA) at 95 °C for 15 min in a heating block. Detection of norovirus RNA in NA eluates was increasingly reduced when lower temperatures of 80, 65, 55, and 45 were evaluated with lack of detection at 22C.

After a brief spin, the 600 μl eluate was removed and the final liquid was obtained by pressing the crumpled disk into the bottom of a microfuge tube. NA was either tested the same day or stored at −70 °C until testing. In some experiments, NA from disks was tested undiluted therefore a 1:10 of original sample or diluted 1:10 (1:100 of original sample), unless otherwise indicated. Ct values were adjusted so that all NA detection results from UNEXP disks expressed as a 1:10 of original sample virus or stool suspension were equivalent to a 1:10 dilution of the original sample.

FTA elute disks of 13-mm size were made by punching from FTA elute micro cards (GE Healthcare) and loaded with 50 μl 10% stool suspension (volume reflecting smaller size than UNEXP disk) and dried for 3 h (similar to the UNEXP disk). For elution of NA from the FTA cards, the published FTA elute protocol was followed (https://www.gelifesciences.co.jp/catalog/pdf/WhatmanFTAElutedatafile_28984402AA.pdf). Disks were placed in 1.7-mL micro centrifuge tubes and washed for 5 s with sterile molecular biology grade water while vortexing several times. After removing excess water, eluted NA was obtained by addition of 500 μl TE, heating at 95 °C for 15 min. The tubes were spun briefly (3 s) and liquid was removed to a fresh tube for storage at −70 °C until analysis or analyzed immediately.

2.3. Clinical samples

Norovirus-positive stool samples (n = 54) were obtained from archived collections at the National Calicivirus Laboratory at CDC; samples had been stored at 4 °C or −70 °C from 3 months to four years. A variety of genotypes were selected as available in CDC archived samples. Of the 54 samples that were tested from the UNEXP disk, 13 were genogroup I (GI) and 41 genogroup II (GII) samples, reflecting that GII is the most commonly circulating genogroup. The 41 samples that were genotyped from the UNEXP disks included 9 different GII types and 6 different GI types. This selection of genotypes is representative of viruses that are currently circulating. CDC Human Research Protection Office reviewed the study and categorized it as public health non-research and that human subject regulations did not apply. Approximately 10% (weight/volume) fecal suspensions were prepared in PBS, vortexed for 1 min, and clarified by centrifugation at 10,000 × *g* for 10 min. MS2, a single-stranded RNA bacteriophage (ATCC, Manassas, VA), was added to each stool sample to obtain a Ct of 27–28 to evaluate potential RT-qPCR inhibitors present in the stool samples. An aliquot of each stool suspension containing MS2 was reserved for liquid UNEX/filter extraction and RT-qPCR along with the UNEXP disk extracts. In all

experiments, MS2 diluted in PBS was also added to UNEXP disks for NA extraction in the absence of stool, simultaneously with the disks containing stool and MS2. Prepared stool suspensions (or dilutions of them) were applied to the UNEXP disks placed on aluminum foil in a biological safety cabinet and allowed to dry. NA was eluted from disks as described above to establish the baseline (0 h) detection of norovirus RNA. For each experiment, duplicate disks of each sample were placed in sealed plastic bags or in 1.7-mL microcentrifuge tubes for storage at room temperature in a dark cabinet until the indicated times for NA elution. NA extracts were tested for norovirus RNA by multiplex RT-qPCR, which included MS2 to assess the presence of PCR inhibitors (Cannon et al., 2017).

2.4. Norovirus genotyping from UNEXP disks

For norovirus genotyping, RNA eluted from the UNEX disks was amplified using hemi-nested conventional RT-PCR (Kitajima et al., 2010) using the OneStep RT-PCR kit (Qiagen, Valencia, CA, USA). PCR products were separated on 2% gels and bands of appropriate size were purified using the QIAquick gel extraction kit (Qiagen) followed by Sanger sequencing (Eurofin, Operon, Louisville, KY). Sequences were edited and aligned using Sequencher (v 5.1, Gene Codes Corporation, Ann Arbor, Michigan) and MEGA7 (Kumar et al., 2016) and genotyped as previously described (Cannon et al., 2017) or using the online Human Calicivirus Typing Tool at https://norovirus.ng.philab.cdc.gov.

2.5. Testing for HAV, adenovirus and poliovirus infectivity after storage on UNEXP disks

Cell culture virus preparations (adenovirus [HAdV 2], HAV, poliovirus [Sabin 1]) were loaded onto cards as described above and processed at the end of the drying period. We evaluated two different approaches to detect infectious virus in UNEXP disk eluates. The first approach was applied for HAV and HAdV-2. Sixty microliters of cell cultured HAV (6×10^5 PFU/disk) was added to UNEXP disk or untreated control disks and allowed to dry for 2–3 h. Discs containing virus were placed individually into separate microfuge tubes, washed once with water, and then 600 μl DMEM 2% FBS (cell culture media) was added. The tubes with the disks were mixed by vortexing and incubated at 37 °C for 1 h (with vortexing every 15 min), after which the supernatant was removed and diluted 1:2, 1:4, 1:10, 1:100 and 1:1000 with Dulbecco's Minimum Essential Medium with 2% fetal bovine serum (DMEM + 2% FBS). Monolayers of Fetal Rhesus monkey kidney cells (Frhk-4) in 24-well plates were inoculated with 5 replicates of 0.1 ml of each dilution of the eluate, incubated at 37 °C and monitored for cytopathic effect (CPE) until day 14 (HAV) or day 6 (HAdV-2) to determine inactivation. HAdV-2 (6.0×10^6 $TCID_{50}$ per disk) was tested using the same protocol, except virus was eluted at room temperature (no 37 °C incubation step), and A549 cells were infected and incubated for 6 days at 37 °C. Control disks without UNEX buffer were also inoculated with HAdv2.

Additional approaches were used for HAV and poliovirus. Liquid inactivation of HAV was tested by addition of equal volumes of virus solution and UNEX buffer. Amicon Ultra-15 centrifugal filter units (50 kDa, Millipore Corporation) were used to remove any residual cytotoxicity from the UNEXP buffer by completely exchanging the eluates of viruses from the UNEXP disks or UNEX liquid buffer containing HAV with MEM. Untreated disk control (those not treated with UNEXP buffer) eluates were processed simultaneously on

the filter units to evaluate loss of the viruses on filters. A solution of 120 μl HAV and 120 μl lysis buffer or 120 μl HAV + MEM-only (control) was made, and each solution was combined with 10 ml MEM in an Amicon ultra-15 unit, spun at 4000 × *g* for 10–15 min to reconstitute to 240 μl (equal to the starting volume) to infect T25 flasks of Frhk-4 cells. Thus, all of the eluate (240 μl) was evaluated for infectivity without dilution. Each experiment was performed in duplicate. Infected cells were examined daily for CPE of HAV for 14 days.

UNEXP disk experiments with HAV and poliovirus were performed with the addition of HAV (6×10^5 PFU/disk) and Sabin 1 poliovirus (6×10^6 $CCID_{50}$ /disk) to UNEXP disks and to untreated disks as described previously. After drying for 3 h, disks containing HAV or poliovirus were eluted for 2 h at 37 °C in MEM. Instead of drying for 3 h, control disks loaded with poliovirus were eluted within a few minutes without drying because 3 h drying abolished infectivity in preliminary experiments (data not shown). Elution of control disks was also for 2 h at 37 °C in MEM. Each eluate (600 μl) (UNEXP disk and untreated disk) was added to the ultra-15 centrifugal filter followed by the addition of 10 ml MEM and concentrated to 600 μl (as described above) for inoculation of HAV or poliovirus in Frhk-4 cells in T25 flasks or RD (muscle tissue taken from rhabdomyosarcoma diseased cell) cells, respectively. Thus, the complete treated eluate was evaluated for infectious virus without dilution. Each experiment was performed in duplicate. Infected cells were examined daily for CPE of HAV and poliovirus for 14 days and 3 days, respectively.

In addition, inactivation of poliovirus on UNEXP cards was evaluated after spiking poliovirus into a poliovirus-negative human 10% stool suspension and incubation for 30 min prior to addition to the disks as described previously. To elute the virus from UNEXP and control disks, they were incubated for 2 h at 37 °C in MEM with vortexing every 15 min. Eluates (600 μl/disk) were then treated with 1/3 vol (200 μl) of chloroform to remove bacteria and mold before a buffer exchange with cell culture medium on an ultra-15 centrifugal filter as described previously. The total filtrate volume eluted from the UNEXP disks or control disks was then inoculated on RD cells to evaluate remaining infectivity. Cells inoculated with eluates from control disks and UNEXP disks were observed microscopically 3 days for CPE, followed by freeze-thawing and inoculation onto RD cells for a second passage.

2.6. Statistical analysis

Significant differences between Ct values of stool suspensions, typed samples, untyped samples and associated MS2 values were calculated using the *T*-test for independent samples (two-tailed results reported) or Mann Whitney two tailed *T*-test (http://vassarstats.net/).

3. Results

3.1. Inactivation of virus infectivity on UNEXP disks

3.1.1. HAV and adenovirus—Eluates from the UNEXP disks at dilutions lower than 1:4 for HAdv-2 on A549 cells, and 1:100 for HAV on FRHK cells were cytotoxic for the

respective cell monolayers; no infectious virus was detected at higher dilutions with HAdV2 or HAV. Infectious HAV or HAdv-2 could be detected in undiluted eluates of the control (untreated) disks.

Undiluted HAV eluates that were buffer-exchanged using Amicon columns did not show CPE at 7 days post infection (dpi) or at 10 dpi, whereas eluates from control disks exhibited 50% CPE at 7 dpi and 100% CPE at 10 dpi. Eluates of HAV-loaded UNEXP disks showed no CPE at 14 dpi. These data confirm that eluates from UNEXP disks do not contain infectious HAV or HAdv-2. HAV treated with liquid UNEX was also completely inactivated.

3.1.2. Poliovirus—Eluates from UNEXP disks loaded with poliovirus that were buffer exchanged using centrifugal filters did not show CPE at 3 dpi. Eluates from non-treated control disks loaded with virus showed complete CPE within 1 dpi. Eluates from control disks loaded with poliovirus-seeded stool suspension showed CPE in 1 dpi whereas those from UNEXP disks gave no CPE after 2 passages on RD cells after 3 dpi of each passage. Chloroform treated poliovirus gave CPE in 1 dpi the same as untreated virus, indicating chloroform treatment of the eluate prior to addition to cells did not contribute to inactivation of virus in the stool. These data demonstrate that 6×10^6 $CCID_{50}$ infectious units of poliovirus Sabin type 1 were not detectable from the UNEXP disks loaded with virus alone or with virus in stool suspension.

3.2. Detection of HAV and AdV2 nucleic acid from UNEXP disks

When Ct values for HAV RNA and HAdv-2 DNA of inocula were compared with Ct values of corresponding card eluates, essentially 100% of viral nucleic acid was detected (Table 1). This high NA detection was observed at all time points and Ct values increased proportionally with dilutions of the virus (approximately 3.3 Ct for each 10-fold dilution).

3.3. Detection and genotyping of norovirus RNA from clinical specimens inoculated onto UNEXP disks

To detect potential of RT-qPCR inhibitors, 10-fold serial dilutions (1:10, 1:100, 1:1000) of three norovirus positive stool suspensions were loaded onto UNEX disks in duplicate. Norovirus RNA was detected consistently for two (GII.4 stool 1 and GI.3) of the three 1:10 inoculums over a 2-week storage period (Table 2). Nucleic acid eluted from the 1:10 GII.4 inoculated (stool 2) disk had progressively higher Ct values throughout the 2-week storage period. At 1:100, RNA detection from the GI.3 specimen remained the similar over the 2 weeks studied. RNA detection from the 1:100 inoculated disks of the other two stool specimens decreased slightly, with a loss of 1 to 2 Ct values. For the 1:1000 inocula, RNA in the GII.4 (stool 2) inoculum was stable over the 2 weeks studied, with slight RNA degradation for the GI.3 specimen. Nucleic acid eluates from the 1:1000 dilution of the GII.4 stool samples had progressively higher Ct values through the 2-week period, culminating in average Ct values that were 12.3 units higher than on day 0. A similar increase of Ct values of MS2 indicated that this sample may have contained RT-PCR inhibitors or substances causing degradation over time.

Nucleic acid from UNEXP disks loaded with 7 undiluted 10% stool suspensions that had been genotyped previously showed that norovirus RNA could be detected up to 3 months after loading (Fig. 1). Six of the seven samples could be correctly genotyped after one month of storage on the UNEXP disk, and two samples could be genotyped at 3 months. Additional NA extracts of UNEXP disks loaded with 10% stool suspensions (n = 47) were evaluated after 2 weeks storage at room temperature on UNEXP disks, providing 54 total stool suspensions that were detectable and candidates for genotyping from the UNEXP disks. Forty-one (75%, 41/54) norovirus-positive stool samples could be genotyped including 16 different genotypes (Fig. 2).

The mean Ct value of the stool suspensions that could be genotyped was 23 (Fig. 2), whereas the mean Ct value was 27 for the corresponding 41 UNEXP NA eluates (Fig. 2). In contrast, the mean Ct value of the NA from the UNEXP disks that could not be genotyped was 31, an increase of 7 Ct from the 24 Ct for the stool sample inocula which was not significantly different from the mean Ct value of 23 for the typed stool samples ($p = 0.3775$, *t*-test). The mean Ct values for the NA extracts that were genotyped compared to those untyped was 27 and 31, respectively, which is significantly different ($p = 0.0006$, *t*-test). The mean MS2 Ct value of the 13 samples that could not be typed was 33 compared to a Ct of 30 for the 41 typed samples (data not shown). A Mann Whitney two tailed *t*-test indicated a significant difference ($p = 0.025$) between MS2 values detected in typed and untyped stools.

The FTA elute cards were compared with the UNEXP disks for the detection and genotyping of norovirus from 12 stool samples (Fig. 3). The same detection frequency and similar genotyping performance was found for both FTA elute cards and UNEXP disks. UNEXP disks yielded genotyping of eight samples and FTA elute genotyping of seven samples; only five were genotyped by both methods. Ten samples were genotyped by either or both methods.

4. Discussion

The UNEXP disk was developed as a simple tool to preserve, store and transport NA from inactivated clinical samples or cultured viral isolates as an alternative to traditional methods, when cold storage and/or shipping on dry ice is not available or affordable, and when biohazard labeling will complicate the process and/or increase the cost. Virus inactivation data from this study indicate that UNEXP disks carrying NA of viral pathogens can facilitate safe shipping of samples as non-infectious materials in small containers (e.g., envelopes) at ambient temperature, resulting in a much lower cost than is typical for infectious and hazardous pathogens that would require biohazard labeling and dry ice to preserve infectivity. The infectivity of three different viruses (a DNA virus [(HAdV-2]) and two RNA viruses [HAV and poliovirus]), was completely eliminated after exposure to the UNEXP disks, which are critical experiments to show that shipping clinical samples on UNEXP disks is safe. HAV and HAdV-2 represent viruses that are resistant to environmental stresses (Pina et al., 1998; Siegl et al., 1984), and with the eradication of poliovirus, many clinical samples collected through surveillance systems of other pathogens, which potentially contain poliovirus (known as potentially infectious material [PIM]), must be destroyed, contained, or transferred to a poliovirus essential facility as per GAPIII (http://

polioeradication.org/wp-content/uploads/2016/12/GAPIII_2014.pdf). Our data suggest that selected samples potentially containing poliovirus could be stored as non-infectious NA on UNEXP disks. We only examined storage for at least 2 weeks at ambient temperature, but storage at 4 °C, −20 °C or −80 °C might provide much longer-term storage for NA-containing UNEXP disks. The complete inactivation of HAV and poliovirus suggests that other small non-enveloped RNA viruses, such as norovirus, will be inactivated on the UNEXP disk as well. In the future, reduction of human norovirus infectivity may be assessed by a recently reported cell culture system (Costantini et al., 2018; Ettayebi et al., 2016).

Viral RNA can be recovered successfully from commercially available FTA classic cards inoculated with Betanodavirus infected material (Krishnan et al., 2016) and rabies virus RNA (Sakai et al., 2015). In the Krishnan et al. study, stability and concentration of Betanodavirus RNA decreased significantly over a 3-week storage period at 4 °C. In the Sakai study, rabies virus RNA was stable at −20 °C and −70 °C over 3 months, although degradation was observed at 4 °C and at room temperature in this study. Foot–and-mouth-disease (FMDV) RNA detection from FTA cards (Madhanmohan et al., 2015) loaded with clinical samples and shipped at varying temperatures and relative humidity, demonstrated recovery of viral RNA, but no quantitation was performed. West Nile virus testing by RT-qPCR in dead bird from oral swabs using FTA cards and RNASound™ demonstrated equally sensitive to the antigen tests of oral swabs (Foss et al., 2016). Therefore, the use of FTA cards was determined a desirable alternative to shipment of dead birds for kidney tissue testing. Rotavirus RNA, eluted at ambient temperature from FTA classic cards, could be detected up to 28 days on FTA cards at varying storage temperatures with 50% loss (Tam et al., 2015) using an alternative card extraction method. The quantitative data from our study suggest equal or better performance for the UNEXP disk in terms of long-term storage of viral RNA at ambient temperature, up to 3 months.

Previous work reported that norovirus RNA eluted from FTA elute cards that had been loaded with a direct stool smear could be detected in 50% of the samples (Lalani et al., 2018, 2015) at up to 6 months. In our study, norovirus GI and GII could be detected in 100% of samples from UNEXP disks at up to 2 weeks, which is adequate time to ship disks from remote locations to testing labs.

Although norovirus RNA from stool suspensions was consistently detectable in NA extracts from UNEXP disks, including those stored for up to 3 months at room temperature, 24% (13/54) of samples could not be genotyped. Possible reasons include a lower sensitivity of the conventional RT-PCR assay used for genotyping compared to real-time PCR assays or the conventional RT-PCR assays were more susceptible to PCR inhibitors than the real-time assay. Stool samples contain several major inhibitors of RT-PCR and PCR reactions, including lipids, bile salts, complex polysaccharides and urate (Schrader et al., 2012). Individual stools may vary in the composition and quantity of inhibitors, as indicated by the variable MS2 values obtained in our experiments. Statistical data on MS2 average values presented in results indicated increased inhibition in the untyped group ($p = 0.025$). The water washes before heat release were incorporated into the procedure to remove UNEX salts on the card and possibly some other inhibitors present in the stool sample on the disk.

We attempted several approaches to remove possible inhibitors in the NA extracts eluted from the UNEXP disks including: i) Zymo RNA Clean and Concentrator with and without DNase, ii) concentration of NA by raising the concentration of NaCl to 2.5 M followed by column purification, iii) RNeasy power pro cleanup kit (Qiagen) and iv) re-extraction with liquid UNEX buffer followed by Qiagen column RNeasy extraction. Although several of these approaches provided at least several additional samples that could be genotyped, no single method provided an adequate increase in the number of samples genotyped to merit further larger-scale evaluation (data not shown). We also used chloroform extraction of stool suspensions, which potentially removes lipids, prior to loading to the UNEXP disks, and included BSA gp32 known to facilitate PCR reactions in the presence of inhibitors, all without resulting in a higher number of samples that could be genotyped.

Because UNEXP disks can be prepared in almost any laboratory using relatively inexpensive, commercially available items, the disks represent a cost-effective alternative to the purchase of commercial cards. UNEXP disks could provide a preservation method for NA present in clinical samples for future analysis, potentially years later if stored at lower temperatures. Current rapid diagnostics to identify and classify pathogens relies increasingly on NA detection and characterization. With the potential to identify DNA or RNA viruses with molecular methods, UNEXP disks could be useful for epidemiologic investigations of disease outbreaks in resource-limited areas by simplifying specimen transport to regional diagnostic laboratories or shipment to international centers without the need to ship samples on dry ice. Additional studies are needed to confirm the usefulness of UNEXP disks for other pathogens and applications.

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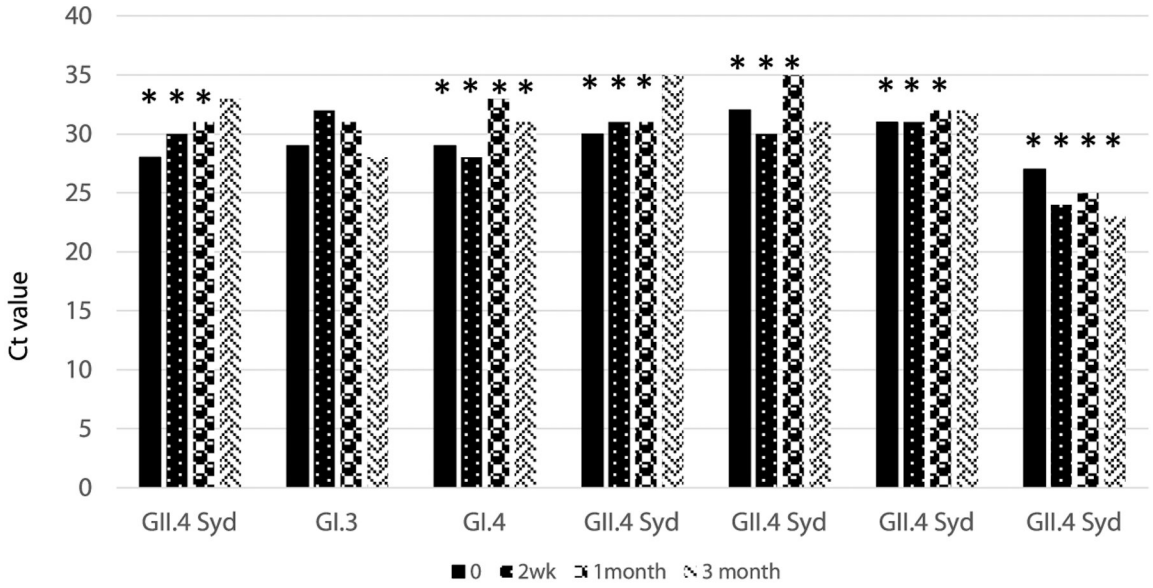

Fig. 1.

Detection of norovirus RNA eluted from UNEXP disks at 0 h, 2wk, 1 mo, and 3 mo after loading of seven different clarified stool suspensions. Ct values are averages of 2 card eluates, tested in duplicate by RT-qPCR. Confirmation of success of obtaining the same genotype as detected in stool is indicated by the * above the bar.

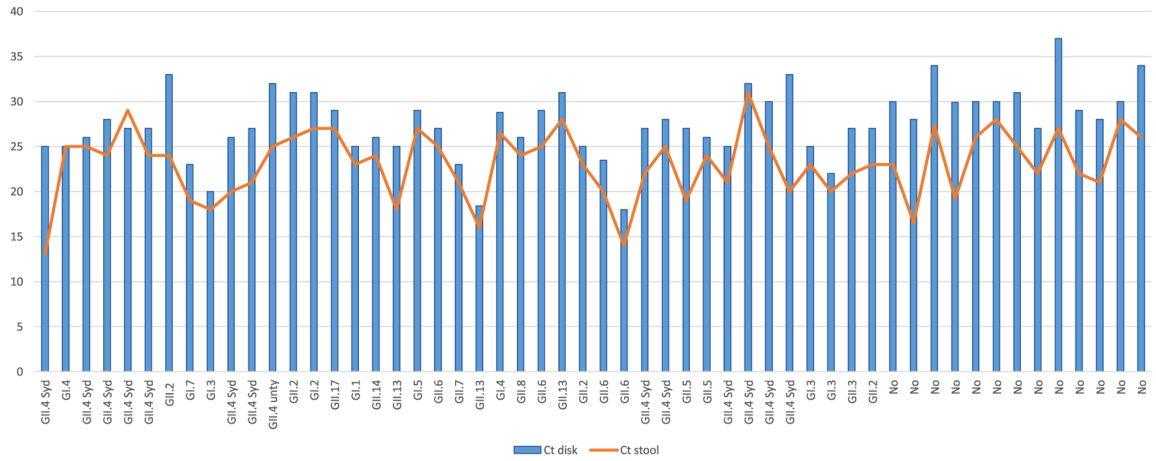

Fig. 2.

Genotyping of stool suspensions (n = 54) from the UNEXP disk eluates. Ct values from 10% stool suspensions are shown by points where the orange line crosses the blue bars and from the UNEXP disk eluates by the blue bars. The bars represent Ct values of disk nucleic acid eluates. Thirteen NA extracts could not be typed indicated by 'No'. GII.4 Syd = GII.4 Sydney (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

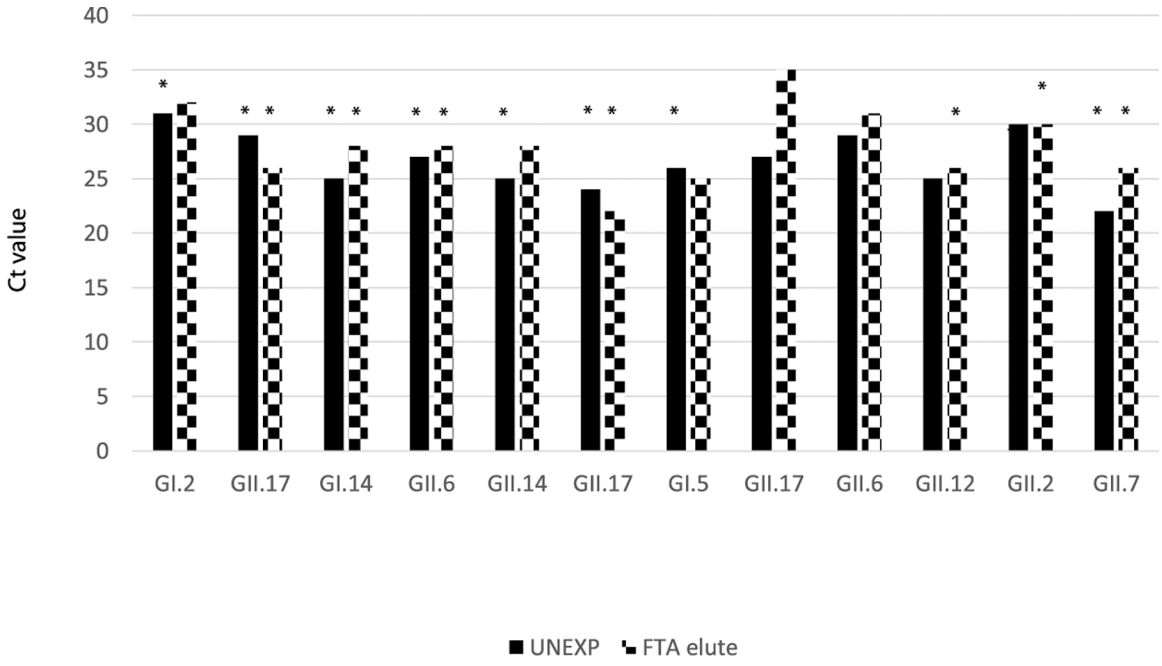

Fig. 3.

Comparison of detection and genotyping of 12 nucleic acid eluates from UNEXP and FTA elute disks after storage at room temperature for 2 weeks. Stars above the FTA and UNEXP bars represent successful genotyping results matching the virus in the 10% stool suspension.

Table 1

Hepatitis A virus RNA and Adenovirus 2 DNA detection from UNEXP disks.

Virus dilution	Ct* virus culture loaded	Ct at 0 h	Ct at day 3	Ct at day 7	Ct at day 14
HAV stock	26.4	26.1	26.2	26.0	26.1
1:10	29.9	29.1	28.0	29.3	29.3
1:100	32.8	32.4	32.5	32.4	32.4
HAdv-2 stock	31.6	31.9	31.6	31.5	31.2
1:10	34.9	34.9	34.8	34.3	34.5
1:100	38.1	37.9	38.0	37.6	37.3

** All Ct values represent the average of 4 replicates.

Ct values are volume equilibrated. The actual volume of the samples from the UNEXP disks are 10X volume of the inoculum, therefore Ct values are shown as 3.3 less than readings to equate to concentration in the viral inoculum.

* HAV = Hepatitis A virus, HAdv-2 = Adenovirus type 2.

Table 2

Detection of norovirus RNA from NA extracts eluted from of UNEXP disks loaded with dilutions of clarified 10% stool suspensions.

Disk incubation time	1:10 of 10% stool suspension			1:100 of 10% stool suspension			1:1000 10% stool suspension		
	GII.4 stool 1	GII.4 stool 2	GI.3	GII.4 stool 1	GII.4 stool 2	GI.3	GII.4 stool 1	GII.4 stool 2	GI.3
0 hour	21.7+/−1.0	21.9+/−0.3	17.3+/−0.7	21.8+/−0.7	24.9+/−0.4	21.1+/−0.2	23.7+/−0-.5	30.2+/−2.2	24.2+/−10.3
3 days	22.7+/−0.3	25.3+/−0.9	17.8+/−0.5	22.2+/−0.7	25.5+/−0.4	21.8+/−0.5	26.9+/−0.2	28.6+/−0.3	25.5+/−0.5
1 week	19.1+/−0.1	31.7+/−0.1	17.1+/−0.4	23.7+/−0.2	25.8+/−0.4	20.9+/−0.3	29.6+/−1.9	30.2+/−2.3	22.3+/−2.8
2 weeks	19.9+/−0.6	32.6+/−0.4	17.2+/−0.2	24.2+/−0.2	26.3+/−0.4	21.5+/−0.3	36.0+/−2.8	29.4+/−0.4	26.0+/−0.1