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Original Article

Use of dried clinical samples for storing and detecting influenza RNA

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Background Most clinical samples collected for diagnostic influenza testing and monitoring require refrigerated or frozen storage or shipment, which imparts logistic and cost burdens. The ability to store and ship dried clinical specimens under ambient conditions for influenza testing would significantly reduce costs and protect samples from improper storage or equipment failure, especially in remote or resource-limited areas.

Objectives To evaluate the collection and storage of dried clinical samples on a transport matrix (ViveST^{TM}, ST) for influenza RNA testing by real-time reverse-transcription PCR (RT-PCR).

Methods Viral transport medium from swab or sputum samples was applied to ST, dried, and stored under ambient conditions from 2 days to 6 months. Additional aliquots of samples were frozen. Testing of frozen and ST-stored samples was performed using the WHO/CDC real-time influenza A (H1N1) RT-PCR protocol and compared to the Luminex xTAG RVP assay.

Results ST-stored samples yielded slightly higher threshold cycle values (median 2·54 cycles) compared to frozen samples tested in parallel. This difference was consistent regardless of viral input. There was no significant difference in signal recovery between samples stored for 1 week versus samples stored for 3 weeks, or from three samples stored for 6 months. Qualitatively, clinical specimens stored on ST were 100% concordant (36/36) with frozen samples for detecting the presence of influenza A RNA.

Conclusion ST-processed dried specimens produced similar rates of seasonal or novel 2009 HIN1 influenza RNA detection compared to conventional sample processing and thus presents a viable alternative to refrigerated or frozen samples.

Keywords Dried samples, influenza, real-time PCR.

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Introduction

A novel H1N1 influenza A virus caused a worldwide pandemic in 2009, but rapid influenza tests that detect the presence of influenza viral proteins that were performed at most clinics did not reliably detect this strain.[1–3] More sophisticated diagnostic tests that are based on analyzing viral RNA obtained from naso- or oropharyngeal swabs, such as the real-time reverse-transcription PCR (RT-PCR) assay developed by the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC), are required to confirm the presence of this influenza strain.[4]

Current recommendations that swab samples, typically stored in liquid viral transport medium, should be held at 4°C for no longer than 4 days prior to testing[5,6] are derived from protocols that require a live virus stock that is generated from the clinical specimen in culture. Little is known about the stability of influenza nucleic acids in refrigerated clinical specimens. While clinical samples may be stored frozen at −20 or −70°C in backlog situations or for future research, acquiring and maintaining freezer space is a logistic and economic burden. In addition, depending on where samples are collected relative to where diagnostic tests are performed, shipping of clinical samples may be required, which can be costly and dangerous to personnel.

Dried clinical samples have been shown to be a simple, safe, and inexpensive alternative to liquid or frozen samples for nucleic acid tests. Dried blood spots (DBS) stored on filter paper have been extensively used for viral RT-PCR-based tests such as diagnosis and monitoring of HIV infection and drug resistance.[7–10] Two studies have found that influenza RNA could be readily detected from dried samples,[11,12] and at least, one public health service has adopted dry swab collection for routine influenza surveillance.[13]

We have previously demonstrated the utility of using ViveST™ (ST), a novel collection and transportation system, to dry and store plasma for detecting and quantifying HIV-1 and HCV viral RNA.[14,15] Herein, we describe the use of ST for collection and storage of respiratory specimens in viral transport medium for detecting influenza RNA.

Methods

Sample collection

The project was approved by the Stanford University and Bronx VA Medical Center institutional review boards. Dacron swabs were used to obtain nasopharyngeal or oropharyngeal specimens from patients suspected of having seasonal or novel 2009 pandemic influenza A (2009 H1N1 flu) infection at VA medical centers between August 2009 and February 2010. The swabs were immediately placed in 3 ml of viral transport medium (Remel Products, Lenexa, KS, USA) and either refrigerated or frozen at −80°C. Sputum samples (200 μl) were diluted into 3 ml of viral transport medium.

Sample processing

One milliliter of sample-containing transport medium was applied to ST matrix and allowed to dry for a minimum of 16 hours at ambient temperature and humidity in a biological safety cabinet. Prior to RNA extraction, each ST was rehydrated and incubated at room temperature with 1·175 ml of reconstitution buffer for approximately 10 minutes before recovery according to the manufacturer's instructions (ViveBio, Norcross, GA, USA). RNA was extracted from 400 μl of ST-recovered samples and from native, frozen samples using MagMax Viral RNA Isolation Kits (Ambion, Austin, TX, USA), or from 140 μl of sample using Viral RNA Mini Kits (Qiagen, Chatsworth, CA, USA). When quantitatively comparing ST versus frozen samples, only Qiagen extractions were used.

To perform quantitative analysis of influenza RNA recovery across a range of viral inputs while maintaining overall sample composition, 12 clinical samples were 10-fold serially diluted into a pool of normal (influenza-negative by RT-PCR) human bronchial-alveolar lavage samples. One milliliter aliquots of each dilution were applied to ST or stored frozen at −80°C, and then tested after 1 or 3 weeks of storage at ambient room temperature. Three samples were tested after 6 months of ST storage at ambient room temperature. Paired ST and frozen samples were extracted and tested in the same assay run.

Sample testing

Samples were originally tested using the xTAG Respiratory Viral Panel (RVP; Luminex Molecular Diagnostics, Toronto, Canada) according to manufacturer's instructions and confirmed with the CDC real-time RT-PCR assay for 2009 pandemic influenza A (H1N1) according to the published protocol.[16] From the CDC assay, the mean threshold cycle (C_T) of the three influenza amplifications (InfA, SW-InfA, and SW-H1) was calculated and used in statistical comparisons of quantitative influenza RNA recovery. When one of the amplifications from a given sample was negative, that sample and its' comparator (ST or frozen) were excluded from the quantitative analysis, but were included in the qualitative analysis (scored as 2009 H1N1 flu-negative).

Statistical analysis

Wilcoxon signed-ranks tests were used to evaluate threshold cycle differences between ST and frozen samples and compare differences between values obtained at 1 and 3 weeks of storage, using InStat (GraphPad Software, La Jolla, CA, USA). Correlation coefficients were determined in Excel (Microsoft, Redmond, WA, USA).

Results

Quantitative analysis of diluted samples

Forty-eight samples (12 samples × 4 dilutions) were stored frozen and on ST to quantitatively compare RNA recovery across a range of viral inputs (estimated based on C_T). Figure 1 shows the results of 31 pairs (ST versus frozen) that both tested 2009 H1N1 flu positive in the CDC assay. Median C_T for frozen samples after both 1 and 3 weeks of storage was 28·6 cycles. ST-stored samples had slightly higher C_T compared to frozen samples (overall median 2·75 cycles higher, range 0·05–4·9 cycles). The C_T difference

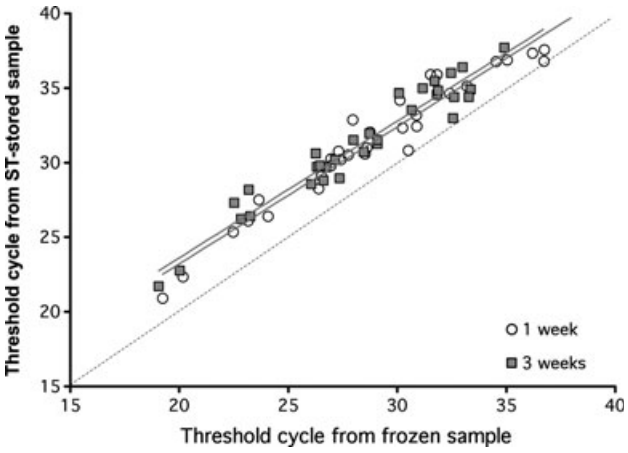

Figure 1. Comparison of real-time RT-PCR threshold cycle values from influenza samples stored frozen or on ViveST™ (ST). Samples were stored for 1 week (open circles) or 3 weeks (shaded squares) before testing. The dashed line represents the line of equality. Lines fitted to 1- and 3-week samples both have a slope of 0·92 and an R^2 value of 0·94.

between ST and frozen samples was not influenced by the amount of virus present (as indicated by the absolute C_T), as the slope of the lines fitting both the week 1 and week 3 data points was 0·92. The differences in C_T between ST and frozen samples after 1 and 3 weeks were not significantly different (2·48 cycles versus 2·92 cycles, respectively, $P = 0{\cdot}11$). The median C_T difference between the three ST and frozen samples stored for 6 months was 2·42 cycles (range 1·4–3·17 cycles).

Qualitative analysis of diluted samples

While the CDC assay is a quantitative RT-PCR assay, for diagnostic purposes, the results are translated into qualitative results. Quantitative data from the 48 diluted samples were scored qualitatively according to assay protocol. Results from 39/48 (81%) samples were concordant between ST and frozen samples after 1 week of storage. Of the nine discordant samples, seven were ST−/frozen+, while two were ST+/frozen−. The median C_T for the discordant samples was 35·5 cycles, indicating a low viral burden in these samples. After 3 weeks of storage, results from 42/48 (88%) samples were concordant. Of the six discordant samples, five were ST−/frozen+, while one was ST+/frozen−. The median C_T for the discordant samples stored for 3 weeks was 34·5 cycles. Therefore, in this constructed set of samples, discordance between ST-stored and frozen samples occurred in only samples diluted to near the limit of detection.

Qualitative analysis of clinical samples

A total of 36 primary (i.e., undiluted) clinical samples that were influenza A positive in the RVP assay were also analyzed by the CDC assay after being applied to and recovered from ST. Results shown in Table 1 indicate that seasonal and non-subtypeable influenza A strains were detected in the ST eluates at the same frequency as from frozen samples, and that identification of subtype from ST-applied samples was consistent with the RVP assay results determined from frozen samples. Four samples that were identified as influenza A positive in both tests but could not be subtyped in either assay appeared to have low amounts of virus (based on each assays' raw output values), and this has been shown to yield equivocal subtyping results in both the RVP and real-time assays.[2,17]

Table 1. Detection of influenza A RNA in dried clinical samples

RVP assay result*	No. of samples	CDC assay results from ST**-stored samples	
		Influenza A	Novel H1N1
Seasonal H1	4	4	0
Seasonal H3	4	4	0
Non-subtypeable	28	28	24

*Tested on frozen samples.
**ViveST™.

Discussion

We demonstrated that dried viral transport medium using ViveST™ devices can be effectively used for the detection of influenza A RNA. Dried specimens, in general, have been employed in other areas of clinical and laboratory research. DBS have been used for over 20 years for newborn screening programs [18] and have been used for therapeutic drug monitoring.[19,20] Advances in RNA extraction methods and molecular tests have allowed dried blood or plasma spots to be used for diagnosis and monitoring of HIV infection,[8–10] assessment of drug resistance mutations,[7,8,21] HCV prevalence by antibody screening or RNA detection,[22,23] hepatitis A virus monitoring,[24] and detection of measles virus RNA.[25] While a typical Guthrie card contains 50–100 μl of blood or plasma per spot (250–500 μl total), an ST can hold up to 1 ml of sample, allowing for multiple analyses from a single specimen. In addition, the tube-and-cap design of the ST allows the dried specimen to be protected from contamination or scraping, thus better preserving the integrity of the sample.

In this study, there was a slight decrease in the amount of influenza RNA detected in the RT-PCR assay from extracts prepared from ST (compared to frozen samples). The qualitative detection of infection rates in low influenza viral load settings, which are influenced by the influenza strain, the immunity of the patient population, and whether samples are collected early in course of infection or before antiviral therapy is begun, could be affected by lowered RNA recovery from ST-stored specimens. However, the high nasopharyngeal influenza virus levels found during the 2009 pandemic influenza A infection from our clinical samples and in other studies[26,27] (all with median C_T values <30 cycles) indicate that a slight loss in RNA recovery (median 2·4 cycles) from ST-stored samples would not affect the overall detection rate. Indeed, compared to frozen samples, the slight signal loss found in ST-stored specimens did not affect the overall influenza detection rate from clinical samples in our study, and only slightly affected influenza detection rates in the samples that were diluted to lower viral load levels. Slightly reduced RNA recovery from ST-stored samples is similar to our previous work demonstrating approximately a 0·5 $\log_{10}$ copies/ml (3-fold) reduction in HIV-1 RNA viral load values

obtained from ST-stored plasma compared to frozen plasma.[14,15]

Typical clinical laboratory guidelines state that nasopharyngeal samples collected into viral transport medium for respiratory virus testing should be stored at 4°C for no longer than 4 days[6,28]; however, these conditions center around the desire to culture virus from specimens. Very little work has been published that describe the best conditions under which to store respiratory specimens for nucleic acid-based tests. The effect of sample refrigeration on recovery of viral nucleic acids from human respiratory samples has not been reported, as most researchers have stored samples at −20 or −70°C. The integrity of avian influenza viral RNA in samples collected from wild birds has been shown to be maintained in viral transport medium for up to 3 weeks at 4°C, but evaluations of later time points were complicated by microbial overgrowth.[29]

A few studies of dried respiratory samples have shown the ability to efficiently recover viral RNA. Respiratory syncytial virus RNA could be recovered from dry cotton or flocked swabs after storage for up to 2 weeks at room temperature.[11] Influenza virus detection rates from dry swabs stored for up to 5 days at room temperature in the same study were higher than rates from paired samples collected in viral transport medium and assayed by cell culture and immunofluorescence.[11] Influenza A RNA could also be recovered from banknotes spotted with virus stock after being held for up to 10 days at room temperature.[12] The work presented in our study indicates that influenza RNA can be recovered from human clinical specimens after 3 weeks of storage on ST devices, and a small data set further suggests equivalent recovery of RNA after up to 6 months of storage. Other matrices for storing dried transport medium (e.g., Guthrie cards) may also be suitable and effective in preserving influenza RNA; larger sample sets than those used in our proof-of-principle study would be needed to effectively compare the performance of multiple methods. Further studies would also be needed to determine how effective dried samples are in preserving other influenza types or other respiratory viruses.

The focus of our study was to examine an alternative method for storing samples for rapid influenza A subtyping according to the CDC-designated real-time RT-PCR method, which employs small (<200 bp) fragments. However, we were also able to amplify, by conventional PCR, 800-bp fragments of the neuraminidase gene for DNA sequencing to determine the presence of drug resistance mutations (data not shown). In previous studies, ST-stored plasma samples have been used to assess genotypic drug resistance via RT-PCR and sequencing of >1000-bp fragments of the HIV protease and reverse transcriptase gene[14,30] and to determine hepatitis C virus subtype in HCV-infected individuals.[15] These findings suggest that significant segments of viral RNA are recovered intact after dry storage.

It is unlikely that influenza virus could be successfully cultured from ST-stored samples, as it has been shown that HIV is inactivated after being dried onto ST.[14] ST-stored samples would therefore be unsuitable for in vitro assays that require live virus to phenotypically characterize viruses and antigens. Therefore, it is possible that dry storage may not be adequate for classical influenza surveillance as performed by some national influenza centers. However, the development of a wide range of sensitive nucleic acid-based tests to detect and subtype influenza strains,[31] as well as identify drug resistance mutations,[32,33] allows non-viable virus-containing samples to provide significant clinical information and epidemiological data.

The use of dried clinical samples is well established in HIV and HCV medicine, and significant data suggests that dried samples would also be suitable for detecting, monitoring, and studying respiratory infections. The advantages of using dried samples include (i) long storage capability (8 weeks at room temperature for HIV[14] and up to 6 months for influenza RNA testing without significant signal degradation); (ii) reduced biohazard risk to laboratory personnel, as ST-stored samples have no spill risk and have reduced infectivity; and (iii) reduced shipping costs through elimination of cold packs or dry ice.[30] While standard clinical laboratory methods for collecting, storing, and testing samples for influenza can be routinely employed when logistically and economically possible, the use of dried specimens may be beneficial for convenient short- and long-term storage of specimens, for simple and inexpensive ambient temperature shipping of specimens, and for collection, storage, and shipping of specimens in isolated and/or resource poor settings where cold storage is limited.

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