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Title

SARS-CoV-2 RNA stability in saliva and dry swabs for storage and transport at ambient temperature for at least 9 days: A cost efficient and practical alternative

Running title

Saliva and dry swabs SARS-CoV-2 RNA long survival for diagnostics

Authors

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Summary

During the current COVID-19 pandemic, different methods have been used to evaluate patients suspected with infection of SARS-CoV-2. In this study, we evaluate the longevity of saliva and dry swab samples to retain SARS-CoV-2 for storage and transport at different environmental settings. Our results show that at ambient temperature of 20°C, SARS-CoV-2 RNA remains stable for up to 9 days giving a long span of time for transport and storage without compromising clinical results. Additionally, this study demonstrates that saliva and dry swabs specimens can also be stored at -20°C and +4°C for up to 26 days without affecting RT-qPCR results. Our data is relevant for low-and middle-income countries, which have limited access to rapid refrigerated transport and storage of samples representing an economical alternative. Finally, our study demonstrates that dry swabs provide clear advantages over using transport medium.

Keywords

Viral survival, COVID-19, surveillance, clinical samples, oropharyngeal, saliva, RNA quantification methods

Main Text (word count 2781)

Introduction

As the number of cases of COVID-19, severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2), are experiencing a slight reduction in the past weeks during the on-going third wave of infection in this pandemic, there are no signs of the disease slowing down. Numbers of clinical samples for detection of SARS-CoV-2 continue to grow worldwide (https://www.ecdc.europa.eu/en/covid-19/data and https://www.cdc.gov/coronavirus/2019-ncov/index.html). The increasing number of samples put pressure on logistics at the various test laboratories and challenges their ability to analyse samples in a timely fashion. While new, innovative and faster methods for screening and diagnosing patients are constantly being developed (e.g. CRISPR-Cas based, and other isothermal amplification methods, conventional antigen-based test for viral proteins, PCR assays for detecting viral amplicons), extraction of nuclei acids, followed by either reverse transcription polymerase chain-reaction (RT-PCR) or real-time RT-PCR (RT-qPCR) approaches, remain the most commonly used due to their high sensitivity and relatively low cost (D'Cruz et al. 2020).

Nasopharyngeal swab (NPS) has been used as the standard reference test method to evaluate patients with suspected respiratory infection caused by virus RT-qPCR (D'Cruz et

al. 2020; Lee et al. 2021). However, other specimen collection methods like oropharyngeal swabs (OPS) and saliva specimen collection have also been accepted by the World Health Organization (WHO) (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance-publications), and the Centers for Disease Control and Prevention (https://www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.html) for SARS-CoV-2 testing during the current COVID-19 pandemic.

Healthcare institutions, research labs and governments have made great efforts to maintain the flow of analytical diagnostics on time to provide results for the adequate detection of positive SARS-CoV-2 clinical cases worldwide.

In Denmark, there are two different test tracks available built upon the current pandemic. Hospitals across the country run the health test track and are responsible for testing people with clear COVID-19 symptoms. Test Centre Denmark (TCDK) at the Statens Serum Institut (SSI) runs the society test track (STT), which allows asymptomatic persons, people with only mild symptoms, or even people who just wants to know their health status to be tested. One of the main premises while establishing TCDK during the early 2020 was that machines, consumables and reagents used at this facility, must not be the same as the ones used at the health track, to avoid the shortage experienced during the early stages of the pandemic across all Europe, and worldwide. Today, TCDK runs in average more than 150,000 COVID-19 tests a day as the government recommends every person going physically to work to be tested at least once a week.

Additionally, the established procedure by SSI requires and demands that all COVID-19 clinical swabs must be analysed within 72 hours after sample collection. In fact, over 90% of the samples are analysed and results released within 19 hours mean time. Any sample received and analysed after this time limit is reported as inconclusive and then discarded. The rationale for the 72-hour deadline is based on two principals. The first principal is that for a testing strategy to be effective results must be available as soon as possible. As the results represent a moment in time, a delayed outcome may no longer signify the actual health status of a patient. Moreover, persons with even slight symptoms are expected to self-quarantine while waiting for the results and this is a substantial burden. The second principal behind the time limit is that when large scale testing was started, it was unknown how long SARS-CoV-2 RNA remains stable on clinical sample swabs. A stability below 72 hours was considered reasonable (Moore et al. 2008). Furthermore, the laboratories for the STT are now centralized within two facilities across the country. Samples are received from

all over the nation and may be delayed in transport, which will subsequently cause them to be excluded from screening diagnostics if they arrived outside this time threshold. This may represent a loss of potential COVID-19 positive cases carrying any of the different SARS-CoV-2 variants, and raises the question to how long does SARS-CoV-2 RNA remains stable in the clinical swab samples during transportation and before arrival to the respective analytical centre?

In the STT samples are collected as OPS and are shipped without transport media as opposed to how samples are shipped in most testing programs worldwide. The logic behind this approach is quite simple, security, stability, and simplicity. There are several practical advantages in using saliva and OPS over NPS such as, it is not an invasive method, it can be performed easily by trained non-health care professionals or individuals themselves. Additionally, this approach has also a directly impact in the economy as less resources are needed in transport medium or expensive cold transportation system and/or refrigerators. After collecting the OPS swab sample from the patient and placed immediately in sealed transport tube with safe screw caps, the sample will only be opened again for the RNA extraction in the laboratory facility by trained personnel, and/or by a robot reducing thus any possibility of cross-contamination. Moreover, while most transport media contain some type of RNA stabilizer; they also disrupt by cell lysis the viral capsid to expose the genetic material. It has been proven that maintaining the virus nuclei acids are more stable in their natural capsid in the absence of transport medium (Moore et al. 2008). During over a year of the pandemic and with over 28 million samples analysed by STT (https://www.sst.dk/en/English/Corona-eng/Status-of-the-epidemic/COVID-19-updates-Statistics-and-charts), which has proven this method to be cost efficient, accurate and safe.

Despite the large volume of documentation generated in 2020 and 2021 about the pathogenicity, infectivity and detection of SARS-CoV-2 and COVID-19, there is, however, only a handful of studies evaluating the longevity and conditions for SARS-CoV-2 RNA particle stability in clinical swab samples for different transport medias (Ren et al. 2020; Perchetti et al. 2020; Rogers et al. 2020). Furthermore, to our knowledge, there is no available data on the stability of SARS-CoV-2 RNA during transportation and longevity in dry swabs (without any preservation buffer). Therefore, in our current study, we add to the previous findings on viral survival and detection by assessing the stability at different environmental temperatures of SARS-CoV-2 RNA particles in swab material without the addition of a preservation media.

Methods

A total of 120 swabs (CLASSIQSwabs™ Dry Swabs, COPAN) collected in December 2020 were spiked with 5 µL of SARS-CoV-2 to the tip of the swab in the lab under two conditions: 60 “dry” swabs and 60 “wet” swabs. The dry swabs were not pre-treated, while the wet swabs had been moisturized by saliva by taking an oropharyngeal swab from one single person. All test samples were placed in transport tubes and kept sealed with screw caps throughout storage. No transport or stabilizing media was added in either of the two conditions, thus, both should be considered dry swabs. Swab samples were stored at three different temperatures (-20°C, +4°C and +20°C), and thereafter analysed in triplicate by RT-qPCR after 1, 3, 5, 8, 9, 15 and 26 days. All incubations took place in dark to overrule the potential effect of light UV radiation in the degradation of RNA molecules.

A well-characterized strain of SARS-CoV-2 (2019-nCoV/Munchen 1-2-2020/964, Charite/Berlin) diluted in PBS, was used to spike the swabs with a C_T -value of 29,4 (~10^3 viral copies/ µL). Dilution series of the original standard sample (C_T -value of 20; ~10^6 viral copies/ µL) allowed the estimation of viral RNA copies/µL.

Samples were analysed at TCDK where 700 µL of PBS were added to the swabs, and the samples were left agitating on a shaker for 10 min (700RPM). Total nucleic acids were extracted from 200 µL of sample using a Biomek i7 (Beckman Coulter) and the RNAdvance Blood kit (Beckman Coulter) following the manufacturer guidelines and eluting in 50 µL DNase and RNase free water. RT-qPCR was performed on a CFX96 (Bio-Rad) using 5 µL eluate in a total reaction volume of 25 µL. The PCR reaction contained Luna Probe One-Step React Mix (New England Biolabs), 0,3% IGEPAL CA-630 (Sigma-Aldrich). Primers and probes targeting the E-gene region, as well as cycling conditions are described in a previous publication (Corman et al. 2020). This standard set-up for analysing the SST swab samples keeps being updated and improved day by day.

Results

No significant variation over time was observed in the C_T values (and viral copy number/ µL) within the two main conditions (see Fig. 1), except for the saliva treatment at +20°C which after 9 days experienced an increase in the mean C_T values (from ~10^3 to ~10^1 viral copies / µL concentration decrease). A direct correlation of higher viral stability in lower temperatures over time for both main conditions was observed (Table 1). Moreover, there was also a slight tendency of saliva swab samples to give lower C_T values and thus, to be a more stable environment for the survival of SARS-CoV-2 RNA during the first 9 days of incubation. Temperature was observed to play a significant role in stabilizing the RNA molecules of the virus for the saliva swabs when compared to the dry swabs (Suppl. Mat. 1). Using linear

regression, there was a statistically significant difference in C_T between -20°C and ambient room temperature (20°C) in the saliva swabs after 3 days (P = 0,003), 15 days (P = 0,003) and 26 days (P = 0,03) of incubation, and in dry swabs after 8 days (P = 0,004) incubation. Moreover, a significant difference was also noticed for the saliva after 15 days (P = 0,006) when compared the +4°C and +20°C temperatures, and in the dry treatment after 8 days (P = 0,03) in between the -20°C and +4°C incubation temperature (Table1). Finally, samples at -20°C were slightly more stable than +4°C and samples at +20°C showed increase in viral particles degradation with lower viral copies (Suppl. Mat. 2).

Discussion

Historically, NPS have been catalogued as the method reaching the highest yield of viral particles, but it is also the most unpleasant method and is disliked by patients, in particularly elderly and children. Other alternatives such as saliva, OPS and nasal have consistently been evaluated during the current pandemic and are in overall less unpleasant than NPS. There is a clear advantage in using saliva swabs, virtually no discomfort at all to the patient during the sampling process. This simple advantage can facilitate sample collection by inspiring people to be tested. A recent systematic review pointed out that while NPS performance is about 97% for viral detection, saliva swabs reach only at 88% detection (Lee et al. 2021). However, in almost all cases transport media was added to the swab samples to inoculate the virus. There is no data available of the stability of saliva and dry samples without transport media.

The design of our study differed from previous investigations that compared swab types and specimen collection methods within a clinical setting (Rogers et al. 2020; Skalina et al. 2020). Studies suggest that saliva may be a suitable and high-yield diagnostic sample type for detection of SARS-CoV-2 considering local viral replication, in addition to the potential mixing of saliva in lower and upper respiratory tract fluids that can carry virus (Chen et al. 2020; Lee et al. 2021). Our approach was laboratory based and no patients were involved. The analysis was only designed as a development study, using the current oropharyngeal national swab method, to provide insight into the stability of RNA viral particles in dry and saliva swabs and not in the disease itself. Even though C_T values provide quantitative information over time, issues such as the relationship between viral load and disease severity could therefore not be assessed and were never the target of the current assay.

A previous study showed that SARS-CoV-2 could be detected using RT-qPCR on swabs after 21 days at room temperature (Skalina et al. 2020). In another study, absolute dry swabs taken from clinical patients showed a slight reduction in utility for several respiratory

viruses, supporting our results (Moore et al. 2008). Although, both conditions in our study should be essentially considered as dry swabs as no preservation buffer were added. The saliva swabs represented better the actual enzymatic environment found in real clinical swab samples, than the absolute dry swabs, in which only 5 µL of the virus was added directly to the tip of the swab. To our knowledge there is no study evaluating the direct effect of oropharyngeal enzymes in the stability of SARS-CoV-2 and thus, we cannot further conclude. Nevertheless, our results suggest that the enzymatic activity of the saliva in the swabs may stabilize the viral capsid particles avoiding its degradation at ambient temperatures up to 9 days. At lower temperatures the stability is even higher to almost no RNA degradation at all up to 26 days, and potentially even longer but this remains to be investigated.

There are several available protocols and regulations for oropharyngeal swab samples collection and the different transport media available (Druce et al. 2012; Rodino et al. 2020). Oropharyngeal and nasopharyngeal swabs are among the most used type of clinical sample specimens collected during the current pandemic (Perchetti et al. 2020). In Denmark, hospitals with the health track use different kinds of transport buffers containing a hydrolysing agent to the swabs right after collection. However, SSI with the STT does not add a transport buffer and swab samples are stored dry in security tubes sealed with screw caps after collection until they reach TCDK for nuclei acid extraction and RT-qPCR analysis. As mentioned above, with more than 28 million samples analysed since the beginning of the pandemic in Denmark, this method has proven to maintain SARS-CoV-2 RNA stable in their natural capsid in the absence of transport medium with no risk of cross contamination or infection. There is evidence supporting that transporting dry swabs do not compromise RNA recovery from clinical samples (Moore et al. 2008). Our results confirm that the current method selected for the STT do not compromise viral stability and retain SARS-CoV-2 RNA up to 26 days, a surprisingly high number of days, without a significant variation or reduction in C_T-values if the samples are kept cold. As such, implementing the use of dry swabs also represents an economical value by reducing the cost of additional preservation buffers, in particular for countries with limited income. The design of our assay was set to a maximum of 26 days for practical reasons, but it is highly likely that SARS-CoV-2 RNA stability may be longer if cold temperature conditions (+4°C, -20°C and below) are available. Furthermore, the swabs used by TCDK were selected based on a preliminary assay that quantified the retention of viral particles by comparing different types of swab materials (Suppl. Mat. 3). Our study and the data provided is particularly relevant for low-and middle-income countries, which have experienced limitations to analyse COVID-19 suspected positive samples immediately and storage for longer periods is required or might be the only alternative as

immediate resources for analysis could be limited. This study demonstrates that swabs specimens can be stored at 20°C ambient temperature for at least 9 days for transportation and storage and at -20°C and +4°C for up to 26 days without clinically affecting RT-qPCR results.

Denmark has become a worldwide reference for performance in COVID-19 detection (Skalina et al. 2020). The approach used in STT, transporting clinical swabs without transport media has simplified large scale testing. It has simplified sample procurement, transport, and storage. Here we present data that demonstrates that this approach provides excellent sample stability. This approach can easily be adopted by testing program in the current pandemic and can also be applied in future events.

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Author contributions

All authors fulfil the criteria for authorship consideration and contributed as follows:
Alonzo Alfaro-Núñez (AAN): conceptualization, writing original draft, analysis and validation, approved final MS
Stephanie Crone (SC): review and edit, statistical analysis, approved final MS
Sofie Holdflod Nielsen (SHN), Michelle Jørgensen (MJ): generation of data approved final MS
Shila Mortensen (SM), Arieh S. Cohen (ASC) and Claus Nielsen (CN): conceptualization, review and edit, approved final MS
Maiken Worsøe Rosenstierne (MWR), Anders Fomsgaard (AF) and Ellinor Marving (EM): review and edit, approved final MS

Patient Consent Statement

No patient or clinical samples were used in this study. Exemption for review by the ethical committee system and informed consent was given by the Committee on Biomedical Research Ethics - Capital region in accordance with Danish law on assay development projects (see Journal-nr.: H-21000338).

Conflict of Interest Statement

The authors declare no competing or conflict of interests.

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Table, Figure and Supplemental materials legends:

Table 1. Statistical comparison of C_T values per conditions. Mean C_T value with SD (standard deviation) values for the two main conditions: “saliva”-moisturized and “dry” swabs. The swabs were spiked with 5 µL of SARS-CoV-2 cultivated virus and quantified after 1, 3, 5, 8, 9, 15 and 26 days. Simultaneously, for each treatment, three different environmental temperature were evaluated (-20°C, 4°C and +20°C). Moreover, three replicas were quantified within each treatment in addition to a negative control. No transport or stabilizing media was added to the saliva or the dry swabs, thus, both should be considered as dry (non-buffered) swabs.

Figure 1. C_T values variation and RNA viral copies / µL concentrations in dry and saliva-moisturized swabs over time. Mean viral RNA concentrations (copies / µL) over time are presented in panel **A** for saliva swabs and panel **B** for dry swabs. Mean C_T values are presented in panel **C** for saliva swabs and panel **D** for dry swabs. Swabs were spiked with 5uL of SARS-CoV-2 cultivated virus and quantified after 1, 3, 5, 8, 9, 15 and 26 days. Simultaneously and for each treatment, three different environmental temperatures were evaluated at -20°C, 4°C and +20°C. Three replicas were quantified within each treatment in addition to a negative control. No transport or stabilizing media was added to the saliva or the dry swabs, thus, both should be considered as dry (non-buffered) swabs.

Supplemental Material 1. Statistical analysis of C_T values.

Supplemental Material 2. Standard curve: C_T vs. viral RNA concentrations in copies / µL.

Supplemental Material 3. Detection of SARS-CoV-2 using three commercial swab types quantifying the swab retention performance.