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RAPID COMMUNICATION

SARS-CoV-2 RNA stability in dry swabs for longer storage and transport at different temperatures

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Abstract

During the current COVID-19 pandemic, different methods have been used to evaluate patients with suspected SARS-CoV-2 infection. In this study, we experimentally evaluate the ability of spiked saliva-moist swabs and spiked swabs without any transport medium to retain SARS-CoV-2 for storage and transport at different environmental settings during different incubation time periods. Our results show that at ambient temperature of 20°C, SARS-CoV-2 RNA remains stable for up to 9 days allowing a long-time span for transport and storage without compromising clinical results. Additionally, this study demonstrates that saliva-moist swabs can also be stored at −20°C and +4°C for up to 26 days without affecting RT-qPCR results. Our data are 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 the practical and economic advantage of using swabs without transport medium.

KEYWORDS
clinical samples, COVID-19, diagnostics, oropharyngeal, RNA quantification methods, saliva, surveillance, transport medium, virus stability

1 | INTRODUCTION

As the number of cases of COVID-19, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), are experiencing a slight reduction during the on-going third wave of the infection in this pandemic, there are no signs of the disease slowing down. Numbers of clinical samples for the 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 large number of samples put pressure on logistics at the various test laboratories and challenges their ability to analyze 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 nucleic 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 collection method to evaluate patients with suspected respiratory infection caused by the virus (D'Cruz et al., 2020; Lee et al., 2021). However, other specimen collection methods such as 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 tracks that have been built in response to the current pandemic, the 'healthcare test track' and the 'society test track'. 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 people with mild symptoms, or even people who just want to know their health status to be tested. One of the main principles when establishing TCDK during the early 2020 was that machines, consumables and reagent used at this facility must not be the same as the ones used at the health track, to avoid the simultaneous 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 that every person going physically to work should be tested at least once a week.

Additionally, the established procedure by SSI requires that all COVID-19 clinical swabs must be analyzed within 72 h after sample collection and any sample received and analyzed beyond this time limit is reported as inconclusive and then discarded. In fact, over 90% of the samples are analyzed and results are released within 15 h on average. The rationale for the 72-h deadline is based on two principles. The first principle 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, individuals with even slight symptoms are expected to self-quarantine while waiting for the results, creating a substantial burden. The second rational behind the time limit is that when large scale testing started, it was unknown how long SARS-CoV-2 RNA remained stable on clinical sample swabs. A stability below 72 h 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 would 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. Therefore, we experimentally investigated the stability of SARS-CoV-2 RNA in clinical swab samples during transportation and before arrival to the respective analytical centre.

In the STT, samples are collected as OPS and shipped without transport media, which is an unusual approach compared to most testing programmes worldwide. The reasoning behind this approach is security, stability and simplicity. There are several practical advantages to use saliva and OPS over NPS such as it is a non-invasive method and can be performed easily by trained non-health care professionals or even individuals themselves. Additionally, this approach has also a direct impact in the economy as fewer resources are needed for transport medium or expensive cold transportation system and/or refrigerators. After collecting the OPS from the individual, it is immediately placed in a sealed transportation tube with a safe screw cap, which will only be opened again for the RNA extraction in the laboratory facility by trained personnel and/or by a robot, thus reducing the possibility of cross-contamination. It has been proven that the virus nucleic acids are more stable in their natural capsids than in the presence of transport medium (Moore et al., 2008). During over a year of the pandemic and with over 32 million samples analyzed by STT (https://www.sst.dk/en/English/Corona-eng/Status-of-the-epidemic/COVID-19-updates-Statistics-and-charts), this approach has been proven 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 clinical features of COVID-19 patients, there is, however, only a handful of studies evaluating the longevity and conditions for SARS-CoV-2 RNA stability in clinical swab samples for different transport media (Parikh et al., 2021; Perchetti et al., 2020; Ren et al., 2020; Rogers et al., 2020). Furthermore, to our knowledge, there are limited data available on the stability of SARS-CoV-2 RNA during transportation and longevity in swabs without any preservation buffer. Therefore, in our current study, we add to the previous findings (Parikh et al., 2021) on viral survival and detection by assessing the stability at different environmental temperatures of SARS-CoV-2 viral RNA in swab material without the addition of a preservation media.

2 | METHODS

A total of 140 swabs (CLASSIQSwabs Dry Swabs, COPAN) were spiked in the lab with 5 μl of SARS-CoV-2 (described below) onto the tip of the swab. Swabs were either dry swabs or saliva swabs (70 swabs of each). The dry swabs were not pre-treated, while the 'saliva swabs' were moisturized by saliva from OPS, all obtained from one single, SARS-CoV-2 RT-qPCR negative individual. The use of saliva from a single individual reduces variability, potential cross-contamination, and infection. No actual nasopharyngeal swab samples were tested in this assay.

A well-characterized strain of SARS-CoV-2 (2019-nCoV/Munchen 1-2-2020/964, Charite/Berlin) was propagated until full cytopathic effect was obtained on Vero E6 Cells. The supernatant was harvested and clarified from cell debris by a brief centrifugation (5 min at 300 x *g*). The clarified supernatant was diluted in phosphate buffered saline (PBS) to a C_T -value of 29.4 ($\sim 10^3$ viral copies/μl), which was used to spike the swabs. Dilution series of the original standard sample (C_T -value of 20; $\sim 10^6$ viral copies/μl) allowed the estimation of viral RNA copies/μl. All test samples were placed in empty transport tubes and kept sealed with screw caps throughout storage.

No transport or stabilizing media was added to the samples during the different storing times below. Spiked swab samples and negative blank controls were stored at three different temperatures (−20°C, +4°C and +20°C) for 1, 3, 5, 8, 9, 15 and 26 days and thereafter analyzed in triplicate by RT-qPCR. All storage took place in dark to avoid

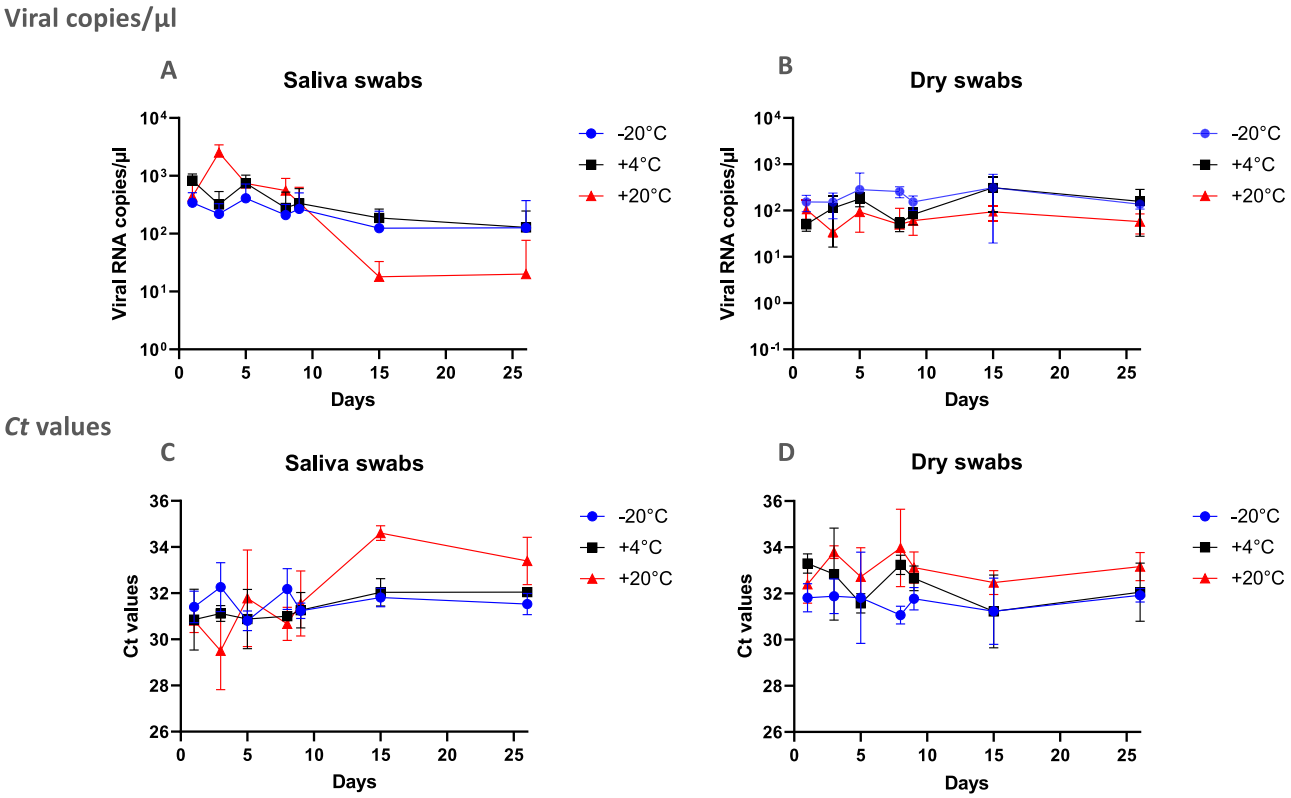

FIGURE 1 C_T values variation and RNA viral copies/μl concentrations in dry and saliva-moisturized swabs over time. Mean viral RNA concentrations (copies/μl) over time are presented in panel A for saliva swabs and panel B for dry swabs. Mean C_T values are presented in panel C for saliva swabs and panel D for dry swabs. Swabs were spiked with 5 μl 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

the potential effect of light UV radiation in the degradation of RNA molecules.

All samples were analyzed at TCDK using their standard SARS-CoV-2 diagnostic pipeline. At the day of analysis, 700 μl of PBS was added to the swabs. Samples were left agitating on a shaker for 10 min (700 RPM) to elute the sample material from the swab. 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's guidelines 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 RT-qPCR reaction contained Luna Probe One-Step React Mix (New England Biolabs) and 0.3% IGEPAL CA-630 (Sigma-Aldrich). Primers and probes targeting the E-gene region as well as cycling conditions are previously described (Corman et al., 2020). This standard set-up for analysing the SST swab samples occurs at ambient temperature in air-conditioned lab facilities

3 | RESULTS

No significant variation over time was observed in the C_T values (and viral copy number/μl) under the two main conditions (see Figure 1), except for the saliva treatment at +20°C which after 9 days experienced an increase in the mean C_T values, reflecting a ~10^3–~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 provide 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 (Supporting information 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 = .003), 15 days (p = .003) and 26 days (p = .05) of incubation, and in dry swabs after 8 days (p = .004) of incubation. Moreover, a significant difference was also detected for the saliva after 15 days (p = .006) when compared the +4°C and +20°C temperatures, and in the dry treatment after 8 days (p = .04) in between the −20°C and +4°C incubation temperatures (Table 1). Finally, samples at −20°C were slightly more stable than +4°C, and samples at +20°C showed increase in viral RNA degradation with lower viral copies (Supporting information 2).

TABLE 1 Statistical comparison of C_T values per conditions. Mean C_T value with SD 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 temperatures 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

SARS-CoV-2 in saliva medium							
	Mean CT (SD) on day						
	1	**3**	**5**	**8**	**9**	**15**	**26**
−20°C	31.4 (0.7)	32.3 (1.1)	30.8 (0.4)	32.2 (0.9)	31.6 (1.4)	31.8 (0.4)	31.5 (0.5)
4°C	30.9 (1.3)	31.1 (0.3)	30.9 (1.3)	31.0 (0.1)	31.2 (0.3)	32.0 (0.6)	32.0 (0.06)
20°C	30.8 (0.5)	29.5 (1.7)	31.8 (2.1)	30.7 (0.7)	31.3 (0.8)	34.6 (0.3)	33.4 (1.0)
	***p*-Values day**						
	1	**3**	**5**	**8**	**9**	**15**	**26**
−20°C vs. 4°C	.76	.32	1	.38	.91	.96	.79
−20°C vs. 20°C	.73	.003	.43	.14	.93	.003	.05
4°C vs. 20°C	1.00	.11	.49	.92	1.00	.006	.21
	***p*-Values day 1 vs. day 9**			***p*-Values day 1 vs. day 26**			
−20°C	1.00			1.00			
4°C	1.00			.73			
20°C	1.00			0.03			
SARS-CoV-2 no medium dry swab							
	Mean CT (SD) on day						
	1	**3**	**5**	**8**	**9**	**15**	**26**
−20°C	31.8 (0.6)	31.9 (0.8)	31.8 (2.0)	31.1 (0.4)	31.8 (0.48)	31.2 (1.4)	31.9 (0.3)
4°C	33.3 (0.4)	32.8 (2.0)	31.6 (0.4)	33.2 (0.4)	32.7 (0.5)	31.2 (1.6)	32.1 (1.3)
20°C	32.4 (0.8)	33.8 (0.3)	32.7 (1.3)	34.0 (1.7)	33.1 (0.7)	32.5 (0.5)	33.2 (0.6)
	***p*-Values day**						
	1	**3**	**5**	**8**	**9**	**15**	**26**
−20°C vs. 4°C	.21	.51	.96	.04	.63	1.00	.99
−20°C vs. 20°C	.77	.08	.54	.004	.34	.33	.32
4°C vs. 20°C	.55	.51	.38	.67	.85	.32	.41
	***p*-Values day 1 vs. day 9**			***p*-Values day 1 vs. day 26**			
−20°C	1.00			1.00			
4°C	.99			.77			
20°C	.98			.97			

4 | DISCUSSION

At the time this study was done, there was only one publication available on the stability of saliva and dry samples without transport media (Parikh et al., 2021). However, Parikh and colleagues tested to a maximum of 7 days incubation period with a limited number of flocked swabs, only 15 in total. This highlights the importance of our findings, which reached the same conclusions as the previous study but used 10 times more flocked swabs and for up to 26 days incubation period (nearly four times longer incubation time).

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 the 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 viral RNA 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 not be assessed and were never the target of the current assay.

Another 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). As no preservation buffer was added to the samples in our study, both conditions were considered as dry swabs. 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. Our results suggest that the enzymatic activity of the saliva in the swabs may stabilize the viral RNA avoiding its degradation at ambient temperatures up to 9 days. At lower temperatures, the stability is even higher as almost no RNA degradation at all is observed up to 26 days, and potentially even longer but this remains to be investigated.

There are several available protocols and regulations for OPS 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 nucleic acids extraction and RT-qPCR analysis. As described above, with more than 32 million samples analyzed since the beginning of the pandemic in Denmark, this method has proven to maintain SARS-CoV-2 RNA stable 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 does not compromise viral stability and retains 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 RNA by comparing different types of swab materials (Supporting information 3, Figure S1).

Our study and the data provided are of particular interest for low- and middle-income countries, which have experienced limitations in their ability to analyze 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 demonstrate that this approach provides excellent sample stability. This approach can easily be adopted by testing programmes in the current pandemic and can also be applied in future events.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors fulfil the criteria for authorship consideration and contributed as follows: *Conceptualization, writing original draft, analysis and validation, approved final MS*: Alonzo Alfaro-Núñez. *Review and edit, statistical analysis, approved final MS*: Stephanie Crone. *Generation of data approved final MS*: Sofie Holdflod Nielsen and Michelle Jørgensen. *Conceptualization, review and edit, approved final MS*: Shila Mortensen, Arieh S. Cohen and Claus Nielsen. *Review and edit, approved final MS*: Maiken Worsøe Rosenstierne, Anders Fomsgaard, Charlotta Polacek Strandh and Ellinor Marving.

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).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in DOI: 10.22541/au.162092670.05340285/v1 at https://www.authorea.com/users/413685/articles/521929-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.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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