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Effect of RNA quality to SARS-CoV-2 RT-qPCR detection from saliva

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Abstract

Saliva is an alternative sample material to nasopharyngeal swab in SARS-CoV-2 diagnostics. We investigated possible aspects to improve the reliability of SARS-CoV-2 detection from saliva. Saliva was collected from asymptomatic healthy subjects (*n*=133) and COVID-19 patients (*n*=9). SARS-CoV-2 detection was performed with quantitative reverse-transcriptase PCR (RT-qPCR) with two viral and one host target serving as an internal control. The use of internal control revealed that in the first RT-qPCR run 25–30% of assays failed. The failure is associated with poor RNA quality. When the amount of RNA was cut down to half from the original amount, the performance of RT-qPCR was greatly enhanced (95% of the assays succeeded). The quality of RNA was not affected by the use of different nucleic acid stabilizing buffers. Our study showed that saliva is suitable material for RT-qPCR based SARS-CoV-2 diagnostics, but the use of internal control is essential to distinguish the true negative samples from failed assays.

INTRODUCTION

The coronavirus disease 2019 (COVID-19) outbreak has created an unforeseen demand for efficient laboratory analytics. Nasopharyngeal (NP) swab is currently the most commonly used sample specimen in SARS-CoV-2 detection by quantitative reverse-transcriptase PCR (RT-qPCR). Sampling with NP swabs occupies a great number of trained medical personnel and increases the demand for personal protective equipment. A nasopharyngeal swab is often painful for the patient and may cause complications such as nasal bleeding [1, 2]. Variation in NP sampling due to the performer's skills and patient's co-operation may cause false-negative results [3].

Diagnostic SARS-CoV-2 tests based on RT-qPCR from saliva have been developed and studied in patient populations [4]. The advantage of saliva is the ease and comfort of the sampling without the need of specifically trained medical personnel, as it may be taken by patients themselves. Studies suggest that saliva can be used as a sample material in the detection of SARS-CoV-2 with at least similar sensitivity compared to that gained with NP swabs [5–7]. On the other hand, some difficulties with saliva specimens as diagnostic material may occur. Saliva contains a variety of electrolytes, proteins, bacterial, cells and traces of blood possibly having PCR-inhibitory qualities leading to false-negative results. Sample collection methods may also contribute to the quality of saliva.

The first COVID-19 infections were reported in Wuhan, China in December 2019 [8]. In Finland, the first patient case of COVID-19 was found in late January 2020 [9]. We studied the presence of SARS-CoV-2 in saliva samples from 133 Finnish subjects collected between September 2019 and February 2020. Saliva samples from hospitalized COVID-19 patients collected between November and December 2020 were used as positive controls. Our aim was to investigate the effect of different stabilizing buffers, RNA quality and the role of internal control for the diagnostic test accuracy of SARS-CoV-2 RT-PCR detection from saliva.

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METHODS

Study population and saliva sample collection

Our study populations included community-dwelling subjects (*n*=133) as well as laboratory-confirmed COVID-19 patients (*n*=9). All participants signed a written consent participation, and the study was conducted according to the guidelines of the Declaration of Helsinki. Study designs were approved by the Ethics Committee of Helsinki University Hospital.

The study population free of respiratory symptoms comprised 133 adult and elderly subjects, who had participated in our previous study Parogene [10]. They were invited for a 13 year follow-up study including oral examination and salivary sampling, which were performed between September 2019 and February 2020, immediately before the first COVID-19 outbreak in Finland. The mean age of subjects was 72.4 years (range 45–89 years) and 82 (62%) were males. Stimulated saliva, minimum of 2 ml, was collected after stimulation by chewing, and 1 ml of saliva was immediately transferred into GFX-01 GeneFix saliva collection tubes (IsoHelix) containing 1 ml of stabilizing buffer. Samples were kept at room temperature until they were stored in −70 °C freezer at the end of the day.

Saliva samples for positive controls were collected from nine COVID-19 patients treated at the COVID-19 ward in Helsinki University Hospital, or at home between November and December 2020 during the second COVID-19 wave in Finland. The SARS-CoV-2 positivity of patients was confirmed from diagnostic NP swabs by PCR at the hospital laboratory (HUSLAB Laboratory, Helsinki, Finland) [11, 12]. The mean age of the COVID-19 patients was 56.9 years and 5 (55%) of them were males. Stimulated saliva was collected into empty collection tubes and transported to the laboratory. The volume of saliva samples varied from 50 µl to 1 ml. If the amount of saliva allowed, the samples were divided into three tubes and mixed with different lysis or stabilizing buffers in 1:1 V/V ratios. The first sample was mixed with lysis buffer from chemagicTM Viral DNA/RNA 300 kit (PerkinElmer), the second sample was pretreated with stabilizing buffer from GFX-01 GeneFix saliva collection tubes (IsoHelix), and the third sample was pretreated with stabilizing buffer from DNA/RNA Shield saliva/sputum collection kit (Zymo Research). Samples were stored at −20 °C.

Viral RNA extraction and RT-qPCR-detection

RNA was extracted from the thawed saliva samples with a chemagicTM Viral DNA/RNA 300 kit (PerkinElmer) according to the manufacturer's instructions. In brief, 300 µl of the thawed sample was combined with 300 µl of lysis buffer containing 10 µl proteinase-K and 4 µl poly(A)-RNA. If the amount of saliva sample was less than 300 µl, lysis buffer was added to obtain the total volume of 600 µl. Extractions were conducted with a chemagicTM 360 device (PerkinElmer) using a chemagic Viral300 360 H96 drying prefilling protocol. RNA was eluted into 70 µl of elution buffer. The efficiency of RNA extractions was monitored by adding extraction controls (ORF1ab and N VLPs from kit 3502–0010, PerkinElmer) on each 96-well plate before DNA extraction. Extraction control was mixed with saliva from healthy volunteers in 1:2, 1:4, 1:8 and 1:16 ratios. RNA concentrations and purity were measured by Nanodrop (Thermo Fisher).

RT-qPCR tests were performed with SARS-CoV-2 RT-qPCR Reagent Kit (PerkinElmer). The assay has two viral targets: nucleocapsid (N) gene and ORF1ab. In addition, the assay contains internal control (IC) targeting human RNA. The reaction mixture contained 5 µl CoV2 Enzyme Mix, 1 µl CoV2 Reagent A and 14 or 7 µl of extracted RNA. The total volume of reactions was 20 µl, PCR-grade water was added to the reaction mixture when needed. RT-qPCR-reactions were run with QuantStudio5 (Thermo Fisher Scientific) using the programme described in the SARS-CoV-2 RT-qPCR Reagent Kit (PerkinElmer). Interpretation of the results was performed according to the manufacturer's instructions. Samples were interpreted as negative if the cycle threshold value (Ct) of both targets were >42 or undetermined and the Ct value of the internal control were ≤40. Sample was interpreted as SARS-CoV-2 positive if either one or both of the target genes showed Ct value ≤42.

RESULTS AND DISCUSSION

Non-invasive and inexpensive SARS-CoV-2 diagnostic tests based on salivary specimens are needed. The sensitivity and specificity of RT-qPCR results obtained from saliva are quite comparable to those from conventional nasopharyngeal swabs [7, 13]. There have, however, still been concerns about the use of saliva in SARS-CoV-2 diagnostics. In this study, we showed that the amount of salivary RNA used in SARS-CoV-2 RT-qPCR analysis is critical if the purity of RNA is not sufficient, which is in concert with general observations on PCR and not limited to SARS-CoV-2. According to our results, salivary RNA is stable and there is no evident difference in the results whether the stabilizing buffers are used or not during sample collection.

Study subjects in the Parogene cohort (*n*=133) were community-dwelling adults and the elderly without flu-like symptoms. In the first RT-qPCR run for the presence of SARS-CoV-2 virus, 100 (75%) samples were confirmed as negative (Fig. 1a) and the rest of the assays failed as there was no amplification of the internal control detected. The extraction control mixed in saliva gave positive signals from both SARS-CoV-2 targets even though the signal coming from ORF1ab was constantly lower (Fig. 1b) compared to the positive RNA control provided in the RT-qPCR kit (Fig. 1d). The failed samples had exceptionally low 260/230 ratios (range 0.16–0.70) deriving from high absorbance at 230 nm. Absorbance at 230 nm is traditionally considered to reflect contamination

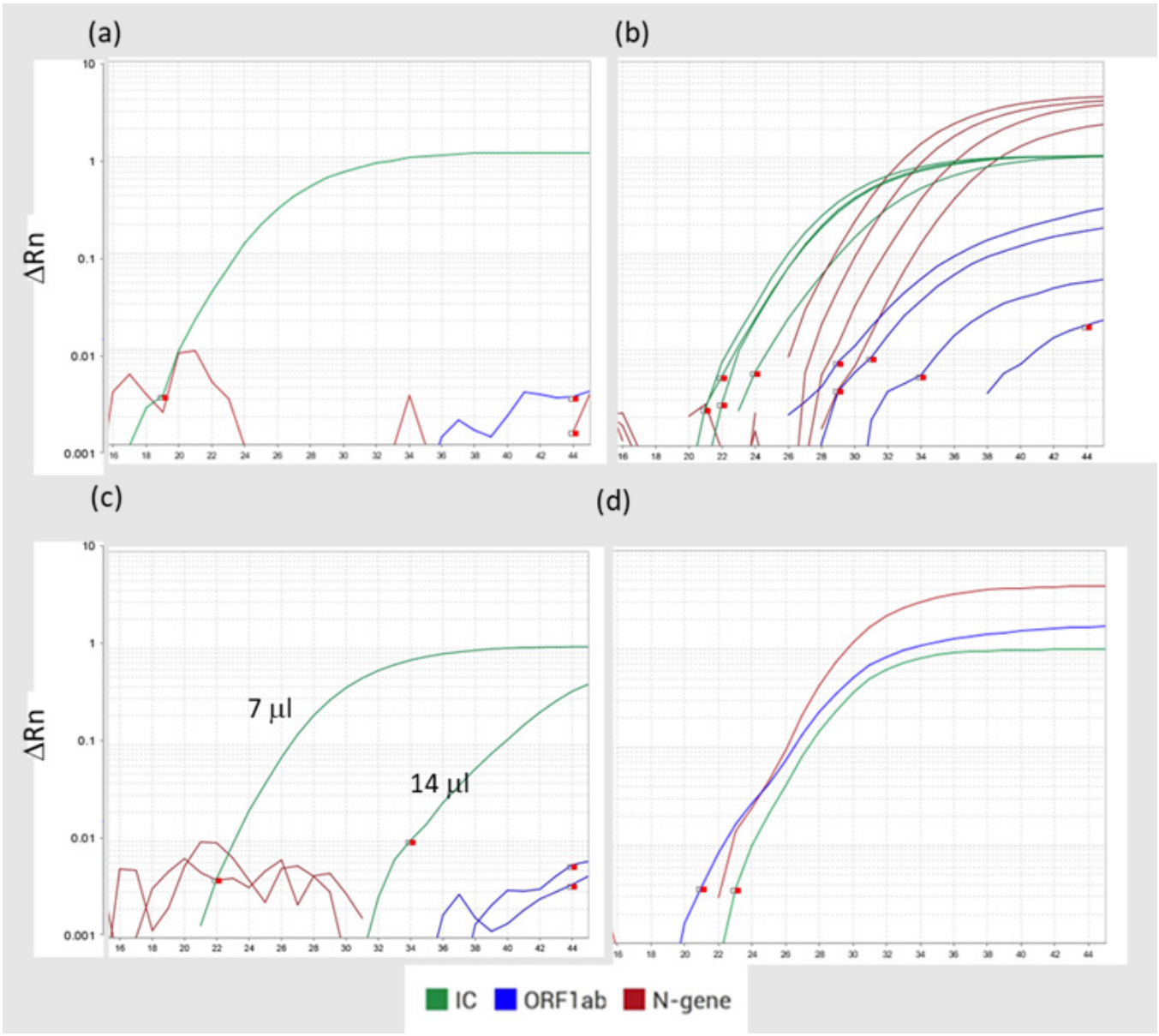

Fig. 1. Results of RT-qPCR from Parogene study population. (a) An example of negative RT-qPCR result; (b) signals coming from extraction control mixed with saliva (1:2, 1:4, 1:8 and 1:16); (c) the effect of the amount of RNA on RT-qPCR performance; (d) signals obtained from the positive control provided in the RT-qPCR kit. IC=Internal control, N-gene=nucleocapsid N gene, Rn=normalized reporter value meaning that the fluorescent signal is normalized to the signal of the passive reference dye for a given reaction.

of the sample with phenolic compounds or proteins. Excess use of lysis buffer may also affect unfavourably to 260/230 ratio. Failed samples were re-run with reaction mixtures containing only half of the RNA used in the original RT-qPCR assays (Fig. 1c). After the second RT-qPCR, only seven (5.3%) assays remained failed. SARS-CoV-2 can be detected in the saliva of asymptomatic persons [14], but we detected no SARS-CoV-2 positive samples among the study population. That was not surprising since the saliva samples for our study were collected during the 5 months before the first COVID-19 outbreak in Finland and the overall number of reported COVID-19 cases in Finland remained relatively low in early 2020 [15]. However, we showed that salivary samples stored at −70 °C can be used for RNA analysis after several months.

The positive control group consisted of confirmed COVID-19 patients (n=9). Using saliva from COVID-19 patients, six out of 20 RT-qPCR reactions (30%) failed when 14 µl of RNA was used in reaction mixtures. When the amount of RNA was cut down to half, only one out of 20 RT-qPCR reactions (5%) remained failed. One COVID-19 patient gave a SARS-CoV-2 negative

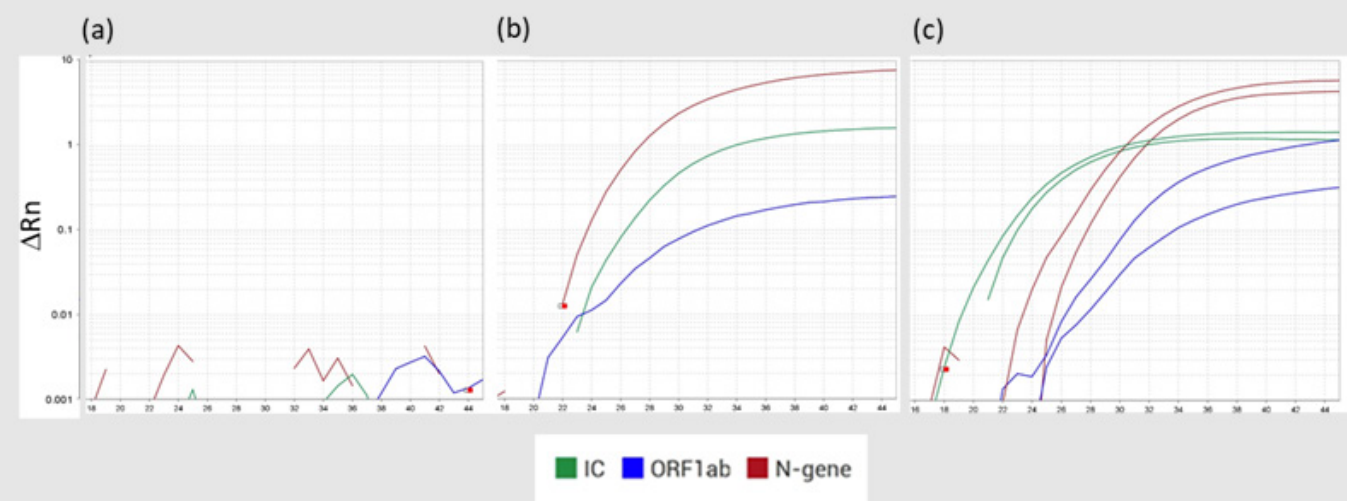

Fig. 2. The effect of the amount of RNA on the performance of RT-qPCR. (a) A third (30%) of the RT-qPCR reactions failed when 14 µl salivary RNA from COVID-19 patient was used as a template but (b) succeeded when only 7 µl RNA was used. (c) With good-quality RNA samples, as determined by 260/230 ratio, the amount of RNA was not critical. IC=Internal control, Rn=normalized reporter value meaning that the fluorescent signal is normalized to the signal of the passive reference dye for a given reaction.

saliva sample; other patients' samples were SARS-CoV-2 positive. Fig. 2 shows the effect of lowering the amount of RNA in the RT-qPCR reaction mixtures.

Salivary samples from COVID-10 patients were used to study if the stabilizing buffers have an effect on the RT-qPCR performance. Several commercial saliva collection kits provide buffers to stabilize nucleic acids for further analysis. The use of stabilizing buffers increases the costs and availability of diagnostic tests. Stabilizing buffers may also have an impact on PCR analysis, since they often contain high amounts of guanidine or SDS. Table 1 presents our results from the RT-qPCR assays. No evident advantage using the stabilizing buffers was seen in RT-qPCR analysis performed from salivary RNA. Our findings corroborate with other studies reporting that RNA stability is not affected by the specific collection devices or preservatives [16, 17]. The interference of RT-qPCR reactions was seen also with the RNA extracted from samples that were not stored in stabilizing buffer indicating that the low 260/230 ratio is not, at least entirely, caused by stabilizing buffers. As a limitation, our COVID-19 positive group

Table 1. RT-qPCR results of saliva samples collected from COVID-19 patients

	Result of saliva RT-qPCR							
	No pretreatment		IsoHelix		Shield			
Sample	RNA 14 µl	RNA 7 µl	RNA 14 µl	RNA 7 µl	RNA 14 µl	RNA 7 µl	Days with symptoms	Days from positive NP PCR-test*
AP9	+	ND	F	+	ND	ND	14	1
AP11	ND	ND	+	ND	+	+	13	5
AP12	ND	ND	ND	ND	+	+	>10	4
AP14	+	ND	F	+	F	F	12	8
AP17	+	+	+	+	+	+	4	2
AP21	F	+	F	+	+	+	6	4
AP23	+	ND	+	ND	+	+	2	1
AP24	F	–	ND	ND	ND	ND	7	6
AP25	+	ND	+	ND	ND	ND	4	1

*The SARS-CoV-2 positivity of the patients was confirmed by NP PCR test at the hospital laboratory.
+, positive RT-qPCR result; -, negative RT-qPCR result; ND, not determined; F, RT qPCR assay failed; NP, nasopharyngeal.

was small and some of the patients had difficulties with salivation, therefore not providing enough sample material for all three tests. Further investigation is needed to confirm our observation.

Several samples (25–30%), both in our study subjects and in positive controls, failed the RT-qPCR analysis performed according to default protocol. Using only half the amount of the RNA in the assay led to successful results with most of the failed samples, indicating the interference of RNA impurities in the enzyme activity in the RT-qPCR-reactions. After this optimization only 5% of saliva samples from both symptomless, healthy patients and COVID-19 patients remained failed. The remaining failed samples may have contained an insufficient amount of RNA. Interestingly, it has been reported that with NP swabs, the amount of false negative RT-qPCR tests is often around 25–30% and even up to 50% [3, 18]. Especially viral target ORF1ab seemed to be sensitive for the interference. The same phenomenon noticed with our present salivary samples may also be seen in negative NP swabs and therefore further studies are warranted. The sample volume as such did not appear to be critical, as the positive RT-qPCR result for SARS-CoV-2 could be determined from only 50 µl of saliva used in RNA extraction.

Development of reliable detection methods of SARS-CoV-2 from saliva samples would lead to lower costs in diagnostic testing, release trained medical personnel from sampling, reduce complications and pain, and altogether simplify the testing protocol, especially from the patient's point-of-view as sampling could be performed at home. Affordable protocol with self-collected samples would also make the method usable for screening purposes and available for larger audiences. This would have an impact on public safety not only in health care and long-term care homes, but also in schools, workplaces, mass gatherings and borders, as long as SARS-CoV-2 and its variants are circulating globally.

In conclusion, our study indicates that saliva is a suitable sample material for the diagnosis of SARS-CoV-2, but the amount of RNA used in RT-qPCR reactions may need some optimization especially when the RNA quality is poor. The use of internal control in SARS-CoV-2 RT-qPCR diagnostics is essential to distinguish the failed samples from the true negative ones. Salivary RNA seems to be relatively stable even without stabilizing buffers or special sampling devices during sample collection, which contributes to the affordability and common availability of the method. The importance of RNA quality and internal control in SARS-CoV-2 RT-qPCR diagnostics should be kept in mind despite the sample material used.

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Conflicts of interest
The authors declare that there are no conflicts of interest. PerkinElmer Finland Oy is Marika Tallgren's employer. No funding from PerkinElmer Finland Oy was received for this study and PerkinElmer Finland Oy was not involved in the manuscript writing, editing, approval or decision to publish.

Ethical statement
Ethics Committee of Helsinki University Hospital has approved this study. Approval numbers (and dates) are HUS/2512/2017(25.12.2017) and HUS/1701/2020(17.01.2020). All participants have signed informed consent.

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