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Coronavirus Pandemic

Evaluation of alternative clinical samples for the detection of SARS-CoV-2 and influenza virus by automated multiplex RT-PCR

Muhammed A Özarslan[1], Ömür M Parkan[2], Mehmet Soylu[1], Oğuzhan Acet[3], Selma Gökahmetoğlu[2], Zeynep Türe Yüce[4], Gamze Kalın Ünüvar[4], Seyfi Durmaz[5], Deniz Akyol[3], Feyza İzci Çetinkaya[4], Pınar Sağıroğlu[2], Gözde Akkuş Kayalı[1], Isabel R Durusoy[6], Ayşin Zeytinoğlu[7], Mustafa A Atalay[2], Meltem Taşbakan[3], Candan Çiçek[1], Orhan Yıldız[4], Hüsnü Pullukçu[3], Şaziye R Sertöz[1], Selda Erensoy[1]

[1] Department of Medical Microbiology, Faculty of Medicine, Ege University, Izmir 35100, Turkey
[2] Department of Medical Microbiology, Faculty of Medicine, Erciyes University, Kayseri 38039, Turkey
[3] Department of Infectious Diseases, Faculty of Medicine, Ege University, Izmir 35100, Turkey
[4] Department of Infectious Diseases, Faculty of Medicine, Erciyes University, Kayseri 38039, Turkey
[5] Department of Occupational Health and Safety Unit, Faculty of Medicine, Ege University, Izmir 35100, Turkey
[6] Department of Public Health, Faculty of Medicine, Ege University, Izmir 35100, Turkey
[7] Department of Medical Microbiology, Faculty of Medicine, Izmir Economy University, Izmir 35330, Turkey

Abstract

Introduction: The aim of this study was to compare the performance of different clinical specimens—nasopharyngeal (NP) swabs collected by healthcare professionals (HCP-NP), self-collected nasal swabs (Sc-N), and saliva samples (S)—in diagnostic tests for investigating severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA and influenza A/B RNA.

Methodology: These clinical samples were collected from 404 symptomatic cases and tested with the SARS-CoV-2 and influenza A/B RNA tests on the cobas 6800 System of Roche Molecular Systems (Roche Molecular Systems, Pleasanton, USA). The SARS-CoV-2 or influenza virus infection status was determined for all patients based on the predefined criteria and corresponding algorithms. Positive and negative predictive values (PPV, NPV), sensitivity, specificity, coefficient of variation (CV), interrater reliability, correlation, ,and days of sample collection of these three sample types were analyzed.

Results: There was almost perfect agreement between the these sample types for the diagnosis of SARS-CoV-2 and influenza A. The overall performance (PPV, NPV, sensitivity) and reproducibility (CV ≤ 6%) were favorable. Additionally, they showed similar trends for days of sample collection.

Conclusions: Diagnostic detection of SARS-CoV-2 and influenza RNA from Sc-N and S samples was comparable to HCP-NP samples. Using these samples would provide an advantage in diagnosing SARS-CoV-2 and influenza A infection, as they can be easily collected without the need for viral transport media.

Key words: SARS-CoV-2; influenza A; RT-PCR; saliva; nasal; nasopharyngeal.

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Introduction

Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), rapidly spread worldwide and became a pandemic [1]. The influenza virus is also an important cause of acute respiratory infections in humans and various animal species. Approximately 20% of the world population is infected with influenza every year [2]. COVID-19 and the ongoing circulation of influenza virus caused a significant increase in morbidity and mortality [3].

Effective microbiological testing is essential for the prevention and diagnosis of COVID-19 and influenza infections. Early detection of infected individuals through a well-structured testing strategy is crucial for slowing viral transmission by enabling timely isolation [4]. Research on infection dynamics and diagnostic tests is necessary to strengthen the clinical evidence of testing strategies.

Real-time polymerase chain reaction (RT-PCR) is the gold standard laboratory method for the diagnosis of SARS-CoV-2 and influenza infections [5,6]. The type of clinical specimen collected is important for early diagnosis and prevention of transmission [7]. Various clinical specimens have been used for SARS-CoV-2 RT-PCR testing. Nasal and nasopharyngeal swabs (NPS), anterior nasal swabs, saliva, and oropharyngeal swabs are commonly used as upper

respiratory specimens. These specimens can be collected by healthcare professionals (HCP) or can be self-collected [5]. Although NPS collected by HCPs are considered the gold standard, self-collected saliva and anterior nasal swabs are preferred for convenience in field studies and community screening [5,8].

Collection of NPS has some challenges and disadvantages, including patient discomfort, rare complications, need for trained and experienced HCPs, requirement of appropriate specimen collection booths, and personal protective equipment (PPE) [5,9]. Therefore, it is important to evaluate the performance of simple, non-invasive, and self-collectable methods as an alternative. Saliva samples (S) are painless, and cost-effective because they do not require swabs or specialized transport media [8,10]. The European Centre for Disease Prevention and Control (ECDC) has stated that S can be used as an alternative to NPS when they cannot be collected from symptomatic patients or in repeat screening of asymptomatic individuals [11]. The Centers for Disease Control and Prevention (CDC) has also identified nasal mid-turbinate swabs (MTS), and anterior nasal swabs, as specimens that can be collected by the patients [12]. A meta-analysis by Hou *et al.* identified the top three sampling methods with higher diagnostic value as nasopharyngeal wash (NPW), MTS, and NPS [13]. However, the effectiveness of each sample type varied with the different virus types due to various pathophysiological mechanisms. For instance, S, NPW, and MTS were the most effective for adenovirus; while NPW, MTS, and nasopharyngeal aspirate were the most effective for respiratory syncytial virus. Sputum, MTS, and NPS were the most effective in the case of other types of coronaviruses. However, sputum has major disadvantages, including the need for pre-processing and the difficulty in obtaining it from children and the elderly, while NPS poses a transmission risk due to coughing [13].

This study aimed to compare the performance of different clinical specimens in diagnostic tests investigating SARS-CoV-2 RNA and influenza A/B RNA using the automated multiplex RT-PCR cobas 6800 System (Roche Molecular Systems, Pleasanton, USA). The specimens included NPS collected by healthcare professionals (HCP-NP), self-collected nasal swabs (Sc-N), and S from symptomatic patients with suspected COVID-19 and influenza virus infections. The verification process, including precision analysis of the three different sample types, can be a guide for the use of alternative specimens for respiratory infection testing with the cobas 6800 System, which is widely preferred in many countries, including Turkey. If S and Sc-N samples can be proven to be effective and reliable in the diagnosis of SARS-CoV-2 and influenza A/B, these specimens will become alternatives that will alleviate the workload in healthcare settings and reduce the risk of transmission. To our knowledge, this is the first study to comprehensively investigate alternative respiratory specimens using the cobas 6800 System for both SARS-CoV-2 and influenza A.

Methodology

Sample collection

A total of 404 clinically suspected COVID-19 and flu patients admitted to the Ege University Hospital (307 patients) and Erciyes University Hospital (97 patients) with symptoms such as cough, sore throat, runny nose, and fever between 11 November 2021 and 25 March 2022 were included in the study. SARS-CoV-2 RNA and influenza A/B RNA were evaluated with 3 different types of samples.

S, Sc-N, and HCP-NP were collected from each patient. S were taken in a sterile container, and mixed with 1cc (mL) isotonic solution, and vortexed for 10 seconds to reduce the viscosity of the samples and homogenize them. After the S were taken, patients self-collected their Sc-N samples with a nasal swab (Hardy sterile standard swab, Hardy Diagnostics, Santa Maria, USA) according to the instructions given by an HCP. These Sc-N samples were transported in tubes containing 2 mL isotonic solution. NPS (Hardy NP swabs, Hardy Diagnostics, Santa Maria, USA) samples were taken from the patients by a trained HCP. These NPSs were transported in BD Media—VIAL Univ Viral Transport PK50 (Becton Dickinson Company, Franklin Lakes, USA).

Demographic data

Demographic data, including gender, clinical conditions (mild, moderate, or severe), SARS-CoV-2 and influenza virus vaccination status, and the date of sample collection (after the onset of symptoms) were recorded. The patients were divided into two groups based on their clinical severity; mild for those who needed outpatient treatment, and moderate/severe for those who needed treatment in the ward or intensive care unit, according to the Turkish Ministry of Health's "COVID-19 (SARS-CoV-2 Infection) Adult Patient Treatment" guidelines [14].

Molecular detection of SARS-CoV-2 and influenza A/B

All clinical samples were tested with the SARS-CoV-2/influenza RNA assay (cobas® SARS-CoV-2

and influenza A/B on cobas 6800 System; Roche Molecular Systems, Pleasanton, USA). This was an automated multiplex RT-PCR assay for the simultaneous qualitative detection and differentiation of SARS-CoV-2, influenza A virus, and/or influenza B virus RNA. The tests were performed according to the manufacturer's instructions. Two hundred microlitres of samples were used for testing. The *human RNA polymerase* (*RP*) gene was used as an internal control. This assay simultaneously detected SARS CoV-2 *ORF1ab* and the structural envelope protein *E* gene (*pan-sarbecovirus* gene) as two targets for SARS CoV-2. Two different channels are used for these targets. In the case of influenza A, the target was the *nuclear export protein* (*NEP*) gene and the *non-structural protein 1* (*NS1*) gene; whereas for influenza B, the targets were the *matrix proteins 1 and 2* (*M1/M2*) genes.

The limit of detection (LOD) according to the manufacturer using USA-WA1/2020 strain were 32 IU/mL for SARS-CoV-2 (0.0063 TCID 50/mL, cycle threshold: 36.1), and 56 IU/mL for pan-sarbecovirus (0.0082 TCID50/mL, cycle threshold: 34.7); (1 TCID50/mL = 7.393 genome equivalents by droplet digital PCR). LOD for influenza A using A/Kansas/14/2017 (H3N2) was 0.086 TCID50/mL (cycle threshold: 37.5), and for influenza B using B/Colorado/06/2017 strain was 0.026 TCID50/mL (cycle threshold: 34.9) [15].

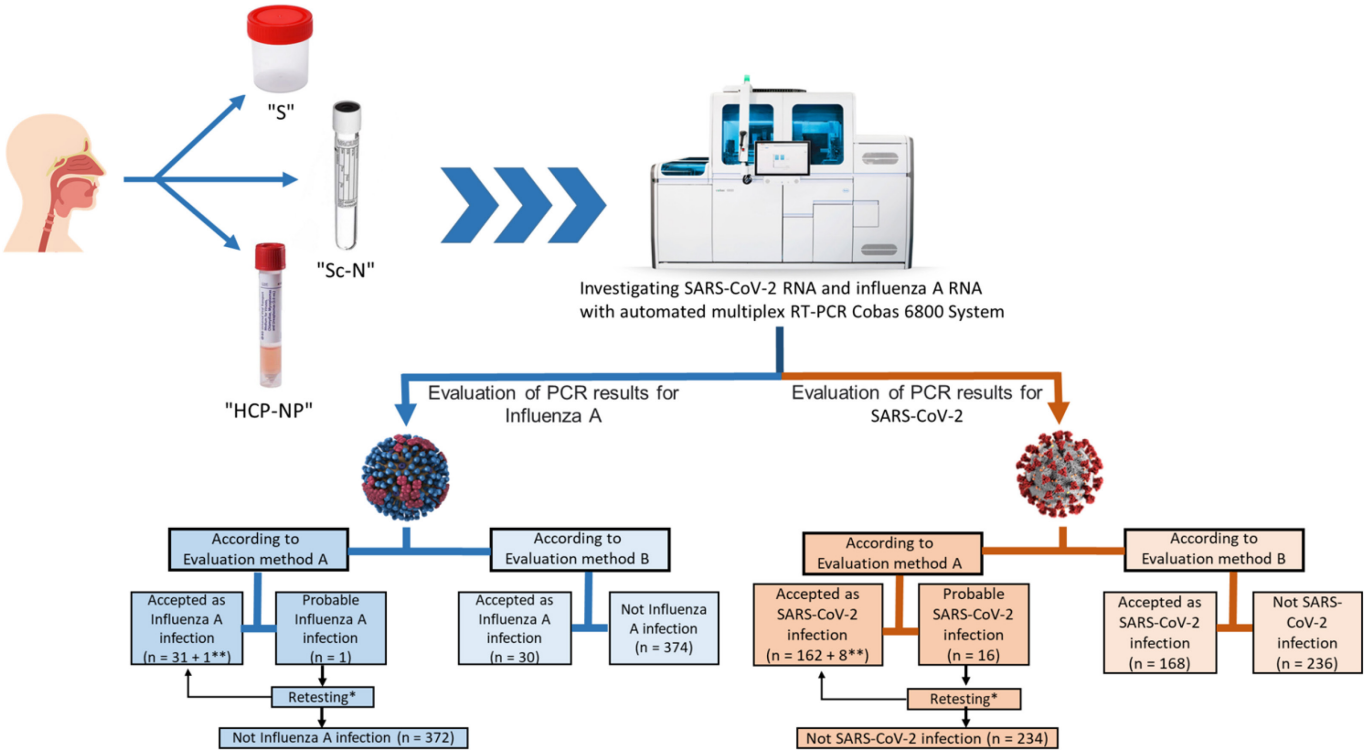
The SARS-CoV-2 RNA kit (cobas® SARS-CoV-2 on cobas6800 System; Roche Molecular Systems, Pleasanton, USA) was used to confirm the samples with indeterminate SARS-CoV-2 results (Evaluation method A in Figure 1 and Table 1). This test also had similar targets and LOD (0.007 TCID50/mL) for SARS-CoV-2 [16].

Evaluation algorithms of test results

The test results for each patient were evaluated with two algorithms using two different reference methods.

Evaluation method A: An extended reference method was identified using an algorithm based on the test instructions, test results, repeated test results, and the correlation of results of different specimens. In this algorithm, decision for the probable cases was made by retesting all 3 samples with the SARS-CoV-2 and influenza A/B RNA test, and with another SARS-CoV-2 RNA kit (cobas® SARS-CoV-2 on cobas 6800 System; Roche Molecular Systems, Pleasanton, USA). The algorithm criteria are described in Figure 1 and Table 1. The criteria were used to clearly define all

Figure 1. Algorithm for evaluation of test results using evaluation method A and evaluation method B.



*: Retesting all 3 samples with the same SARS-CoV-2/influenza RNA test, and with another SARS-CoV-2 RNA kit. **: Cases accepted as SARS-CoV-2 and influenza-A infection as a result of retesting of the probable group. HCP-NP: nasopharyngeal swab collected by healthcare professional; Sc-N: self-collected nasal swab; S: self-collected saliva; RT-PCR: real time polymerase chain reaction; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

Table 1. Criteria for the decision on SARS-CoV-2 and influenza A/B infection.

1. Accepted as SARS-CoV-2 infection	**2. Probable SARS-CoV-2 infection (indeterminate result)**
a) Both SARS-CoV-2 and pan-sarbecovirus target channels are positive in at least two specimen types	a) One target channel (SARS-CoV-2 or pan-sarbecovirus) is positive only in one specimen type
b) Both target channels are positive in at least one specimen type and one channel (SARS-CoV-2 or pan-sarbecovirus) in another specimen type	b) One target channel (SARS-CoV-2 or pan-sarbecovirus) is positive in two specimen types
c) Both target channels are positive in HCP-NP swab	c) Both target channels are positive in one specimen type other than HCP-NP swab
1. Accepted as influenza A or influenza B infection	**1. Probable influenza A or influenza B infection (indeterminate result)**
a) HCP-NP swab sample is positive for influenza A or influenza B	a) Influenza A or influenza B channel is positive only in one specimen type other than HCW-NP swab
b) Influenza A or influenza B channel is positive in at least two specimen types	

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; HCP-NP: nasopharyngeal swab collected by healthcare professional.

cases as SARS-CoV-2 or not, and influenza A/B or not (Figure 1). Performances of different clinical samples were analyzed by taking this case definition as the reference.

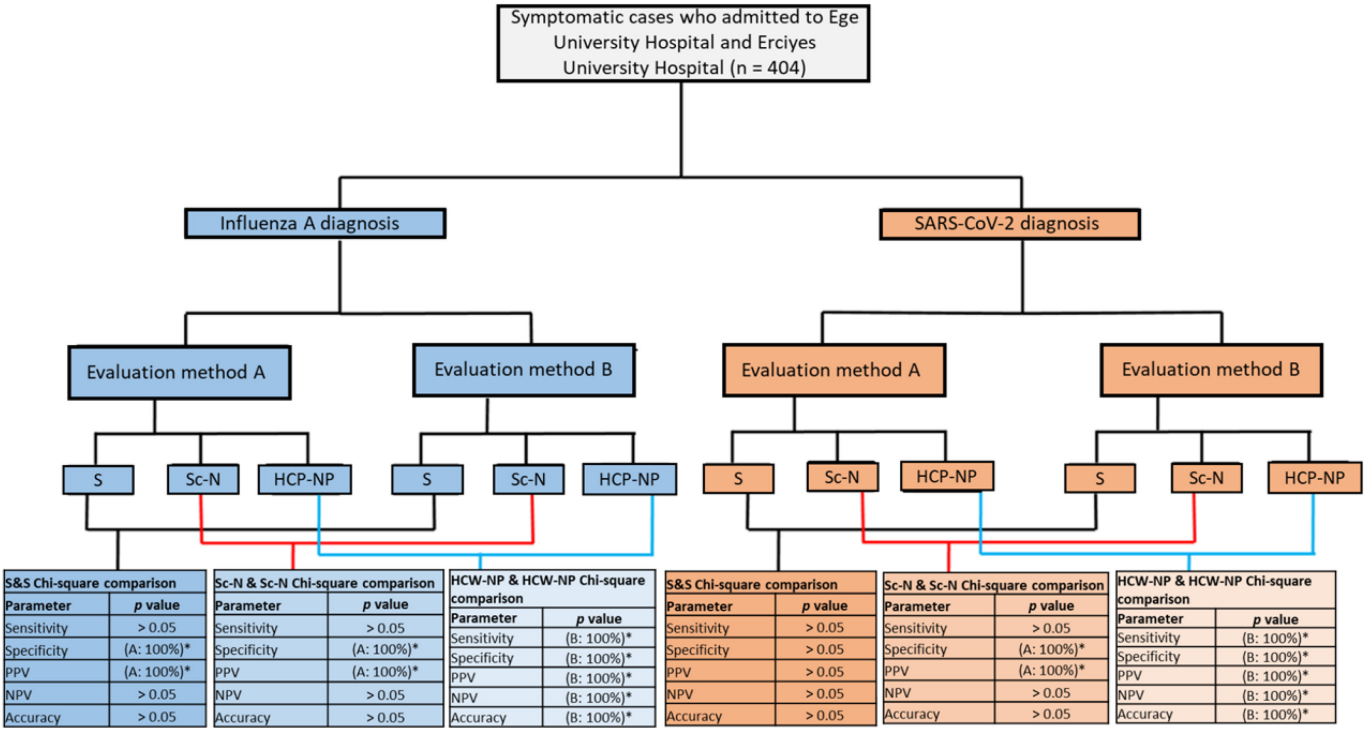
Evaluation method B: The results obtained from the NPS collected by an HCP were considered as the reference standard for decision on the patient's diagnosis.

Analysis of specimen performance

The results of 3 clinical sample types were compared with each other in terms of performance values such as sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV); according to the criteria-based "evaluation method A" and the HCP-NPs result based on "evaluation method B". The performance values of each sample type obtained from the two evaluation methods (A and B) were compared using Chi-square analysis (Figure 2).

All three clinical specimens of the patients considered to be probable SARS-CoV-2 infection according to the evaluation method A were tested again with SARS-CoV-2/influenza RNA test and SARS-CoV-2 RNA test. The patient was considered positive if the re-test result met the SARS-CoV-2 infection criteria (Table 1), or if any of the samples tested positive in the SARS-CoV-2 RNA test. If the influenza A or

Figure 2. Comparison of performance of the evaluation methods.



(A: 100%)*, Could not be compared because the result was 100% in evaluation method A; (B: 100%)*, Not convenient for the comparison because HCP-NP was taken as the reference in evaluation method B, where the results were already 100%. S: self-collected saliva; Sc-N: self-collected nasal swab; HCP-NP: healthcare-professional-collected nasopharynx swab; PPV: positive predictive value; NPV: negative predictive value; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

influenza B channel was positive again (repeated reactivity) in any sample, the patient was considered as positive for influenza A/B (Table 1 and Figure 1).

In summary, the evaluation method B grouped patients based solely on the results from the valid reference method, HCP-NP; while the evaluation method A assessed the results of all samples more comprehensively using a specific algorithm. The performances of these two methods were then compared.

Statistical analysis

IBM SPSS for Windows version 25.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. Descriptive findings were presented as numbers, percentages, means and standard deviations. Student's t test was applied to normally distributed (parametric) measurement variables, and Mann-Whitney U test was applied to nonparametric measurement variables. Pearson correlation and Cohen's Kappa coefficient were used in the concordance and correlation analysis [17]. In addition, the Chi-square analysis was applied for categorical variables.

Inter-assay, intra-assay precision analysis

When calculating the coefficients of variation (CV) of Ct values obtained from 3 channels (SARS-CoV-2, pan-sarbecovirus, and influenza A/B) in all 3 sample types, 3 high positive, 3 low positive, and 3 negative samples were repeated 3 times within the same run for intra-assay precision; and 2 high positive, 2 low positive, and 2 negative samples were run 3 days consecutively for inter-assay precision [18]. High positive samples (n = 9; including HCP-NP, Sc-N, and S separately for inter-assay and intra-assay) were selected with Ct values between 18–26 in the SARS-CoV-2 channel (mean: 22.9 [SD: ± 3]), between 18–25 in the pan-sarbecovirus channel (n = 9; mean: 22.7 [SD: ± 2.9]), and between 18–28 in the influenza A channel (n = 9; mean: 25.5 [SD: ± 1.2]). Low positive samples (including HCP-NP, Sc-N, S, separately for inter-assay and intra-assay) were selected with Ct values between 26–32 in the SARS-CoV-2 channel (n = 9; mean: 28.1 [SD: ± 2], between 26–31 in the pan-sarbecovirus channel (n = 9; mean: 27.8 [SD: ± 1.8]) and between 28–32 in the influenza A channel (n = 9; mean: 29.7 [SD: ± 1.1]).

Days of sample collection

Distribution of positive samples and Ct values by day of sample collection for each sample type were illustrated on descriptive charts.

Ethical considerations

Institutional ethics committee approval for this research was obtained from the Ege University Clinical Research Ethics Committee (decision no: 20-12T/6). Informed consent was obtained from all participants prior to sample collection and data analysis. Patient confidentiality was strictly maintained throughout the study.

Results

Among the total of 404 patients included in the study, 53% were female (n = 214), 47% were male (n = 190), and the median age was 35 years (interquartile range: 26–46). SARS-CoV-2 vaccination rates (n = 372; 92.1%) were significantly higher than influenza vaccination rates (n = 24; 5.9%) ($p < 0.05$).

Table 2A. Positive numbers and ratios of targets in 3 sample types.

Positive numbers and ratios of targets			
Sample type (n)	SARS-CoV-2 n (%)	Pan-sarbecovirus n (%)	Influenza-A n (%)
S (404)	162 (40.1%)	161 (39.8%)	29 (7.2%)
Sc-N (404)	135 (33.4%)	130 (32.2%)	23 (5.7%)
HCP-NP (404)	162 (40.1%)	166 (41%)	30 (7.4%)

Table 2B. Positive and negative predictive values (PPV and NPV), sensitivity, and specificity of 3 sample types with two different evaluation methods.

	SARS-CoV-2 RNA evaluation method A			SARS-CoV-2 RNA evaluation method B			Influenza-A RNA evaluation method A			Influenza-A RNA evaluation method B		
	S (n = 404)	Sc-N (n = 404)	HCP-NP (n = 404)	S (n = 404)	Sc-N (n = 404)	HCP-NP (n = 404)	S (n = 404)	Sc-N (n = 404)	HCP-NP (n = 404)	S (n = 404)	Sc-N (n = 404)	HCP-NP (n = 404)
Sensitivity %	94.7	80	96.4	92.8	79.7	100[a]	90.6	71.8	93.7	90	73.3	100[a]
Specificity %	98.7	100	98.2	96.6	99.1	100[a]	100	100	100	99.4	99.7	100[a]
PPV %	98.1	100	97.6	95.1	98.5	100[a]	100	100	100	93.1	95.6	100[a]
NPV %	96.2	87.3	97.4	95	87.3	100[a]	99.2	97.6	99.4	99.2	97.9	100[a]
Accuracy %	97.03	91.58	97.52	95.05	91.09	100[a]	99.26	97.77	99.5	98.76	97.77	100[a]
Prevalence %	42.08 (n = 170)			41.58 (n = 168)			7.92 (n = 32)			7.43 (n = 30)		

[a]These values are 100%, since HCP-NP was taken as the reference in evaluation method B. S: self-collected saliva; Sc-N: self-collected nasal swab; HCP-NP: healthcare-professinal-collected nasopharyngeal swab; PPV: positive predictive value; NPV: negative predictive value.

Test results

Based on the criteria algorithm (evaluation method A), 162 (40%) patients were identified as SARS-CoV-2 positive and 31 (7.7%) patients as influenza A positive. Influenza B positivity was not detected in any of the patients. In addition, 16 (4%) patients were considered as probable SARS-CoV-2 infection. After repeat testing of the 3 samples from each of these patients with two different test kits (SARS-CoV-2/influenza RNA and SARS-CoV-2 RNA test), 8 patients were evaluated as SARS-CoV-2 positive, while the other 8 patients were evaluated as not SARS-CoV-2 because they did not meet the SARS-CoV-2 positivity criteria (evaluation method A), and the SARS-CoV-2 RNA test result was negative in all clinical samples (Figure 1). All 8 patients who were considered SARS-CoV-2 positive after repeat testing were positive for SARS-CoV-2 RNA in at least 1 of the 3 sample types; only 1 of them had a SARS-CoV-2/influenza RNA test positivity showing direct SARS-CoV-2 infection according to the algorithm.

The mean Ct values of the SARS-CoV-2 channel (n = 11; mean: 33.8 ± 4.9; min–max: 20.1–37.2) and the pan-sarbecovirus channel (n = 14; mean: 33.5 ± 4.3; min–max: 20–36.8) of patients with probable SARS-CoV-2 were close to the LOD. When calculating the Ct mean value of this group, the lowest Ct values of each patient in SARS-CoV-2 and pan-sarbecovirus channels among the 3 samples were included in the evaluation. There was no sample with probable SARS-CoV-2 result in repeat testing with the same kit and negative result with the other SARS-CoV-2 RNA test kit.

One (0.2%) patient was probable influenza A (only S positivity). When 3 samples from this patient were repeated, only S was positive again with a Ct value above LOD (Ct: 27.2). The patient was accepted as having an influenza A infection.

Performance of samples and agreement of results

The positivity rates, sensitivities, specificities, PPV, NPV, prevalence, and accuracy percentages of the 3 sample types in both " evaluation method A" and " evaluation method B" are summarized in Table 2. When the sensitivities of the samples in evaluations A and B, with the exception of HCP-NP, were compared among themselves (S with S, Sc-N with Sc-N), it was observed that neither sample type was significantly different for SARS-CoV-2 or influenza A ($p > 0.05$; Figure 2 and Table 2).

Agreement analysis among the different sample types

There was almost perfect agreement between the 3 sample types (Cohen kappa values: 0.81–1.00; $p < 0.001$) for the diagnosis of SARS-CoV-2. Almost perfect agreement was found between SARS-CoV-2 and pan-sarbecovirus target channels in 3 sample types (Cohen kappa values: 0.81–1.00; $p < 0.001$). Almost perfect agreement was found between S and HCP-NP, and between Sc-N and HCP-NP (Cohen's kappa values: 0.81–1.00; $p < 0.001$), and substantial agreement was found between S and Sc-N (Cohen's kappa values: 0.61–0.80; $p < 0.001$) in the diagnosis of influenza A.. While substantial agreement reflects an acceptable level of consistency, almost perfect agreement demonstrates a powerful and reliable correlation. Inter-rater reliability results are shown in more detail in Table 3.

Analysis of Ct values

The Ct values and their inter-assay and intra-assay CV results of the 3 sample types in all 3 target channels (SARS-CoV-2, pan-sarbecovirus, influenza A), and the correlation of Ct values between sample types, and between SARS-CoV-2 and pan-sarbecovirus channels are shown in Table 4A, 4B, and 4C. When the variation was evaluated qualitatively, all repeats of high and low positives were positive and all repeats of negatives were negative (0% CV).

Table 3A. Interrater reliability values (Cohen's kappa statistic analysis; based on qualitative results) between sample types. B) Between SARS-CoV-2 targets.

		Influenza A		SARS-CoV-2	
Reference sample type	**Other sample type**	**Kappa**	***p***	**Kappa**	***p***
HCP-NP (n = 404)	S (n = 404)	0.909	< 0.001	0.898	< 0.001
HCP-NP (n = 404)	Sc-N (n = 404)	0.818	< 0.001	0.811	< 0.001
S (n = 404)	Sc-N (n = 404)	0.770	< 0.001	0.821	< 0.001

Table 3B. Interrater reliability values (Cohen's kappa statistic analysis; based on qualitative results) between SARS-CoV-2 targets.

Sample type (n)	Kappa	*p*
S (n = 404)	0.979	< 0.001
Sc-N (n = 404)	0.961	< 0.001
HCP-NP (n = 404)	0.964	< 0.001

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; HCP-NP: nasopharyngeal swab collected by healthcare professional; Sc-N: self-collected nasal swab; S: self-collected saliva. Cohen's kappa agreement levels: ≤ 0 (no agreement): 0.01–0.20 (none to slight): 0.21–0.40 (fair): 0.41–0.60 (moderate): 0.61–0.80 (substantial): 0.81–1.00 (almost perfect).

Inter-assay and intra-assay precision analysis of Ct values of 3 different sample types

CV values (%) of intra- and inter-assay precision analysis for high and low positive samples were 0.55, 0.06, 0.38, 0.63 for S; 2.72, 0.17, 1.29, 0.93 for Sc-N; and 1.02, 0.58, 1.76, 5.43 for HCP-NP samples; for the SARS-CoV-2 target respectively. In the case of the pan-sarbecovirus target, these CV values were 2.10, 0.49, 2.47, 1.22 for S; 2.30, 0.23, 1.06, 0.61 for Sc-N; and 1.11, 0.22, 1.22, 6.05 for HCP-NP. In the case of the influenza A target these values were 1.33, 0.66, 0.92, 0.89 for S; 2.2, 0.53, 1.69, 1.1 for Sc-N; 0.87, 0.34, 1.22, 1.5 for HCP-NP, as shown in Table 4C.

Among the patients, 12 had moderate or severe COVID-19, and no significant difference was observed between the mean Ct values of patients with mild, moderate or severe clinical status in all 3 sample types ($p > 0.05$) (Table 4D). Since all influenza A patients had a mild clinical condition, a comparable analysis could not be performed.

Sample collection day analysis

The mean sample collection day was 3.8 days (SD: 3.4; min–max: 1–25) for all patients. It was 4.1 days (SD: 3.8; min–max: 1–19) after the onset of symptoms for SARS-CoV-2 positive patients, 3 days (SD: 1.2; min–max: 1–7) for influenza A positive patients, and 4 days (SD: 3.4; min–max: 1–25) for SARS-CoV-2 and influenza A negative patients.

Distribution of positive samples and Ct values according to the day of sample collection for each sample type is shown in Figures 3 and 4. All three sample types showed similar trends.

Most of the samples were collected within the first 3 days after the onset of symptoms. Specifically, the percentages of samples collected within this time frame were 65.8%, 64.8%, 67.4% for NP, S, and Sc-N respectively for SARS-CoV-2 positives; and 80%, 81.25%, 78.26% for NP, S, Sc-N respectively for influenza A positives.

Table 4A. Analysis of cycle threshold (Ct) results of three different positive specimen types.

Analysis	INF A [S] (n = 29)	INF A [Sc-N] (n = 23)	INF A [HCP-NP] (n = 30)	SARS-CoV-2 [S] (n = 162)	SARS-CoV-2 [Sc-N] (n = 135)	SARS-CoV-2 [HCP-NP] (n = 162)	PanSarb [S] (n = 161)	PanSarb [Sc-N] (n = 130)	PanSarb [HCP-NP] (n = 166)
Mean	31.73	33.32	31.02	28.88	28.28	25.82	28.29	27.84	25.72
Median	31.43	34.13	31.06	29.36	28.34	25.17	28.99	28.07	25.17
Standard deviation	± 3.69	± 3.63	± 4.71	± 4.71	± 5.27	± 6.15	± 4.40	± 4.83	± 6.04

Table 4B. Pearson correlation coefficient values of three different positive specimen types of cycle threshold (n = 404).

Between three different specimen types						
Reference	**INF A (n = 32)**		**SARS-CoV-2 [a] (n = 170)**		**PanSarb [a] (n = 170)**	
	r	***p***	**r**	***p***	**r**	***p***
S and Sc-N (n = 404)	0.31	0.171	0.325	< 0.001	0.246	0.005
S and HCP-NP (n = 404)	0.32	0.103	0.369	< 0.001	0.362	< 0.001
Sc-N and HCP-NP (n = 404)	0.164	0.465	0.542	< 0.001	0.554	< 0.001
Between SARS-CoV-2 and pan-sarbecovirus targets (for each sample type)						
Sample types	**r**	***p***				
S (n = 404)	0.994	< 0.001				
Sc-N (n = 404)	0.973	< 0.001				
HCP-NP (n = 404)	0.998	< 0.001				

Table 4C. Coefficient of variation values (%) based on precision analysis [b].

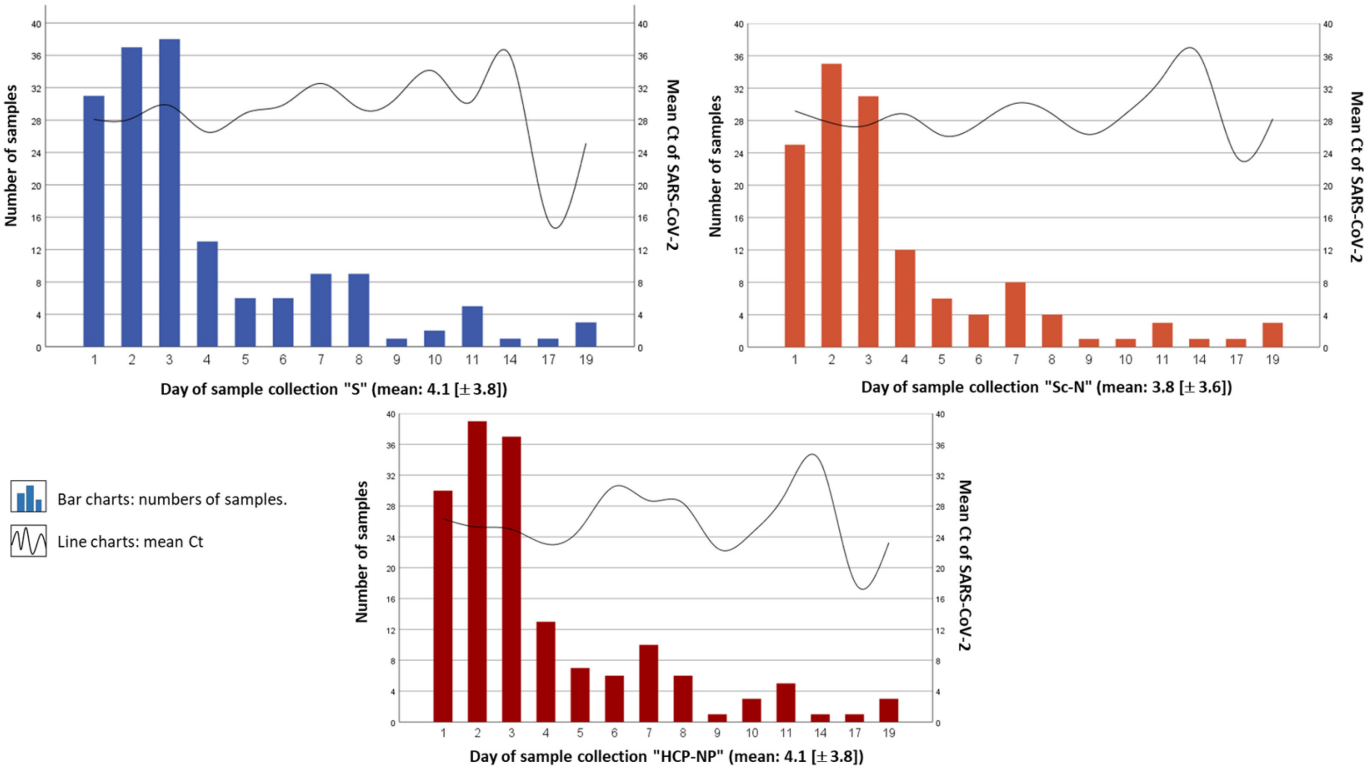
Sample type	SARS-CoV-2 target				PanSarb target				Influenza A			
	High positive		Low positive		High positive		Low positive		High positive		Low positive	
	Inter-A	Intra-A	Inter-A	Intra-A	Inter-A	Intra-A	Inter-A	Intra-A	Inter-A	Intra-A	Inter-A	Intra-A
S	0.55	0.06	0.38	0.63	2.1	0.49	2.47	1.22	1.33	0.66	0.92	0.89
Sc-N	2.72	0.17	1.29	0.93	2.3	0.23	1.06	0.61	2.2	0.53	1.69	1.1
HCP-NP	1.02	0.58	1.76	5.43	1.11	0.22	1.22	6.05	0.87	0.34	1.22	1.5

Table 4D. Cycle threshold analysis according to clinical situation.

Parameter	Clinical situation [c] (n)	Mean (SD)	*p* value
S Sars-CoV-2	Mild (150)	28.7 (± 4.6)	> 0.05
	Moderate/severe (12)	30.1 (± 5.3)	
Sc-N Sars-CoV-2	Mild (127)	28.2 (± 5.2)	> 0.05
	Moderate/severe (8)	28.9 (± 5.9)	
HCP-NP Sars-CoV-2	Mild (150)	25.7 (± 5.9)	> 0.05
	Moderate/severe (12)	27.1 (± 8.4)	

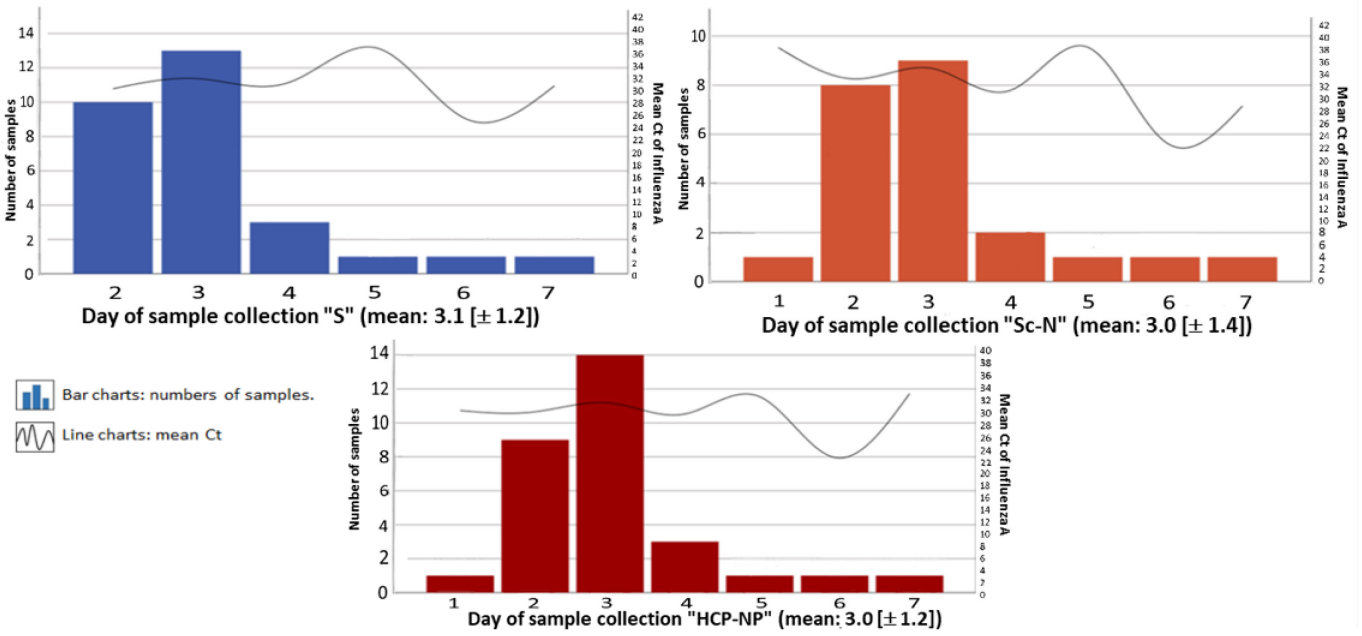
[a] Ct values of the three different sample types showed low to moderate correlation (r) with each other. [b] All coefficient of variation values were analyzed from Ct values based on precision analysis. [c] There was no moderate/severe influenza-A patient. HCW-NP: nasopharyngeal swab collected by healthcare professional; Sc-N: self-collected nasal swab; S: self-collected saliva; PanSarb: pan-sarbecovirus; INF A: influenza-A; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; r: Pearson correlation coefficient; Inter-A: inter-assay; Intra-A: intra-assay.

Figure 3. Distribution of collection days and Ct values in SARS-CoV-2 positive samples.



Bar charts: numbers of samples. Line charts: mean Ct of SARS-CoV-2. HCP-NP: nasopharyngeal swab collected by healthcare professional; Sc-N: self-collected nasal swab; S: self-collected saliva; Ct: cycle threshold; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

Figure 4. Distribution of collection days and Ct values in influenza A positive samples.



HCP-NP: nasopharyngeal swab collected by healthcare professional; Sc-N: self-collected nasal swab; S: self-collected saliva; Ct: cycle threshold; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

Discussion

Population

The demographic characteristics of the study population, including mean age and gender distribution, were consistent with the national demographic profile (mean age: 33.5 years, 50.1% male in Turkey) [19]. The SARS-CoV-2 vaccination rate was significantly higher than the influenza vaccination rate ($p < 0.05$), likely due to the study period coinciding with a phase of widespread SARS-CoV-2 vaccination in Turkey in response to the pandemic [20,21].

Evaluation methods A and B

The evaluation method A included an extended algorithm based on the criteria including repeated test results and the correlation of results among different specimens. In this way, the range of definition for patients that could be detected by S and Sc-N samples was widened. In addition, the specificity, PPV, and NPV of the three sample types were mostly increased, compared to evaluation method B, because of restricted description based solely on HCP-NP sample result (Table 2, Figure 2). Although the sensitivities of S and Sc-N samples were higher in the evaluation method A algorithm than in the evaluation method B, the sensitivity rates of all 3 samples were not significantly different ($p > 0.05$).

However, in the evaluation method A, the specificity, PPV, and NPV were all higher than 97.3%; while the sensitivity of HCP-NP was 93.7% for influenza A and 96.4% for SARS-CoV-2 (Table 2). All these values, along with the fact that the accuracy percentages were > 97.5% for HCP-NP and not significantly different between evaluations (A and B) for both influenza A and SARS-CoV-2 in S and Sc-N, indicate that the performance of clinical samples in evaluation method A was not significantly different from evaluation method B. This supported the appropriateness of evaluation method A to identify patients with insufficient HCP-NP results in our study. Evaluation method B had limitations in terms of analysis of alternative samples, but it was based on validated HCP-NP results.

As a result, the high specificity, PPV, and NPV contribute to reducing false positive and false negative results, improving patient management, and minimizing the risk of unnecessary isolation or treatment. Large-scale rapid testing during epidemics requires accurate and swift results. The reliable performance of inexpensive and easily accessible alternative samples means that tests can be applied to a wider population. This plays a critical role in public health control. Rapid and accurate tests offer opportunities for early intervention to prevent the spread of infection and reduce pressure on the healthcare system.

Evaluation of indeterminate results with repeated testing

Indeterminate results may lead to a missed diagnosis, posing a risk in terms of transmission. Therefore, care should be taken for these patients during the treatment and isolation process. Additional measures should be implemented such as repeating the sample or using another test.

In this study, the samples that were positive for one of the SARS-CoV-2 channels, but could not be directly considered as SARS-CoV-2 positive were retested with the same, or, if possible, with a second test kit. Half of the probable SARS-CoV-2 cases (indeterminate results) were identified as SARS-CoV-2 positive when retested. The fact that Ct values of these patients were closer to the LOD indicated a low viral load which may have been the cause of the indeterminate results. Cases which could not be identified as SARS-CoV-2 positive after the repeat testing (the other half of those that were retested, Figure 1) were considered false positive. False positivity may arise from technical issues or the presence of another unknown pathogen [15,22]. They may also be caused by a mutation in the target regions of the test kit [15,23]. All these conditions could be observed in all three sample types. False negativity with any sample type from a SARS-CoV-2-positive patient may be due to the quality of the sample or due to the amount of viral load [23].

In this study, one patient was identified as probable influenza A according to the initial influenza A test results. After detailed evaluation, the patient was accepted as influenza A positive, since the Ct was 27.2 and the repeat test was also positive with S. As a result, no false positivity for influenza A was observed in the study.

Compatibility of sample types

The almost perfect agreement among the three sample types in the diagnosis of SARS-CoV-2 indicates that all three sample types can be used as alternatives to each other to provide qualitative results. The Ct values of the target channels, which showed weak to moderate correlation among the three sample types, did not influence the almost perfect agreement of the qualitative results (Tables 3 and 4). The almost perfect agreement between S and HCP-NP, as well as between Sc-N and HCP-NP, in the diagnosis of influenza A,

suggests that S and Sc-N can be used as an alternative to HCP-NP (Tables 3 and 4). A meta-analysis by Hou *et al*. [13], concluded that sample types such as NPW, MTS, and NPS were effective for detecting respiratory viruses in general. However, alternative sample types are particularly valuable in situations where traditional methods are difficult to implement or when patient comfort is prioritized. Saliva, in particular, stands out due to its non-invasive nature, ease of collection, and cost-effectiveness compared to nasal or throat swabs. Saliva collection is more convenient for pediatric and elderly patients and improves the safety of HCP by reducing transmission risk. Additionally, it offers logistical advantages for large-scale screenings and mass testing, enabling faster and more cost-efficient processes. Therefore, alternative sample types represent a critical tool for improving diagnostic workflows and patient management. Sample types that are more easily obtainable and compatible with automated systems like the cobas 6800 will eliminate the need for complex pre-processing. This can also facilitate the routine workflow of laboratories.

Precision and sensitivity analysis

SARS-CoV-2 and pan-sarbecovirus target channels exhibited excellent agreement across all three sample types for both qualitative and Ct results. The sample type did not affect the compatibility of these two complementary channels. The CV's (Table 4) were all ≤ 6% for all three samples, indicating that reproducibility was also favorable with influenza A and SARS-CoV-2 for Ct values. In the qualitative precision analysis, a high level of reproducibility was observed for SARS-CoV-2 in all three samples with a CV of 0% since all results (high positives, low positives, and negatives) were identical. In addition, the accuracy of S and Sc-N was > 90% throughout the analysis. Sensitivity of > 90% and specificity of > 98% were detected in S for both SARS-CoV-2 and influenza A. In the case of Sc-N, 80% sensitivity for SARS-CoV-2, 71.8% sensitivity for influenza A, and 100% specificity for both were determined.

The difference in sensitivity between S and Sc-N suggests that S may be a better alternative than Sc-N. This may be due to the patients' hesitation in taking nasal samples and their ability to give saliva relatively more easily. In the meta-analysis by Duncan *et al.* [24], the sensitivity of S was 87% and the specificity was 99%. In our study, the detection of higher sensitivity with cobas 6800 system provided additional evidence for the usability of S in this system.

For saliva to serve as an alternative sample, pathogen survival in droplets is an important factor affecting its detectability. This situation may vary depending on the type of virus [13]. In this study, precision analysis of the S with the cobas 6800 automated multiplex PCR system for the diagnosis of SARS-CoV-2 and influenza A was successful. Additionally, in a study comparing the automated multiplex system with SARS-CoV-2 specific PCR in S, multiplex showed higher sensitivity than SARS-CoV-2 specific PCR (91.9% and 87.9%, respectively) and 80% agreement between the two PCR methods was reported [25]. Lee *et al.* reported that an automated PCR system developed by them and referred to as "point of care tool kit" was able to reliably detect SARS-CoV-2 and influenza RNAs in S samples at concentrations as low as 50 copies/μL with colorimetric analysis [26].

These results support the advantage of multiplex PCR systems such as cobas 6800 which was found successful in terms of sensitivity and precision in this study for the detection of SARS-CoV-2 and influenza A.

Correlation of test results with clinical status

The clinical status of the patients (mild to moderate/severe) was not associated with the Ct values of SARS-CoV-2 and pan-sarbecovirus target channels in all three sample types. All the patients with moderate or severe clinical status (n = 12) were hospitalized individuals who needed oxygen due to pneumonia. Previous studies have reported that false negatives of up to 33% were found in patients with pneumonia who could be diagnosed early with lung computed tomography [27]. In this study, the difference in the viral load detectable in pneumonia patients with moderate or severe clinical status based on Ct values was investigated. However, no significant difference was observed between mild infection and pneumonia patients, in the mean Ct values in with three sample types ($p > 0.05$).

Sample collection days after the onset of symptoms

The mean day of sample collection following symptom onset among the 404 patients was 3.8 days, although it extended up to 25 days. Most of the positive samples were collected in 3 days, as expected. The latest sample collection day for all three sample types after the onset of symptoms among the SARS-CoV-2 positive patients was the 19th day, and it was the 7th day for influenza A patients. Wang *et al.* noted that symptoms typically emerge 1–7 days after exposure to the influenza A virus; while in the case of COVID-19, symptoms usually appear within the first 11.5 days,

including a 5-day incubation period [28]. In this study, a narrower distribution of days and a lower mean "sample collection days" were observed in patients with influenza A. It has been demonstrated that symptoms became more pronounced at a faster rate in the case of influenza A. No clear directional increase or decrease in Ct values was observed over the days for either agent. Particularly, similar viral loads were detected in the first week, when the most samples were collected. These results support the recommendation that the upper respiratory specimens collected in the first week are efficient for the success of RNA tests [4–6].

Strengths and limitations of the study

This study is the first to demonstrate the utility of different sampling methods, including saliva, in diagnosing SARS-CoV-2 and influenza A infections using the automated multiplex RT-PCR cobas 6800 system, supported by real-world data and analysis of sampling times. A comprehensive verification process, including precision and qualitative accuracy analyses, was performed for the three sample types. Additionally, Ct values were evaluated. The GPower 3.1 software was used to analyze the kappa coefficient with two evaluators and two categories on 404 tests, and the statistical power calculated for "kappa coefficient of 0.770" (calculated for the minimum kappa value in this study) was found to be 0.999 [29]. This shows that the study had high statistical power. All data obtained and presented in this report will be helpful to laboratories with intensive routine workload. Analysis of experiences from the COVID-19 pandemic is a valuable source of information to be prepared for potential future pandemics, especially caused by respiratory viruses. In this sense, effective testing algorithms and easily collected samples are important.

In this study, the method for providing alternative samples was only verbally explained by the HCP. Apart from this, the HCP did not make any additional contributions. Since the effectiveness of this method has been demonstrated, a similar written instruction can be implemented in routine practice without the need for HCP involvement, thus eliminating any direct contact with the HCP.

However, our research had some limitations. Viral load and analytical sensitivity analyses of different samples based on RNA copy numbers or cell culture methods could not be performed. Comparisons with other respiratory samples (such as NPW, sputum) or various transport media could not be made.

Conclusions

The excellent compatibility of S and Sc-N results with HCP-NP results in the case of SARS-CoV-2 and influenza A/B RNA testing with automated multiplex RT-PCR cobas 6800 System in symptomatic SARS-CoV-2 and influenza A infected patients demonstrated that these samples may be suitable alternatives to provide qualitative results.

S and Sc-N, which are comfortable and easy to collect, offer greater convenience for performing SARS-CoV-2 and influenza A RNA testing in home care, outpatient, crowded places in the community, and other community-serving settings. Poor and uncorrelated cycle threshold agreement rates did not affect the qualitative result agreement. In fact, SARS-CoV-2 and influenza A RNA results are reported qualitatively. The defined diagnostic algorithm based on criteria in evaluation method A was prepared to evaluate the performances of the alternative specimens, but it is not a recommendation for reporting the results. However, it was observed that retesting or collecting an additional sample could be effective in resolving indeterminate results.

The clinical status of SARS-CoV-2 patients (whether mild, moderate, or severe) did not significantly impact the Ct values across all three sample types. It was observed that Sc-N and S can be used alternatively, regardless of whether the patient was an outpatient or hospitalized due to moderate/severe illness.

Finally, the Sc-N swab in isotonic solution and the S sample can be used as alternatives to HCP-NP for SARS-CoV-2/influenza RNA testing; and their use offers valuable advantages. These samples are easy to collect, do not require a viral transport medium, and can be processed collectively on the cobas 6800 System, providing significant benefits for laboratory testing.

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Authors contributions

Conceptualization, MAÖ, AZ, SG, MAA, SE; methodology, MAÖ, PS, MS, OA, ZTY, FIÇ, SD, ÖMP, DA, AZ, GKÜ, MT, SG, MAA, GAK, İRD, CÇ, HP, OY, SRS, SE; software, SD, MAÖ; validation, MAÖ, ÖMP, MS, OA, ZTY, FİÇ, SD, PS, DA, AZ, GKÜ, MT, SG, MAA, İRD, CÇ, HP, OY, SRS, SE; formal analysis, MAÖ, PS, MS, ÖMP, SG, MAA; investigation, MAÖ, ÖMP, MS, OA, SG, ZTY, GKÜ, SD, DA, FİÇ, PS, GAK, İRD, AZ, MAA, MT, CÇ, OY, HP, SRS, SE; resources, SG, SE; data curation, MAÖ, ÖMP, SD, ZTY, MAA, SE; manuscript—original draft preparation, MAÖ,

SE; manuscript—review and editing, MAÖ, SD, AZ, SG, MAA, SE; data visualization, MAÖ, MS, SD; funding acquisition, SG, SE; supervision, SG, SE; project administration, SG, SE. All authors have read and agreed to the published version of the manuscript.

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Corresponding author

Muhammed Alper Özarslan, MD.
Ege University Faculty of Medicine Hospital, Department of Medical Microbiology,
35100 Bornova-Izmir, Turkey.
Tel: + 90 (232) 390 33 03
Fax: + 90 (232) 390 29 83
Email: alperozarslan@hotmail.com

Conflict of interests

No conflict of interests is declared.

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