

Conjunctival polymerase chain reactions in COVID-19 patients and its correlation with clinical and paraclinical indexes

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ABSTRACT

Background and Objectives: This study aimed to detect SARS-CoV-2 in conjunctival samples of COVID-19 patients to investigate the transmission route of COVID-19 and its correlation with laboratory indexes.

Materials and Methods: In this cross-sectional study, 44 COVID-19 patients were tested for conjunctival PCR in Ayatollah Rouhani hospital of Babol, Iran, in January and February 2021. The conjunctival samples were collected using a conjunctival swab and suspended in a viral transport medium. After RNA extraction and cDNA synthesis, real-time PCR was performed to investigate the SARS-CoV-2 genome in samples. The ocular manifestations and laboratory indexes were evaluated for all patients.

Results: Among 44 COVID-19 patients, 6 samples (13.63%) were positive in terms of conjunctival PCR. The mean \pm SD age of conjunctival PCR-positive patients was 76.17 ± 16.61 -year-old, while conjunctival PCR-negative COVID-19 patients were aged 57.54 ± 13.61 -year-old ($p < 0.05$). D-dimer serum level is significantly higher in conjunctival PCR-positive COVID-19 patients ($4001.00 \pm 3043.36 \mu\text{g/ml}$) compared to normal individuals ($496.80 \pm 805.92 \mu\text{g/ml}$, $p < 0.01$).

Conclusion: Our study showed that the conjunctiva and tear contain the SARS-CoV-2 in COVID-19 patients as a possible transmission route.

Keywords: Conjunctiva; SARS-CoV-2; Real-time polymerase chain reaction; COVID-19; Eye manifestations

INTRODUCTION

According to the World Health Organization (WHO), coronavirus infectious disease 2019 (COVID-19) has infected more than 167 million people worldwide and more than 3.4 million mortality

by May 2021 (<https://covid19.who.int>). Since the beginning of the COVID-19 pandemic, there have been numerous reports of new complications in patients with the disease (1). Gastrointestinal complications (2), joint and muscle pain (3), fever and cough (4), respiratory distress (5), and ocular manifestations (6)

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are the most common of these complications (7).

Coronaviridae is the most prominent family of RNA viruses that can cause some ocular manifestations. Before the COVID-19 pandemic, there were conflicting reports on the ocular effects of coronaviruses in humans (8). Different parts of the eye are affected differently by infectious diseases associated with the coronaviruses. Also, because of the connection between the mucosa of the eye and the upper respiratory tract through the nasolacrimal duct, the eye can be a way for respiratory viruses such as coronaviruses to enter the respiratory tract. The presence of the severe acute respiratory syndrome-associated coronavirus 2 (SARS-CoV-2) in the conjunctiva has been confirmed using molecular detection techniques (9, 10). PCR is mainly used for SARS-CoV-2 detection in conjunctival swab samples. In a recent study, Lu et al. showed that SARS-CoV-2 could be transmitted through conjunctiva (9). In the current study, we aimed to detect SARS-CoV-2 in conjunctival swab samples to investigate the transmission route of COVID-19 and its correlation with laboratory indexes.

MATERIALS AND METHODS

Patients. In this cross-sectional study, 44 COVID-19 patients were tested for conjunctival PCR in Ayatollah Rouhani Hospital of Babol, Iran, in January and February 2021. This study is confirmed by ethical committee of Babol University of Medical Sciences (IR.MUBABOL.REC.1399.391).

Sample collection and preparation. Conjunctival swabs were collected from patients, stored in 1 ml viral transport medium (Pasteur institute of Iran), and were shipped with ice to Ayatollah Rohani Hospital laboratory affiliated to the Babol University of Medical Sciences. All samples were processed immediately under a class II biosafety cabinet without further steps of dilution or heat inactivation according to standard laboratory biosafety guidelines and good laboratory practice (GLP) regulations. After processing, samples were divided into small volume aliquots and frozen at -80°C until examination.

Viral nucleic acid isolation. Viral RNA was freshly isolated from 200 μL of swab-storage media using the Behperp Viral Nucleic Acid Extraction Kit (Be-

hGene Biotechnology, Shiraz/Fars, Iran) according to the manufacturer's instructions. Briefly, for virus dissociation and purification of viral nucleic acid, 200 μL of LB lysis buffer, 25 μL Proteinase K and 6 μL of carrier RNA (2 $\mu\text{g}/\mu\text{L}$) were added to each swab-storage media containing microcentrifuge tube. Samples were subsequently incubated at 56°C for 10 minutes until the virus particles were lysed properly. RNA cleanup was done by mini spin column (silica matrix) according to the manufacturer's instructions.

One step real-time-reverse transcription- PCR for SARS-CoV-2 detection. After viral RNA extraction, samples were promptly subjected to one-step real-time RT-PCR analysis using COVID-19 One-Step RT-PCR Kit (Pishtaz Teb Diagnostic, Iran) with resuspended master mix and RdRp/N/internal control (IC) primers & TaqMan probes which could detect SARS-CoV-2 (sensitivity= 200 copies / mL). The reaction mixture (20 μL) includes the following reagents: 9 μL of resuspended master mix, 1 μL of primers & TaqMan probes mix, 5 μL of RNase-free water, and 5 μL of isolated RNA. Experiments were performed in a QIAquant 96 5plex (Qiagen, Hilden, Germany) real-time PCR instrument. The amplification program was adjusted as follow: 1) Reverse transcription at 50°C for 15 min, 2) cDNA Initial Denaturation at 95°C for 3 min, 3) 45 cycles of denaturation at 95°C for 15 sec and amplification at 55°C for 40 sec. The reporter dye channel sets as 5' Fluorescein Amidite (FAM) for the viral RdRp gene; 5' hexachlorofluorescein (HEX) for the viral N gene; and 5' carboxyrhodamine (ROX) for IC gene.

The ophthalmic and laboratory examinations. The ophthalmic examination was performed for all participants, and the ocular manifestations were recorded by an ophthalmologist. Also, to the investigation of the possible association of biochemical and hematological parameters with conjunctival PCR results, laboratory indexes, i.e., the oxygen saturation (SPO₂), the differential white blood cell (WBC), red blood cell (RBC), and platelet (Plt) counts, hemoglobin (Hb), mean cell volume (MCV), prothrombin time (PT), partial thromboplastin time (PTT), international normalized ratio (INR), D-dimer, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), electrolytes, interleukin-6 (IL-6), lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), alkaline phosphatase (ALP) were measured.

tase (ALP), fasting blood sugar (FBS), blood urea nitrogen (BUN), creatinine (Cr), procalcitonin, and pro-B-type natriuretic peptides (proBNP) were evaluated in the laboratory of Ayatollah Rouhani hospital.

Statistical analysis. Mann-Whitney U test was used to investigate the correlation of positive conjunctival PCR results and laboratory indexes. SPSS ver.25 was used for statistical analysis. The significance level was considered as 95% ($p < 0.05$). The receiver operating characteristic (ROC) curve was plotted to indicate the sensitivity and specificity of significant laboratory indexes.

RESULTS

Demographic data of conjunctival PCR-positive COVID-19 patient. Out of 44 COVID-19 patients, 6 samples were positive in terms of conjunctival PCR. The mean \pm SD of conjunctival PCR-positive patients was 76.17 ± 16.61 -year-old, while conjunctival PCR-negative COVID-19 patients were aged 57.54 ± 13.61 -year-old ($p < 0.05$). All descriptive data of conjunctival PCR-positive COVID-19 patients are listed in Table 1.

Potassium and D-dimer association with the positivity of conjunctival PCR. To investigate possible associations between laboratory indexes and positivity of conjunctival PCR, biochemical and hematological parameters were evaluated for conjunctival PCR-positive and negative COVID-19 patients (Table 2). The results showed that potassium level in serum of conjunctival PCR-positive COVID-19 patients (3.98 ± 0.53 mmol/L) is significantly lower compared to conjunctival PCR-negative COVID-19 patients (4.50 ± 0.51 mmol/L, $p < 0.05$). Also, D-dimer serum level is significantly higher in conjunctival PCR-positive COVID-19 patients (4001.00 ± 3043.36 μ g/ml) compared to normal individuals (496.80 ± 805.92 μ g/ml, $p < 0.01$).

Sensitivity and specificity of potassium and D-dimer in the presence of positive result of conjunctival PCR. The area under the curve (AUC) was calculated in the ROC curve to indicate the sensitivity and specificity of potassium and D-dimer in the presence of positive results of conjunctival PCR. The AUC of D-dimer is 0.965 ± 0.040 . The result of the Coordinates of the Curve showed that the value of equal and greater than 178.50 μ g/ml indicates the positive conjunctival PCR result for COVID-19 patients

Table 1. Demographic data of conjunctival PCR-positive COVID-19 patients

Patients No.	Gender	Age	Ocular manifestation	Symptoms when referring	Background disease
1	Female	74	Redness, Pinguecula, Dried eye, Burning eye	Lethargy, Fever, Caught, Myalgia, Anorexia	Ischemic Heart Disease
2	Male	83	Discharge and exudate, Redness, Dried eye, Burning eye	Lethargy, Caught, Dyspnea	Ischemic Heart Disease
3	Male	89	Blurred vision, Pinguecula,	Fever, Dyspnea	-
4	Female	76	Dried eye, Burning eye,	Lethargy, Fever, Caught, Vomiting, Anorexia	Hypertension, Diabetes Mellitus, Hyperlipidemia, Coronary artery bypass grafting
5	Female	90	Redness, Itchy eye, Pinguecula, Burning eye	Fever, Caught, Myalgia, Dyspnea	-
6	Female	45	Dried eye, Burning eye	Fever, Caught, Headache, Myalgia	Diabetes Mellitus

with 100% sensitivity and 52.6% specificity. Also, the AUC of potassium is 0.061 ± 0.054 , and sensitivity and specificity are not meaningful (Fig. 1).

Correlation of positivity of conjunctival PCR and the ocular manifestation. The ophthalmic examination was applied for conjunctival PCR-positive and negative COVID-19 patients to find the possible association of ocular manifestation and the result of conjunctival PCR. Our results showed that the ocular manifestation of COVID-19 in conjunctival PCR-positive patients is not different from conjunctival PCR-negative patients.

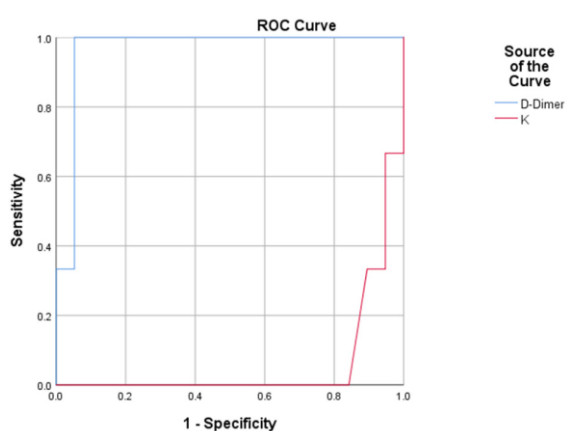


Fig. 1. The ROC curve of potassium and D-dimer in the positivity of conjunctival PCR in nasopharynxal PCR positive COVID-19 patients. The AUC of D-dimer is 0.965 ± 0.040 . The Coordinates of the Curve showed that the value of equal and greater than $178.50 \mu\text{g/ml}$ indicates the positive conjunctival PCR result for COVID-19 patients with 100% sensitivity and 52.6% specificity.

DISCUSSION

It seems that the eyes are an important organ for the transmission of respiratory viruses, i.e., SARS-CoV-2, through the nasolacrimal duct, which connects the conjunctiva and eye cavity to the respiratory tract. The investigation of the viral genome content of SARS-CoV-2 in conjunctival samples is vital in determining the ocular transmission route of COVID-19. In this study, the PCR test was used to investigate the SARS-CoV-2 RNA in conjunctival samples.

Our results showed that 6 patients (13.63%) from 44 patients with nasopharyngeal-positive SARS-CoV-2 results had a positive result for conjunctival PCR

of SARS-CoV-2. Atum et al. found positive results of conjunctival SARS-CoV-2 PCR in three patients through 40 (7.5%) (10). A study by Xia et al. showed that just 6.67% of SARS-CoV-2 patients have positive PCR results for SARS-CoV-2 (11). Also, Wu et al. reached 5.2% positive results of conjunctival PCR in nasopharyngeal PCR-confirmed patients (7). Kaya et al. showed that conjunctival SARS-CoV-2 PCR is positive for 15.6% of nasopharyngeal PCR-confirmed COVID-19 patients (12). Li et al. found 8.2% of COVID-19 patients with conjunctival SARS-CoV-2 PCR positive results (13), while their study was conducted on COVID-19 patients without ocular symptoms. In the study of Güemes-Villahoz et al., 5.5% of COVID-19 patients had positive PCR results of conjunctival samples (14). In our study, compared to other studies, more positive results of conjunctival SARS-CoV-2 PCR were obtained. In the first setting-up, we used 2 ml of viral transport medium while the results showed no positive results for conjunctival PCR. Then, we stored the swabs in 1 ml of viral transport medium, reaching six positive results. It seems that it is better to use a less volume of viral transport medium for low viral-loaded samples.

The nasolacrimal duct gets tighter through aging (15). Our results showed that the average age of conjunctival PCR-positive SARS-CoV-2 patients is about 20 years more than conjunctival PCR-negative SARS-CoV-2 patients. It seems that the tightness of the nasolacrimal duct through aging leads to the entrapment of SARS-CoV-2 in the eyes, which led to more positive results of conjunctival PCR in older patients. The results of Kaur et al. and Atum et al. did not show any significant correlation between age and results of conjunctival SARS-CoV-2 PCR (10, 16).

D-dimer is a substance of fibrin degeneration. A four-fold increase in serum level of D-dimer is significantly correlated with poor prognosis and mortality in COVID-19 patients (17). We found a significant correlation between the serum level of D-dimer and positive results of conjunctival SARS-CoV-2 PCR in COVID-19 patients. D-dimer serum level in conjunctival PCR-positive COVID-19 patients is about 8-fold more than in conjunctival PCR-negative COVID-19 patients. As an indirect conclusion, the positive results of conjunctival SARS-CoV-2 PCR in COVID-19 patients is potentially correlated with poor prognosis and mortality in COVID-19 patients. More studies are needed for the establishment of an association of poor prognosis and mortality in

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Table 2. Association of hematologic and biochemical laboratory indexes with the positivity of conjunctival PCR.

Parameter	Conjunctival PCR	Mean	Std. Deviation	Z-score	p-value
SPO2 (%)	Negative	93.27	4.85	-0.359	0.719
	Positive	93.25	5.50		
WBC (cell/ μ l)	Negative	14751.28	39742.69	-0.167	0.867
	Positive	8833.33	4742.85		
Neutrophil (%)	Negative	77.66	9.65	-1.009	0.313
	Positive	80.16	14.72		
Lymphocyte (%)	Negative	18.40	10.61	-1.186	0.236
	Positive	14.68	13.76		
RBC (106cell/ μ l)	Negative	4.31	0.71	-0.284	0.777
	Positive	4.25	0.59113		
Hb (mg/dl)	Negative	12.55	4.01	-0.919	0.358
	Positive	11.58	0.86		
MCV (fl)	Negative	85.46	15.73	-0.100	0.920
	Positive	86.26	9.37		
PLT (cell/ μ l)	Negative	236666.66	112925.06	-0.868	0.385
	Positive	270333.33	91957.96		
ESR (mm/hours)	Negative	34.11	22.03	-0.811	0.417
	Positive	23.25	8.06		
CRP (mg/L)	Negative	61.41	65.16	-0.139	0.889
	Positive	87.80	99.11		
FBS (mg/dl)	Negative	174.83	129.88	-0.866	0.386
	Positive	163.60	43.20		
PT (sec)	Negative	12.89	1.28	-0.506	0.613
	Positive	12.61	0.98		
PTT (sec)	Negative	41.03	20.64	-0.086	0.932
	Positive	48.50	35.39		
BUN (mg/dl)	Negative	22.91	20.99	-0.665	0.506
	Positive	22.16	10.60		
Cr (mg/dl)	Negative	2.78	10.73	-0.084	0.933
	Positive	0.95	0.24		
INR	Negative	2.13	6.14	-0.685	0.493
	Positive	1.08	0.13		
LDH (IU/L)	Negative	666.26	217.81	-0.126	0.900
	Positive	657.40	370.71		
ALT (IU/L)	Negative	52.94	69.11	-0.418	0.676
	Positive	41.00	19.67		
AST (IU/L)	Negative	55.44	64.26	-0.067	0.947
	Positive	44.83	28.93		
ALP (IU/L)	Negative	200.19	155.32	-0.036	0.971
	Positive	165.50	56.69		
Ca (mg/dl)	Negative	8.64	0.59	-0.676	0.499
	Positive	8.56	0.23		
Mg (mg/dl)	Negative	2.26	0.33	-1.162	0.245
	Positive	2.13	0.21		
P (mg/dl)	Negative	3.63	0.63	-0.112	0.911
	Positive	4.10	1.44		
Na (mEq/L)	Negative	136.59	3.81	-1.287	0.198

Table 2. Continuing...

K (mmol/L)	Positive	138.66	2.58	-1.969	0.049 a
	Negative	4.50	0.51		
D-Dimer (µg/ml)	Positive	3.98	0.53	-2.667	0.008 a
	Negative	496.80	805.92		
IL-6 (pg/ml)	Positive	4001.00	3043.36	-0767	0.767
	Negative	11.55	8.70		
Pro-Calcitonin (ng/ml)	Positive	64.72	101.57	-0.265	0.791
	Negative	0.36	0.40		
Pro-BNP (ng/ml)	Positive	0.16	0.09	-1.866	0.062
	Negative	395.10	644.20		
	Positive	1942.33	2502.57		

COVID-19 patients and the positive results of conjunctival SARS-CoV-2 PCR. Regarding our results, Kaur et al. showed that the mean serum level of D-dimer is significantly higher in patients with positive results of conjunctival SARS-CoV-2 PCR (16). The elevated D-dimer level in COVID-19 patients is due to the immunothrombosis, in which neutrophil entrapments are enrolled (18). The presence of SARS-CoV-2 in conjunctiva may indicate more viral load in conjunctival PCR-positive COVID-19 patients. The more viral load indicates a more severe infection and then more neutrophil entrapment. Therefore, our finding, the significant correlation between the presence of SARS-CoV-2 in the conjunctiva (confirmed by conjunctival PCR) and higher D-dimer, may be related to more neutrophil entrapment. Contrarily, Atum et al. did not find any significant correlation between the serum level of D-dimer and positive results of conjunctival SARS-CoV-2 PCR in COVID-19 patients (10).

Also, our findings indicated the predictor role of D-dimer in the positive result of conjunctival PCR ($AUC = 0.965 \pm 0.040$). On the other hand, D-dimer is correlated with poor prognosis and high mortality in COVID-19 patients (17). Therefore, positive results of conjunctival SARS-CoV-2 PCR in COVID-19 patients may indicate a poor prognosis. For this aim, we suggest a long-term investigation regarding with correlation of results of conjunctival SARS-CoV-2 PCR and the prognosis of COVID-19.

In our study, other laboratory indexes (i.e., SpO₂, WBC, RBC, Plt, Hb, MCV, PT, PTT, INR, ESR, CRP, IL-6, LDH, ALT, AST, ALP, FBS, BUN, Cr, proBNP) were not significantly correlated with the positive result of conjunctival SARS-CoV-2 PCR. In the study of Kaur et al. mean total leukocyte count

was significantly higher in conjunctival SARS-CoV-2 PCR-positive patients than in negative patients (16). Also, Atum et al. showed no significant difference between the positive result of conjunctival SARS-CoV-2 PCR and laboratory indexes (i.e., CRP, Albumin, Cr, ALT, AST, ferritin, BUN, WBC, Hb, and Plt) (10).

Finally, due to the positive PCR results of conjunctival samples in 13.6% of patients, it seems that the eyes are vital in the transmission route of SARS-CoV-2. It seems that conjunctival PCR of SARS-CoV-2 was falsely negative in COVID-19 patients due to the ocular dehydration of hospitalized COVID-19 patients and passing the time from infection; therefore, we suggest investigating the SARS-CoV-2 genome in the conjunctiva in the first days of infections. For further researches, also we suggest follow-up the COVID-19 patients to investigate the correlation of positive results of conjunctival SARS-CoV-2 PCR and prognosis. Also, the quantification of viral load may be useful to determine the correlation between conjunctival viral load and laboratory indexes and ocular manifestations.

CONCLUSION

In this study, we found that the SARS-CoV-2 genome is detectable in 13.63% of COVID-19 patients. Also, the positive result of conjunctival PCR was directly correlated to age, serum D-dimer, and potassium levels. Therefore, the ocular transmission of SARS-CoV-2 may have an impact on COVID-19. So, personal protection equipment is essential to inhibit transmission of SARS-CoV-2, especially for medical staff.

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