Research Article

COVID-19 AND HUMORAL IMMUNE RESPONSE IN CONVALESCENT PLASMA DONORS IN PAKISTANI COHORT – ANALYSIS FROM CONVELECENCE PLASMA TRIAL

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ABSTRACT

Current COVID-19 pandemic has affected the entire globe. While there was no vaccine neither any specific treatment, investigational use of convalescent plasma has been explored in clinical trials. A prospective multicenter study of convalescent plasma was conducted. Donors were tested for total Anti-SARS-CoV-2 antibodies by electrochemiluminescence (ECLIA) and RT-PCR for COVID-19. Enzyme Linked Immunosorbent Assay (ELISA) was used to detect semi-quantitative and quantitative IgG anti-SARS-COV-2 antibodies. IgG Immunofluorescence-based lateral flow immunoassay (LFIA) was used to recheck seronegative donors. A total of 400 donors were enrolled. Twelve donors were SARS-CoV-2 positive by RT-PCR. Nine of 12 donors had developed SARS-CoV-2 IgG antibodies, while in 3 donors antibodies were not developed. A total of 70 donors (17.5%) were deferred due to seronegative status; 64 (16%) of them did not develop antibodies when plasma collection was planned. The IgG semiquantitative ELISA was positive in 282 and quantitative in 284 of 330 donors with a mean value of >1:160 and 44.10±39.22 IU/ml respectively. A total of 116 (29%) donors did not show IgG humoral response to COVID-19 even 28 days from the onset of illness. Subsequently, LFIA method was able to detect IgG antibodies in 20 of 48 (41.6%) seronegative donors and in 20 of 34 (58.8%) ECLIA positive ELISA negative donors. Viral RNA detection in recovered asymptomatic patients with concomitant IgG antibodies indicates recovery. Inability to detect antibodies by different testing kits may be due to their different antigenic targets or sensitivity. Significance of a positive COVID-19 RT-PCR in asymptomatic recovered patients is yet to be determined.

Key Words: Anti-SARS-CoV-2, Convalescent plasma, Covid-19 pandemic, Passive immunization

INTRODUCTION

World Health Organization (WHO) declared COVID-19 as a pandemic and as of 29th June 2020, with 10,004,707confirmed cases and 500,000 deaths reported in 216 countries around the world (1). In Pakistan, around 206,512 people have been diagnosed with COVID-19 and 4,167 deaths have been

reported as of now, with the mortality rate of around 2.0%(2). Given the absence of any approved treatment for COVID-19 and vaccine, investigational treatment regimens have emerged as therapeutic options to be considered as a cure.

Passive immunization refers to a process of transferring antibody preparations derived from sera or secretions of immunized donors via systemic or mucosal route to non-immune individuals. Plasma collected from recovered patients of a given infectious illness during convalescence period is referred to as convalescent plasma. It has been used over many decades for a variety of different infectious agents such as pneumococcal pneumonia(3), poliomyelitis(4), measles, influenza(5) in the past century, and H1N1 influenza(6), Ebola(7), SARS(8)and MERS(9) in this century. In the current SARS-COV-2 pandemic, anecdotal reports have shown efficacy of convalescent plasma(10-12).

A number of published studies have reported the detection of viral RNA of SARS-COV-2 many weeks after documented recovery(13-18,19-22). Clinical significance of persistence or re-emergence of virus is not well understood. Utilization of convalescent plasma, as a way of providing neutralizing antibodies to severely ill patients, has been approved by regulatory authorities in many countries including Pakistan in a setting of clinical trial or as expanded access program. Seronegativity of recovered COVID-19 patients have also been reported in different studies; its clinical significance remains to be seen. This may be due to lower sensitivity of the testing kits, different antigenic targets used or different techniques used. With this background, this study was designed to find out the rate of anti COVID-19 antibodies and to check seroconversion in recovered COVID-19 patients after convalescence period and compare different antibody detection methods in seronegative patients.

METHODS

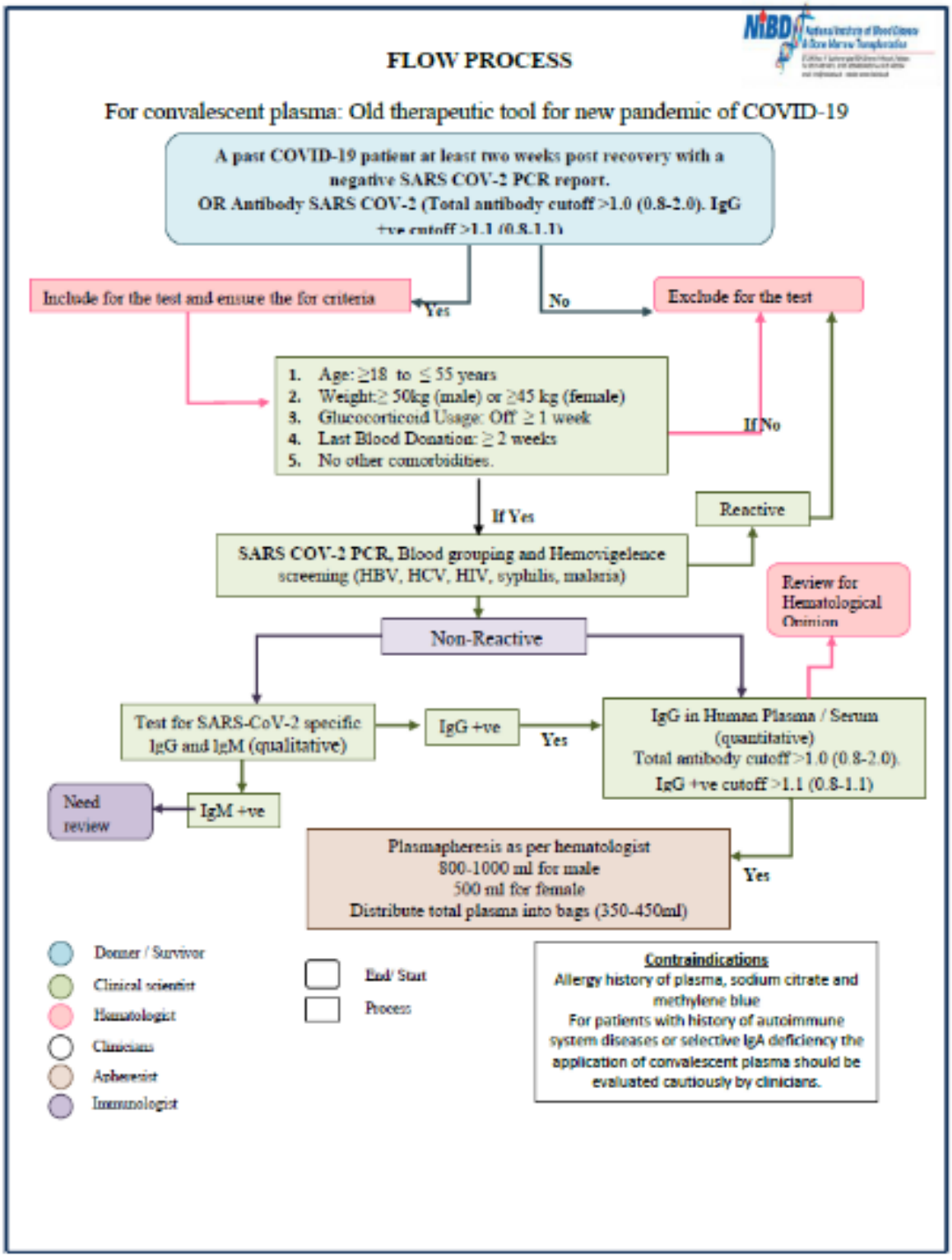
The trial was approved by National Bioethics Committee (NBC) and Drug Regulatory Authority of Pakistan (DRAP). It was conducted in accordance with ICH-GCP guidelines. This trial protocol was registered at www.clinicaltrials.gov (trial number: NCT04352751) as experimental use of COVID-19 convalescent plasma for the purpose of passive immunization in current COVID-19 pandemic in Pakistan 2020. Convalescent Plasma Donors were selected according to WHO criteria as given in Figure 1. COVID-19 recovered patients who volunteered to donate convalescent plasma were selected. The patients had a history of COVID-19 during last 4-8 weeks, followed by negative RT-PCR for SARS-COV-2 RNA on 2 consecutive samples 24 hours apart. They had recovery at least 2-weeks before they donate. The participants were between 18-60 years of age weighing >50kg for men and > 45kg for women. At least a week been passed since last use of glucocorticoids. They all must met all the criteria for a regular blood donor (i.e. negative for hepatitis B, C, HIV, Syphilis and RT-PCR negative for SARS-CoV-2). Once they fulfill all the criteria informed consent was signed before donation. Moreover, multiparous female donors were excluded from the study. The flow-chart of the patients selection is given in Figure 1.

Specimen Collection and Transportation:

A. Blood Collection:

Whole blood (10 ml) was collected in lavender top (containing K_3EDTA for plasma) and gel tube and plain tube (for serum). Serum or plasma was separated by centrifugation of respective tubes and distributed to 2 to 3 aliquots and sent to respective departments.

Figure 1: Flow Process for convalescent plasma donors



B. Respiratory Specimens Collection for RT-PCR

Nasopharyngeal swab was collected as per CDC guideline; specimens were sent in viral transport media sealed in zip lock bags to the molecular department. All specimens were transported through maintenance of cold chain in reference center for qualitative testing of antibodies against SARS-COV-2.

Lab Investigations:

Complete blood count (CBC) and routine biochemistry investigations were performed as per institutional standard laboratory protocol. Serum samples were tested for qualitative detection of antibodies against SARS-COV-2, using Elecsys® assay kits using ECLIA immunoassay on Cobas e411 immunoassay analyzer by Roche Diagnostics International Ltd, (CH – 6343 Rotkreuz, Switzerland). Qualitative ELISA for IgG antibodies against SARS-CoV-2 was done using Omega Diagnostics IVD, (Genesis Diagnostics Ltd, Cambridgeshire, UK). For quantification, first quantitative kit is developed by AESKULISA SARS-CoV-2 NP IgG (AESKU Diagnostics GmbH & Co., Wendelsheim, Germany), and CE marked. It was used for quantitative measurement of antibodies against SARS-CoV-2. It detects nucleo-capsid protein.

ELISA tests were performed as per suppliers' instructions. Briefly, controls and diluted patient plasma/serum were incubated into individual microplate wells. In a positive test, the specific IgG will bind to the recombinant antigen. Addition of enzyme linked conjugate (anti human IgG) bound the antigen-antibody complex. Chromogen/substrate solution was added in each well for catalyzing a colored reaction. Stop solution was added into each well to inhibit the enzymatic catalyzation. Photometric measurement of the color intensity was made after adding stop solution.

Lateral Flow Immuno-fluorescent Assay kit detects anti-SARS-CoV-2 IgG and IgM (Lateral flow immunoassay) (Shenzhen Lifotronic Technology Co., Ltd, Shenzhen, China). LFIA was used later when the kit became available. ECLIA positive and quantitative ELISA negative as well as both negative donors with LFIA method were tested. RNA extraction was done using dry swab RNA kit FavorPrep viral nucleic acid extraction kit-1 (Favorgen Biothech Corps, Ping-Tung, Taiwan). Amplification was carried out using manufacturer's instructions (Bosphore, Novel Coronavirus (2019-nCoV) detection kit v2, Anatolia geneworks, Istanbul, Turkey). The kit employed multiplex PCR targeting two regions i.e. orf1ab (acquired through FAM filter) and E gene (acquired through HEX filter). For amplification and acquisition of fluorescence Rotorgene –Q (Qaigen) was used.

Statistical analysis

Data was analyzed using SPSS version 21.0. Descriptive statistics including mean and SD were computed for continuous variables. Frequency and percentages were evaluated for categorical variables. Independent *t*-test was applied to identify the difference between the means in two unrelated groups and Chi-square test was used to test a relationship between categorical variables.

RESULTS

A total of 400 donors were enrolled for convalescent plasma donation. There were 304 (76%) males (male to female ratio was 3.1:1), mean age of donors was 36.4±11.3 years. 332 (83%) of them remained home quarantine for a mean duration of 17.7±6.4 days. Follow up COVID-19 RT-PCR was done on a mean of 16.3±6.4day after their first RT-PCR positive test. Viral RNA was not detected on this testing. Plasma collection was done on day 15±14.2 after last negative RT-PCR. Table 1 shows the demographic details of these donors. Of 400 donors, 70 (17.5%) had a travel history abroad as shown in F*igure 2*. Their laboratory investigations were within normal range as shown in Table 1. After all screening and baseline testing 200 patients were excluded and finally 200 donors were recruited for antibody analysis. All selected donors were negative for transfusion transmitted infections such as Hepatitis B, C, HIV, syphilis and malaria parasite.

At the time of donation, 376 out of 400 donors were negative for SARS-COV-2 by RT-PCR. However, out of remaining 12, RT-PCR detected the presence of viral RNA, they were all male. Two of these 12 were hospitalized for a week when COVID-19 was diagnosed while 10 of these 12 were home quarantined. None of these 12 had any associated comorbid. Nine of these 12 showed seroconversion i.e., presence of concomitant anti-SARS-CoV-2IgG antibodies. A total of 70 (17.5%) donors were deferred due to absence of anti-SARS-CoV-2 antibodies in 64 (16%) and detection of viral RNA in 3 (0.75%) without any evidence of seroconversion. In 60

seronegative cases, there were 52 males. Eight of these 60 needed hospitalization while 52 were quarantined at home.

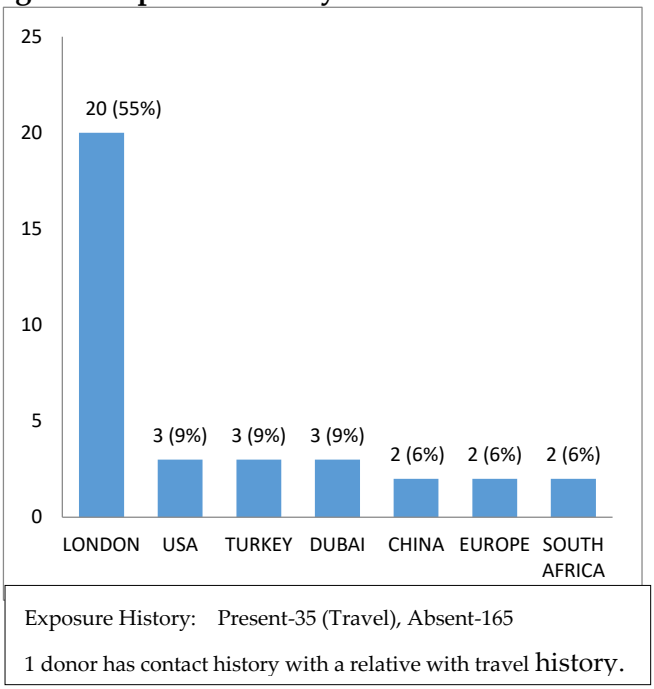
Table 1: Demography & Laboratory Parameters

DEMOGRAPHIC DATA n(%)	
Male Female	152 (76%) 48 (24%)
Low Medium High	8 (4%) 188 (94%) 4 (2%)
Age (years)	36.42 ± 11.34
HEMATOLOGICAL PARAMETERS (Mean±S.D)	
[1]Hemoglobin (g/dl)	14.0 ± 1.5
[2]Hematocrit (%)	40.3 ± 4.6
[3]Red blood cell count (x10^{12}/L)	4.9 ± 0.54
[4]White blood cell count (x10^{9}/L)	7.6 ± 1.5
[5]ALC* (x10^{9}/L)	2.6 ± 0.76
[6]ANC*(x10^{9}/L)	3.9 ± 1.1
[7]Reticulocyte Count (10^{9}/L)	56.2 ±32.3
[8]Platelets (10^{9}/L)	267 ±65.4
[9]IPF® (%)	6.0 ±3.2
BIOCHEMICAL PARAMETERS (Mean±S.D)	
[10]Albumin (g/dl)	4.5 ± 0.27
[11]Calcium (mg/dl)	9.7 ± 0.40
[12]Lactate Dehydrogenase (U/L)	167 ± 32.0
[13]Urea (mg/dl)	24.3 ± 8.3
[14]Creatinine (mg/dl)	0.87 ± 0.16
[15]Sodium (mEq/L)	138.8 ± 2.6
[16]Potassium (mEq/L)	4.1 ± 0.3
[17]Bicarbonate (mEq/L)	25.2 ± 2.52
[18]Chloride (mEq/L)	101.0 ±13.9
[19]Bilirubin Total (mg/dl)	0.56 ± 0.34
[20]Bilirubin Direct (mg/dl)	0.25 ± 0.63
[21]Alkaline Phosphatase (U/L)	47.06 ± 18.8
[22]SGPTº (U/L)	23.2 ± 9.1
[23]Random Blood Sugar (mg/dl)	103 ± 21.7
MOLECULAR ASSAY	
SARS-CoV-2 RT-PCR	Not Detected : 188
Anti-SARS-CoV-2 IMMUNOLOGICAL ASSAYS (Mean ± S.D) in COVID-19 recovered patients	

*Absolute Neutrophil counts, Absolute Lymphocyte counts,®Immature Platelet fractions,ºSerum glutamate pyruvate transaminase ¥Electrochemiluminesence ≠Enzyme Linked immunosorbent Assay **Reference ranges:**[1](M: 13.0-16.5,

[24] ECLIA¥	35.93 ± 31.4 (165/200) Sensitivity: 82.5% Specificity: 100%
[25]ELISA≠ Qualitative / semi-quantitative	142/165 (86%) Mean: >1:160 fold serum dilution. Range: >1:180 - >1:320. Sensitivity: 71% Specificity: 100%
[26]ELISA Quantitative	141/165 (85.4%) 59.74 ± 35.90 Sensitivity: 70.5% Specificity: 100%

Figure1: Exposure History



Exposure History: Present-35 (Travel), Absent-165

1 donor has contact history with a relative with travel history.

Table 2: Recurrence of SARS-COV-2 in published studies, worldwide

Studies	Diagnostic Tools	Covid-19 Recurrence
Chan D et al(13)	RT-PCR	1 patient
Zhang J Fen et al(14)	RT-PCR	1 patient
Huang P et al(15)	RT-PCR	1 patient
Xiao AT, Tong X, Zhang S et al(17)	RT-PCR	15/70patients
Jian M, Li Y, Han M et al(18)	RT-PCR	6/29 patients
Lan L, Xu D, Ye G et al(16)	RT-PCR	4 patients
Li et al(21)	RT-PCR	18/610 patients
Zhou L, Liu Q et al(22)	RT-PCR	14% patients
Current study	RT-PCR	12*

Mean value of anti-SARS-CoV-2 immunoassay by ECLIA was 35.93±31.4 (This is a qualitative test; significance of this numerical value is not known). Qualitative ELISA for IgG antibodies were positive in 284 out of 330 donors (86%). Mean IgG antibody titer was >1:160-fold serum dilution (range >1:80 - >1:320). Quantitative ELISA for IgG was detected in 282 donors (85.4%) with a mean 59.74 ± 35.90 (cut off: 8.0 U/mL) and range (9.98 - 100 U/mL). This means that further 46 donors did not develop IgG anti-SARS-CoV-2 antibodies. Subsequently, LFIA method was able to detect IgG antibodies in 20 of 48 (41.6%) seronegative donors and in 20 of 35 (58.8%) ECLIA positive ELISA negative donors.

Table 3: Comparison of COVID-19 seropositive and seronegative donors

	ELECYS RESULTS		
	Positive	Negative	*p* value
Age(Mean± SD)	36.43 ± 10.7	34.10 ± 12.3	0.266[a]

Sex			
Male (n)	126	26	0.660[b]
Female(n)	25	04	
Facility where admitted/Quarantine			
Hospital (n)	17	4	
Home(n)	134	26	0.746[b]
Days of Quarantine (Mean± SD)	16.05 ± 6.3	15.50 ± 4.4	0.236[a]
Comorbids			
Yes	13	3	0.806 [b]
No	138	27	

[a] = Independent *t*- test, [b]= Chi- square test

Table 4: Performance of LFIA technique in Seropositive and Seronegative COVID-19 Patients

ECLIA Positive & IgG Negative (n=17)	IgG +ve& IgM –ve (n=14)
	IgG -ve& IgM –ve (n=3)
ECLIA Positive & IgG Positive (n=4)	All 4 were IgG +ve and IgM -ve
ECLIA Negative & IgG Negative (n=24)	IgG -ve& IgM –ve (n=12)
	IgG +ve& IgM –ve (n=10)
	IgG -ve& IgM +ve (n=2)

SARS-CoV-2 Assay kit (Lateral flow immunoassay), Shenzhen Lifotronic Technology Co., Ltd, Shenzhen, China

DISCUSSION

Convalescent plasma donation is one of the experimental treatment options for COVID-19 and has been approved as a trial in a number of countries like United States of America and Britain. The therapeutic benefits of convalescent plasma were studied formally in animal models in early 20th century. In 1916, convalescent plasma from polio survivors was administered to poliomyelitis patients(27) to determine its efficacy followed by influenza(31)and measles(28, 29) and recently in SARS(34), MERS(35), and Ebola virus diseases(33).

In Pakistan, this trial for experimental use of COVID-19 convalescent plasma for the purpose of passive immunization was approved by national bioethics committee on April 4 and by Drug Regulatory Authority of Pakistan (DRAP) on April 9th, 2020 and the first donation was taken on April 15th.

Detection of COVID-19 RNA was found in a number of published studies, in which after RT-PCR negativity on 2 consecutive samples (Table 2), subsequent RT-PCR testing showed viral RNA again. Our study was designed to make sure that at the time of plasma donation, all the donors should not have any evidence of the presence of COVID-19 RNA and there should be documented evidence of the presence of anti-SARS-CoV-2 antibodies in their serum. Plasma donations were acceptable from the donors who have at least 2 negative PCR reports 24 to 48 hours apart almost 2 weeks prior to donation, as per FDA guidelines(6) .

Total antibodies are considered to be the most sensitive and earliest serological markers and increment in their levels start to appear after the first week of symptoms onset(8). Test using nucleocapsid antigens and receptor binding domain combined are the most sensitive(12). Seventy of our 400 donors did not show IgG antibodies after≥2 weeks of PCR negative results, which is in contrast to the findings in others studies which state that higher levels of IgG and IgM ELISA occur in the second and third week(9,10), and may persist for

2 years(11), however they can be positive as early as on fourth day after onset of symptoms. In contrast, according to CDC's (center for disease control) current guidelines, some patient's body's immune response may take longer time to develop immunity(23), resulting in a negative antibody result.

Another interesting finding was when subsequently, LFIA kit became available, seronegative donors' samples were analyzed who were either seronegative with ECLIA and ELISA kits or showed a positive reaction to ECLIA but no evidence of IgG on ELISA, a good number of them detected presence of IgG in those sera. This may be due to different sensitivity of these kits, a false positive/negative result or their differing target antigens in the testing system.

In our study, viral RNA for SARS-COV-2 was detected in 12 donors. Out of these, 9 had concomitant quantitative IgG antibodies (ELISA) for SARS-CoV-2 and 3 showed negative antibody results. According to a study RT-PCR has been detected even beyond week 6 following the first positive test(7).

Our results showed a lower number of seroconversions (82.5%) as compared to Chinese studies. If we add up LFIA detected IgG results in the initial cohort, then IgG positivity has risen to 89%. This may be due to the fact that most of our donors (89%) had milder disease and did not require hospitalization. In contrast, Chen D *et al(*13), Zhang J Fen *et al(*14), Huang P *et al(*15) reported recurrence of RT-PCR positivity on oropharyngeal swab specimen in one patient each, after 2 consecutive negative PCR results. Another study reported 4 patients with COVID-19, who met criteria for hospital discharge or discontinuation of quarantine, to be positive on RT-PCR, 5 to 13 days later(16). Other studies, however reported a higher trend of recurrence, as 21.4% (15/70) and 20% (6/29) by Xiao AT (17)and Jiang M (18), respectively.

All these studies suggested that initial negative results may be due to various reasons such as variable viral load, sample site, technical expertise, effect of antiviral drugs, hormonal therapy taken, sensitivity of nucleic acid detection kit, false negative results or prolonged nucleic acid conversion. Given the chance of recurrent positive SARS-CoV-2 RNA in the clinical course and to minimize the risk of spread in other COVID-19 cases, together they suggested that different specimen types to be analyzed at a time, such as oropharyngeal/nasopharyngeal, etc, larger samples to be taken, more than one method like serology testing should be considered combined with RNA testing. The patients in recovery phase should also be regularly tested for assessment of infectivity, all discharged patients should be ensured for at least 14 days home quarantine and RT-PCR test results of pharyngeal swab specimens should not be considered as the only one indicator for diagnosis, treatment, isolation, recovery or discharge and transferring for hospitalized patients. RT- PCR detection of viral traces cannot always be correlated with the ability of transmission (13,14,18,21,22).

In a report on 9 patients, viral isolation attempts in culture were un-successful beyond day 8 of onset of illness , which points towards decline of infectivity beyond the first week(24). That is why the "symptom-based strategy" of the Centers for Disease Control and Prevention (CDC) indicates that health care workers can return to work, if "at least 3 days (72 hours) have passed since recovery defined as resolution of fever without the use of fever-reducing medications and improvement in respiratory symptoms (e.g., cough, shortness of breath); and, at least 10 days have passed since symptoms first appeared".(25)

This is a large scale study with concomitant antibody testing with RT-PCR, use of 2 different assays for the detection of anti-SARS-CoV-2 antibodies, selection of patients at least 14 days after last PCR negative or 28 days from start of symptoms.

However, use of single nasopharyngeal/oropharyngeal swab sample only in each patient, current RT-PCR techniques for SARS-COV-2 detection has almost 25-30% chance of false negative results were the limitations of the study. Inability to check for neutralizing antibodies at the time of this paper submission and a possible patient selection bias cannot be completed excluded, as all the donors were healthy volunteers recovered from COVID-19.

CONCLUSION

We conclude that 29% recovered COVID-19 patients did not show IgG humoral immune response at least 2 weeks after negative RT-PCR result in our cohort. Although, additional testing with LFIA kit reduced it to

11% only. However, a majority of them had concomitant IgG antibodies and only 0.75% had isolated PCR positivity in asymptomatic recovered patients from COVID-19 donors. Current serological diagnostic kits have limitations as they are first generation kits. COVID-19 is a novel disease and scientific data is adding up on daily basis. Better kits are needed to accurately diagnose seroconversion status for COVID-19 in general population.

Conflict of Interest statement

All the authors declared no conflict of interest.

Trial Registration: DRAP Registration No: F.NO.17-8/2020 DD (PS), NBC Registration No: NBC-472 COVID19-03, NIH ID: NCT04352751

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