Original Research Article

EVALUATION OF C-REACTIVE PROTEIN LEVELS IN THE SALIVA OF COVID RECOVERED PATIENTS

Running title: Salivary C-reactive protein level COVID recovered patients

Abstract:

Introduction

The acute phase reactant synthesized by the liver. CRP is an annular (ring- shaped) metameric protein set up in plasma, whose circulating immersion rise in response to inflammation. The idea of the study is to estimate the C-reactive protein situations in the salivary samples of COVID- 19 recovered cases and healthy controls.

Materials and methods

An experimental study on salivary samples of COVID recovered cases. The study was non-invasive and easy to perform without important vexation to cases. The samples were acquired from cases who came to the clinics of Saveetha Dental College and Hospitals. An aggregate of 20 saliva samples was collected from recruited cases 10 of whom were healthy controls and 10 were collected from cases who had made complete recovery from COVID infection.

Results

C-reactive protein (CRP) could be generally used as a biomarker of systemic inflammation, routinely measured in serum blood samples. Still salivary samples offer a non-invasive and simply accessible preference which might upgrade point of care (POC) testing for inflammation. This study illustrates the group of healthy controls and COVID recovered cases.

Conclusion:

Within the limitations of our study, we were capable to interpret the difference of CRP levels between COVID recovered cases and healthy individualities.

Keywords: C-reactive protein; COVID recovered cases; healthy individualities; innovative technique.

Introduction:

C-reactive protein (CRP) is an acute phase reactant synthesized by the liver. CRP is an annular (ring-shaped) pentameric protein found in plasma, whose circulating concentrations rise in response to inflammation (1). The function of CRP is to be associated with the role within the innate system. CRP levels within the blood increase when there's a condition causing inflammation within the body(2).

CRP is a test which is useful in medicine, reflecting the presence and intensity of inflammation, although an elevation in C-reactive protein isn't the telltale diagnostic sign of anybody's condition(3). A CRP test measures the quantity of CRP within the blood to detect inflammation in acute conditions or to observe the severity of disease in chronic conditions(4). Since inflammation is believed to play a significant role within the development of coronary artery disease, markers of inflammation are tested in relevance to heart health(5). CRP is also accustomed to stratify risk for coronary disease additionally to traditional factors like high vital sign or elevated cholesterol(5,6). Also, recently it has been found that a significant increase of C-reactive protein was found with levels on the average 20 to 50 mg/L in patients with COVID-19.

Elevated levels of CRP were observed up to 86% in severe COVID recovered patients(7). The symptoms of elevated CRP levels include unexplained exhaustion, pain, muscle stiffness, soreness and weakness, low-grade fever, chills, headache, nausea, loss of appetite and indigestion, difficulty sleeping or insomnia and unexplained weight loss(7,8).

Recent studies have demonstrated the association between oral health status and systemic diseases, including systemic infections, disorder, pregnancy outcomes and respiratory diseases. Moreover, the impact of excellent oral care on risk reduction of viral acute respiratory diseases has been reported in numerous studies(9)(10) This study is a reflection of data in the Indian population when compared to the various other studies which are done in China.

This research is needed to know the C-reactive protein biomarkers are helpful to diagnosis the inflammation and healing process. The main deficiency it fulfill that other study was mostly done China population but this research is going to be done in Indian population .Our team has extensive knowledge and research experience that has translate into high quality publications (11).(12–25) ,(26–30) .The aim of the study is to evaluate the C-reactive protein levels in the saliva of COVID-recovered patients

MATERIALS AND METHODS

Study design and setting:

An observational study on saliva samples of COVID recovered patients. The study was non-invasive and easy to perform without much inconvenience to patients. However, the sample size was limited. Before the initiation of the study, clearance was obtained by the Scientific Review Board with Ethical approval number IHEC/SDC/UG-1999/21/213. The samples were obtained from patients who came to the clinics of Saveetha Dental College and Hospitals. The number of samples collected was 20 in which 10 patients of whom were healthy controls and 10 were collected from patients who had made complete recovery from covid infection at least three months ago. The samples were collected in an unbiased manner using randomized sampling. Validation was done by an expert pathologist.

Patients Selection and Recruitment:

The samples were recruited from the COVID recovered patients. Clinical history was taken from COVID recovered patients in this study. It was also ensured that patients with systemic comorbidities or terminally ill patients were not included for the study. All the patients included in the study belonged to the same ethnic group of Tamil Nadu. Informed consent was obtained from the patients for inclusion in the study and it was also ensured that the patients anonymity was maintained. All the patients completed a questionnaire covering medical, residential, and occupational history.

Variables:

Dependent variables were C-reactive protein levels whereas independent variable was age and sex of the patients. C-reactive protein and age were expressed as pg/ml and years, respectively.

Sample collection:

A total of 20 saliva samples were collected from recruited patients 10 of whom were healthy controls and 10 were collected from patients who had made complete recovery from covid infection at least three months ago. Unstimulated saliva from the patients was collected according to the protocol. Participants were initially asked to rinse their mouth with tap water

prior to sampling, followed by collection of at least 5ml saliva from the mouth floor, deposited for 30 seconds and were stored in a sterile Eppendorf tube at -20°C.

Estimation of C-reactive protein:

Enzyme Linked Immunosorbent Assay was based on the competitive binding technique in which the C-reactive protein level present in the sample competes with a fixed amount of horseradish peroxide (HRP) - labeled C-reactive protein on a human monoclonal antibody. Standards and samples are pipetted into the wells and salivary present in a sample are bound to the wells by the immobilized antibody. The wells were washed and a biotinylated anti-human salivary C-reactive protein antibody was added. After washing away the unbound biotinylated antibody, Horseradish Peroxidase (HRP) conjugated streptavidin is pipetted to the wells. The wells were washed again, a Tetramethylbenzidine (TMB) substrate solution was added to the wells and color developed in proportion to the amount of salivary bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Reagent Preparation:

All reagents and samples were brought to room temperature (18-25°C) before use. Also, Assay Diluent B (Item E) should be diluted to 5-fold with deionized or distilled water before use. For dilution of sample Assay Diluent, a (Item D) should be used for dilution of serum and plasma samples. The suggested dilution for normal serum/plasma is 2 - 20 fold. For the preparation of the standard, a vial of Item C was briefly spun. 400 µL of Assay Diluent A (for serum/ plasma samples) was added into Item C vial to prepare 50ng/ml standard. The powder was dissolved thoroughly by a gentle mix. 15 µL C-reactive protein standard (50 ng/ml) was added from the vial of Item C, into a tube with 485 µL Assay Diluent A or 1X Assay Diluent B to prepare a 1,500 pg/ml standard solution. 400 µL Assay Diluent A or 1X Assay Diluent B was pipetted into

each tube. 1,500 pg/ml standard solution was used to produce a dilution series (shown below). Each tube was mixed thoroughly before the next transfer. Assay Diluent A or 1X Assay Diluent B served as the zero standards (0 pg/ml). If the Wash Concentrate (20X) (Item B) contained visible crystals, it was warmed to room temperature and mixed gently until they dissolved. 20 ml of Wash Buffer Concentrate was diluted into deionized or distilled water to yield 400 ml of 1X Wash Buffer. Detection Antibody vial (Item F) was briefly spun before use. 100 µL of 1X Assay Diluent B (Item E) was added into the vial to prepare a detection antibody concentrate. This was then pipetted up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The

detection antibody concentrate should be diluted 80-fold with 1X Assay Diluent B (Item E) and used in relevant prior steps. The HRP-Streptavidin concentrate vial (Item G) was briefly spun and pipetted up and down to mix gently before use, as precipitates may form during storage. HRP- Streptavidin concentrate should be diluted 200-fold with 1X Assay Diluent B (Item E).

Assay Procedure:

All reagents and samples were brought to room temperature (18-25°C) before use. Samples were running in duplicate. Removable 8-well strips were labeled as appropriate for the experiment. 100 µL of each standard and sample was added into appropriate wells. These wells were then covered and incubated for 2.5 hours at room temperature with gentle shaking. The solution was discarded and washed 4 times with 1X wash solution. Each well was filled and washed with Wash Buffer (300 µl) using a Pipette. Complete removal of the liquid at each step is essential for good performance. After the last wash, any remaining wash buffer was removed by aspirating or decanting. The plate was inverted and blotted with clean paper towels. 100 µl of 1x prepared biotinylated antibody was added to each well. This was then incubated for 1 hour with gentle shaking. The solution was discarded and the wash was repeated. 100 µL of prepared Streptavidin solution was added to each well. This was then incubated for 45 minutes at room temperature with gentle shaking. The solution was discarded and the wash repeated. 100 µL of TMB One-Step Substrate Reagent (Item H) was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. 50 µl of Stop Solution (Item I) was added to each well and read at 450 nm immediately. The mean absorbance was calculated for each set of

duplicate standards, controls, and samples, and the average zero standard optical density was subtracted. The standard curve was plotted using Sigma plot software, with standard concentration on the x-axis and absorbance on the y- axis. The best-fit straight line was drawn through the standard points. The minimum detectable dose of Human CRP was determined to be 3pg/ml. The minimum detectable dose is defined as the analytic concentration resulting in an absorbance that is 2 standard deviations higher than that of the blank (diluents buffer).

Statistical analysis:

In this study two statistical tests have been carried out, one is Student's t-test analysis to evaluate the C-reactive protein levels in COVID recovered patients. Statistical tests were performed using Statistical Package for the Social Sciences (IBM SPSS statistics for windows version 23.0, Armonk, NY: IBM Corp. Released 2015). Values were expressed as Mean and SD. The post COVID recovered patients (3 months) under age group of 18-23 years (6 males and 4 females)

were included in the study. The patients under medication and other systemic diseases are excluded from the study**.**

Results:

C-reactive protein (CRP) could be commonly used as a biomarker of systemic inflammation, routinely measured in serum blood samples. However salivary samples offer a non-invasive and simply accessible alternative which might improve point of care (POC) testing for inflammation. This study illustrates the group of healthy controls and COVID recovered patients. The group are expressed in pg/ml in which Mean±SD value for healthy controls is 34.40±13.87 and value for COVID-recovered patients is 48.60±9.913. P-value for healthy controls and COVID recovered patients is 0.0105(Table 1).This study also illustrates the assessment of Salivary C - reactive protein levels in healthy controls and covid-19 recovered patients. Each bar represents the Mean±S.D of 20 samples of which 10 are healthy controls and 10 are COVID recovered patients. The CRP levels were measured by sandwich ELISA and levels are expressed in pg/ml.The P-value is 0.0105 and significance was considered at the levels of $p<0.05$. *: Compared with healthy controls (Figure 1).

Table 1: Depicts the association between healthy controls and COVID -19 recovered patients with the P-value of 0.0105 which is statistically significant.

Groups	Mean±SD	P value
Healthy controls (pg/ml)	34.40±13.87	0.0105
Covid-19 Recovered patients (pg/ml)	48.60±9.913*	

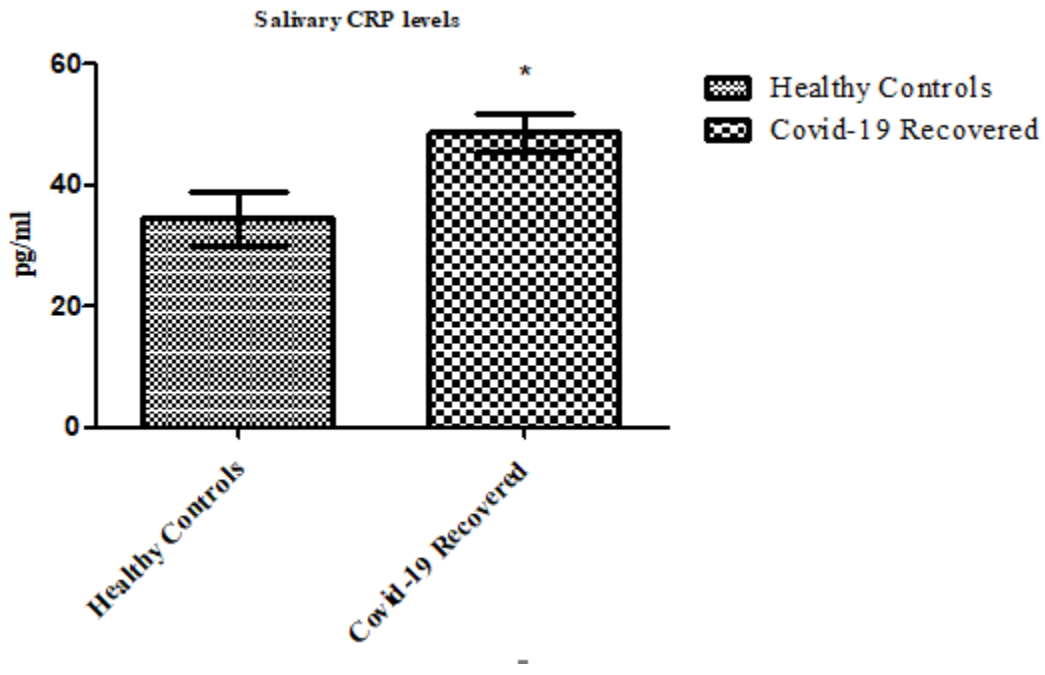

Figure 1: The above error bar graph illustrates the assessment of Salivary C - reactive protein levels in healthy controls and covid-19 recovered patients. Each bar represents the Mean±S.D of 20 samples from each group (n=20). The CRP levels were measured by sandwich ELISA and

levels are expressed in pg/ml. The significance was considered at the levels of $p<0.05$. *: Compared with healthy controls.

Discussion:

In our study we have found that C- reactive protein is increased for COVID recovered patients when compared with the healthy individuals. We have collected 10 samples from COVID recovered patients who have recovered at least 3 months before the initiation of the study. The patients were affected by COVID and they have been home quarantined and recovered uneventfully 3 months ago. The salivary sample is collected in the month of August and it has found that C-reactive protein level is high when compared to healthy controls.

In a study by Sultana et al they confirmed the association of two main, inflammatory and biochemical covariates with COVID-19 severity for the primary time in Bangladeshi patients. Their study can help us to thoroughly understand the complications caused and predict the progression of the disease with way more confidence by studying CRP level in blood(31). Assessment of the severity of COVID recovered patients has been somewhat unclear, but guidelines from different disease centers, just like the Centre for Disease Control and prevention (CDC), World Health organization (WHO), National Health Service (NHS) and National Institute for Health and Care Excellence (NICE), used an equivalent criteria to classify and assess COVID-19 severity in saliva levels of high-Sensitivity C-reactive Protein in Acute Myocardial Infarction(32,33). However, all available scores which classified pneumonia or COVID-19 severity were obsessed on face-to-face consultations and examination, which weren't applicable within the COVID era and a few examination tools have also been prohibited which compares the analysis of C-Reactive protein levels in the saliva and Serum of dogs with various diseases(34). Some trials showed good outcomes in assessing patients using phone calls, video calls or filling hospital forms. As we compare with the previous study, therefore we came to the conclusion that dental health may have an effect on the severity of COVID-19 sickness in childhood trauma which increases the C-reactive protein(34,35). Furthermore, poor dental health

was linked to higher CRP levels during the primary week of sickness, indicating a significant disease status.

Liver secretes CRP which elaborates the wide range of inflammatory cytokines. Levels of CRP increase very rapidly in response to trauma, inflammation, and infection and reduce even as rapidly with the resolution of the condition(9,10). Thus, the measurement of CRP is widely accustomed to monitor various inflammatory states(36). Fernandez R et al., in their study aimed to investigate the effect of C-reactive protein levels on the severity of COVID illness in recovered patients as well as previous access to health data through a nationwide database of results through corresponding participant health records(36,37).

As we compare with the study done by Zonca V et al, which demonstrates the serum CRP and ESR levels in the cases compared to controls and found that there is statistically significant increase in the levels of CRP and ESR in the cases when compared to the controls. Hence it may be concluded that inflammation is a crucial risk factor of endothelial damage and atherosclerosis. Measures to pull back the inflammation of endothelium and atherosclerotic plaque of blood vessels, in the long-term can significantly reduce the incidence of stroke and its consequences in predisposed individuals (35). In our study, we found that C-reactive protein level is increased.

Limitations of this study include a limited sample size and short duration of study. However, they can be extrapolated to arrive at a scientific understanding of the interrelationship between the Salivary C-reactive protein levels of the COVID recovered patients. To improvise further, a cross sectional nature of this study can be done using D-dimer levels in the blood and the consequent missing clinical data can be worked upon. Also, a larger sample size would be used to obtain more appropriate salivary findings.

CONCLUSION:

Within the limitations of our study, we were able to elucidate the difference of CRP levels between COVID recovered patients and healthy individuals. The difference was statistically

significant proving that in spite of complete uneventful recovery from COVID infection the individual's inflammatory markers are seen to be on rise.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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