Evaluation of Salivary Interleukin-1 Beta in Covid Recovered Patients

ABSTRACT

Introduction: The COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The epidemic started in Wuhan in mid-December 2019 and quickly spread across the world as a pandemic. Saliva is emerging as a promising alternative to nasopharyngeal/oropharyngeal swabs for COVID-19 diagnosis and monitoring.

Aim: To evaluate the Interleukin-1 Beta in the salivary samples of COVID-recovered patients and healthy controls.

Materials and Methods: An observational study on saliva samples of COVID recovered patients. The study was non-invasive and easy to perform without much inconvenience to patients. The samples were obtained from patients who came to the clinics of Saveetha Dental College and Hospitals. 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 Student T test 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. An observational study on saliva samples of COVID recovered patients.The salivary Interleukin-1 levels were analyzed using ELISA.

Results: Within the limitations of the study, we conclude that salivary Interleukin-1 level is increased in COVID recovered patients. 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 at our rise.

Conclusion: Salivary Interleukin-1 levels are increased in COVID recovered patients. Further prospective studies with the limited sample size of the salivary levels of IL-1 can effectively assess disease severity and predict outcome in patients with COVID-19.This study illustrates the group of healthy controls and COVID recovered patients.

1. INTRODUCTION

The COVID-19 is caused by illness ranging from the common cold to more severe diseases such as the Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS). In humans, coronaviruses cause respiratory tract infections that can range from mild to lethal. A novel coronavirus (COVID-19) was identified in 2019 in Wuhan, China [1]. The most common symptoms of COVID-19 are fever, dry cough and tiredness. Other symptoms that are less common and may affect some patients include loss of taste or smell, aches and pains, headache, sore throat, nasal congestion, red eyes, diarrhea or skin rashes. The infection caused by this novel virus was named coronavirus disease 2019 (COVID-19) by the World Health Organization (WHO) [2]. The fast spread of this disease is related to its highly infectious nature and the disease is suggested to be transmitted through saliva droplets and nasal discharge [3]. Interleukin-1 (IL-1) is one among the family of biologically active small protein molecules known as cytokines. Interleukin (IL) are a type of cytokine first thought to be expressed by leukocytes alone but have later been found to be produced by many other body cells [4]. They play essential roles in the activation and differentiation of immune cells as well as proliferation, maturation, migration and

adhesion. IL-1 has been implicated in the progression of several viral infections [5].

Saliva is a biological fluid in which SARS-CoV-2 can be found and for this reason saliva has been taken into consideration in the diagnosis of COVID-19. Saliva-based testing can be an alternative to the more widely used nasopharyngeal swabs for COVID-19 diagnosis and disease monitoring [6]. Collection of saliva can be done in several ways. The spitting out technique is the cheapest one and the saliva sample thus collected also includes nasopharyngeal/airway secretions. Salivary biomarkers associated with the development and progression of COVID-19 can allow a better distinction between asymptomatic, mild, moderate or advanced status of the disease [7]. Diagnosis of saliva-based viral infections depends on the presence of viral DNA, RNA, microRNA, antigens or host antibodies in saliva. The use of saliva-based SARS-CoV-2 testing offers several clinical advantages and is scientifically well-founded [8].

Interleukin-1 (IL-1) is the prototypic pro-inflammatory cytokine. There are two forms of IL-1, IL-1alpha and IL-1beta and in most studies, their biological activities are indistinguishable. IL-1 affects nearly every cell type, often in concert with another pro-inflammatory cytokine, tumor necrosis factor (TNF) [9].IL-1β is a pro-inflammatory cytokine that not only mediates immune responses during infection and inflammation, but also has a role in acute and chronic auto inflammatory diseases. Cytokines are vital in regulating immunological and inflammatory responses. Among them, IL-1 is the most important type. IL-1 levels are closely linked to the severity of COVID-19 infection [10]. An increase in IL-1 levels signifies respiratory dysfunction and cytokine-mediated lung damage caused by COVID-19 infection. This study is needed as IL-1 levels may be significant in identifying disease progression among COVID-19-infected patients and IL-1 has been proven to be a good biomarker [11]. The aim of the study is to evaluate the variation in the salivary Interleukin-1 levels among the COVID recovered patients and healthy controls.

2. MATERIALS AND METHODS

2.1 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. The samples were obtained from patients who came to the clinics of Saveetha Dental College and Hospitals. 20 samples were collected in an unbiased manner using randomized sampling. Validation was done by an expert pathologist.

2.2 Patients Selection and Recruitment

The samples were recruited from the COVID recovered patients. Clinical history was collected from COVID recovered patients. 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.

2.3 Variables

Dependent variable was salivary Interleukin-1 level whereas independent variables were age and sex of the patient. Salivary Interleukin-1 and age were expressed as pg/ml and years respectively.

2.4 Sample Collection

A total of 20 saliva samples were collected from selected patients , ten were healthy controls and another ten were completely recovered from COVID 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.

2.5 Estimation of Salivary Interleukin-1

Enzyme Linked ImmunoSorbent Assay was based on the competitive binding technique in which the Interleukin-1 present in the sample competes with a fixed amount of horseradish peroxide (HRP) - labeled salivary Interleukin-1 on a human monoclonal antibody. Standards and samples are pipetted into the wells and saliva present in a sample binds to the wells by the immobilized antibody. The wells were washed and a biotinylated anti-human salivary Interleukin-1 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 Interleukin-1. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

2.6 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 Interleukin- 1 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).

2.7 Assay Procedure

All reagents and samples were brought to room temperature (18-25°C) before use. samples were run 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 Interleukin-1 was determined to be 3pg/ml. The minimum detectable dose is defined as the analyte concentration resulting in an absorbance that is 2 standard deviations higher than that of the blank (diluents buffer).

2.8 Statistical Analysis

In this study two statistical tests have been carried out, one is Student's t-test analysis to evaluate the Salivary levels of Interleukin-1 beta 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 the 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.

3. CLINICAL PRESENTATIONS OF THE PATIENT

Clinical Presentations of COVID-19 range from asymptomatic/ mild symptoms to severe illness and mortality. Common symptoms include fever, cough, and shortness of breath. In our present study, the patients show symptoms like cough, myalgias and headache are the most commonly reported symptoms.The majority of patients presenting with coronavirus disease (COVID-19) experience a mild illness that can usually be managed in the community. Patients require careful monitoring and early referral to hospital if any signs of clinical deterioration occur. Around 80% of COVID-19 infections present as a mild respiratory illness in a patient who is ambulatory and can generally be managed outside the hospital. Around 15% typically need hospital care (usually for moderate to severe pneumonia), and another 5% have critical illness requiring more intensive support.

4. RESULTS

Salivary Interleukin-1 beta 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 38.60±7.106 and value for COVID-recovered patients is 31.20±11.50. P-value for healthy controls and COVID recovered patients is 0.118(Table 1).This study also illustrates the assessment of Salivary Interleukin-1 beta 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 Salivary Interleukin-1 beta levels were measured by sandwich ELISA and levels are expressed in pg/ml.The P-value is 0.118 The significance was considered at the levels of $p<0.05$. No statistical significance among healthy and covid-19 recovered patients (Fig. 1).

Table 1. The tabular column illustrates the group of healthy controls

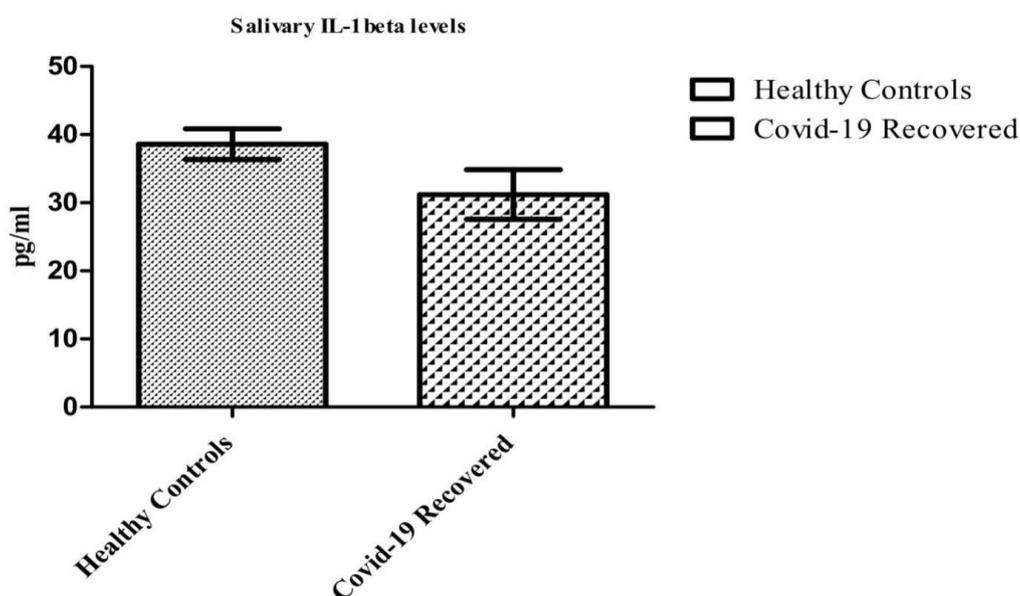| Groups	Mean±SD	P value
Healthy controls (pg/ml)	38.60±7.106	0.118 NS*
Covid-19 Recovered patients (pg/ml)	31.20±11.50	

Fig.1. The bar diagram shows that assessment of Salivary IL-1β 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 IL-1β levels were measured by sandwich ELISA and levels are

expressed in pg/ml. The significance was considered at the levels of p<0.05. No statistical significance among healthy and covid-19 recovered patients

5. DISCUSSION

In our study we have found that Interleukin-1 is increased for COVID-19 recovered patients when compared with the healthy individuals.We have collected 10 samples from patients who were affected by COVID-19 who have been home quarantined and recovered uneventfully at least 3 months prior to the study and 10 samples were collected from the healthy individuals The salivary sample is collected in the month of August. It has found that Interleukin-1 levels are high.

The cytokine interleukin-1 (IL-1) is a key mediator of the inflammatory response. IL-1 is produced mainly by one type of white blood cell, the macrophage, and another type, the lymphocyte, to fight infections. Anakinra (Kineret) is an IL-1 receptor antagonist that blocks the biologic activity of IL-1. IL-1 receptor antagonist anakinra and remdesivir can be used in the treatment of severe COVID-19 cases. The role of IL-1 in the cytokine storm of anakinra as a potential therapeutic in severe coronavirus infection is established [12].The salivary levels of IL-1 are significantly elevated in the setting of severe COVID-19 disease.IL-1 is a multifunctional cytokine that transmits cell signaling and regulates immune cells. This factor has a strong proinflammatory effect with multiple biological functions and plays an important role in inflammation, tumorigenesis and haematological diseases.IL-1 response may suggest that part of the pathogenesis of complicated disease involves a dysregulated and excessive host inflammatory response [13]. IL-1 is the primary trigger for cytokine storms pointing out that peripheral blood IL-1 levels could be used as an independent factor to predict the progression of COVID-19 [14] which is consistent with the results of this study. Therefore, the role of IL-1 in this disease deserves special attention. IL-1 levels are significantly elevated in the setting of complicated COVID-19 disease and increased IL-1 levels to be in turn significantly associated with adverse clinical outcomes [15].

Studies demonstrate that salivary levels of IL-1 are significantly elevated in the setting of severe Covid-19 disease [16]. It is increasingly recognised that a dysregulated host immune response to foreign infectious pathogens is integral to the development of target organ dysfunction and a major contributor to morbidity and mortality [17]. Another study suggests that IL-1 is a pro-inflammatory mediator, inducing both its own production and the synthesis of several secondary inflammatory mediators [18]. Increased IL-1 released in viral infections results in lung and tissue inflammation and fever.The salivary levels of IL-1 can effectively assess disease severity and predict outcome in patients with COVID-19 [18].The inflammatory response plays a critical role in COVID-19 and inflammatory cytokine storm increases the severity of COVID-19. From previous study [19], we understand that IL1-beta, thus produced, results in the inflammation of the lungs, fever and fibrosis to lead to respiratory complications in the infected host. The present study is concordant with other study which says that IL-1 levels could be used as an independent factor to predict the progression of COVID-19 [20].Therefore, the evaluation of salivary levels of IL-1 have a great significance in assessing the severity of COVID-19 and can be used as an independent factor to predict disease risk.

In our study, we found that Interleukin-1 level is increased in Covid recovered patients. Limitations of this study is a limited sample size. However, this concept may be extrapolated to arrive at a scientific understanding of the significance of Salivary Interleukin-1 levels in the COVID recovered patients. In the future, a larger sample size would be used to obtain improved results. Our team has extensive knowledge and research experience that has translate into high quality publications [21].[22–35] ,[36–40].From the results, we can conclude that the amount of salivary Interleukin-1 of COVID-19 patients to be further studied and need to understood better and explore. We have observed a statistically significant increase in the salivary levels of Interleukin-1 in COVID 19 recovered patients.This study has a limited sample size and the duration of time is less.In future, studies can be done with more sample sizes and the biomarker of Interleukin-6 of salivary and serum levels COVID-19 recovered patients can be included.

6. CONCLUSION

Within the limitations of our study, we were able to elucidate the difference of Interleukin-1 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 at our rise.

DISCLAIMER

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.

CONSENT

Informed consent was obtained from the patients.

ETHICAL APPROVAL

Before the initiation of the study, clearance was obtained by the Scientific Review Board with Ethical approval number IHEC/SDC/BDS/1990/01.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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