# Spotting and characterization of biomarkers in serum samples of COVID-19 patients: study protocol of case-control study.

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**ABSTRACT**

*Background: The COVID-19 disease is a viral infection that is now turning into a health threat all over the globe, caused by newly discovered Coronavirus 2, which is several acute respiratory syndrome coronaviruses 2 (SARS-Cov 2). Identification of potential markers in COVID-19 infection will be the key to understanding the complicated pathogenesis of the infection by using advanced proteomics techniques. As this study is based on hospitalized patients the role of the relationship between the anti and pro-inflammatory cytokines can also play a major role in the disease prognostic. Thus, by targeting IL-6 and IL-10 levels throughout the infection and focusing on the molecular approach, we might find a way to balance the level of anti and pro-inflammatory cytokines and further, the in silicon molecular docking for potential drug development with IL-6 and IL-10 will also help in to prevent the severity of the COVID-19 infection.*
*Objective: To identify potential biomarkers in serum samples of COVID-19 patients admitted to COVID-19 dedicated hospital and to check the expression and negative association of inflammatory factors IL-6 and IL-10 in COVID-19 infection.*
*Methodology: The study type is a case-control study. This study aims to identify and characterize potential biomarkers in serum samples of COVID-19 patients for use in the prognosis, pathogenesis, and the treatment of the infection by highly advanced proteomics techniques like mass spectrometric analysis and polyacrylamide gel electrophoresis. To detect the negative association of IL-6 and IL-10 in different stages of the infection by serology technique ELISA and to further validate the potential drug target for COVID-19 disease by in silico molecular docking.*
*Expected results: This study expects to detect the unique biological changes in severe patients of SARS-Cov-2 infection in the form of biomarkers. These molecular changes can provide information on COVID-19 disease prognosis. Even targeting the anti and pro-inflammatory factors can prevent the progression of the SARS-CoV-2 infection.*
*Discussion: The overall conclusion of this study is to identify biomarkers for COVID-19 infection. New knowledge generated through proteomics techniques with references to novel biomarkers for COVID-19 infection can be useful for the four arms prognosis, diagnosis, pathogenesis, and treatment. IL-10, an anti-inflammatory cytokine, has the potential to be used as a drug molecule in the treatment of COVID-19 infection.*

**Keyword**: *SARS-CoV-2, Inflammation, Pro and anti cytokines, COVID-19 biomarkers.*

## 1. INTRODUCTION:

The COVID-19 disease is caused by newly discovered several acute respiratory syndrome coronaviruses 2 (SARS-Cov 2). The World Health Organization (WHO) declared a global pandemic as an unusual case of pneumonia type infection was found in Wuhan, China in 2019 which was later identified as a novel coronavirus (SARS-Cov 2) infection, and the disease was named COVID-19 [2]. SARAS-Cov 2 is an enveloped positive-sense single-stranded RNA virus with a diameter of about 80 nanometers and a length of about 30,000 bases. Coronavirus has four major

structural proteins: crown-like spike (S-) glycoprotein (150-200kDa), membrane (M-) glycol protein (25-30kDa), envelops (E-) protein (8-10kDa) on the viral surface, and nucleocapsid (N-) protein (45kDa) [10]. Like all the other virus, SARAS-CoV-2 invade the host cell through the interaction between the spike protein (S-) protein on the viral particle surface and a receptor protein on the membrane of the host cell. The spike (S-) protein binds to the ACE2 (angiotensin-converting enzyme 2) receptor, a protein found on the surface of the host cell. ACE2 is normally involved in blood pressure regulation. As the coronavirus binds to it, chemical modifications occur that essentially fuse the membranes surrounding the cell and the virus, allowing the virus's RNA to penetrate the cell. The virus then uses the host cell's protein-making machinery to replicate itself [4]. As the disease progresses, the patient, depending on the type of infection, exhibits mild, severe, critical, and asymptomatic symptoms including fever, dry cough, loss of taste and smell, and tiredness. Serious symptoms include difficulty in breathing, chest pain, loss of speech or moments, and pneumonia. Asymptomatic patients contribute to disease transmission. The first case of COVID-19 was reported on 31$^{st}$ December 2019 in Wuhan, China till then over 53.4Cr people have been affected by COVID-19 infection worldwide and 4.32Cr people in India.

In the context of COVID-19, proteomics can help to reveal novel biomarkers and define point-of-care procedures that could be cost-effective. Proteomics profiling of plasma from COVID-19 patients has the potential to reveal several new proteins and biomarkers to study and analyze COVID-19 infection. Many studies are conducted to this date that supports the identification of biomarkers and possible therapeutic targets to help clinical decision-making and, as a result, enhance COVID-19 prognosis. These countries have different demographic and economic profiles when compare to India, proteomics situation in India is yet to be studied widely. The second wave, which begins in March 2021 in India, is even more destructive, with an increase in the number of cases and mortality rate. As a result, the identification of crucial biomarkers linked to COVID-19 patients with an Indian profile should be a top priority for diagnostics, treatment, and prognosis of COVID-19 disease.

The role of cytokines in COVID-19 is related to a series of physiopathological mechanisms that mobilized a variety of biomolecules, in the most severe cases, the prognosis can be marked worsened by the hyper production of pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, INF-Y, TNF-α [14]. The immunology reaction caused in SARS-CoV-2 infection mostly by the pro-inflammatory factor changes is correlated with disease severity. The detection and control of pro-inflammatory response are crucial in the early stages of SARS-Cov 2 viral infection. Interleukin 6 (IL-6) is one of the key cytokines which controls the immune response. IL-6 is a pro-inflammatory cytokine that contributes to the severity of the disease's progression, while IL-10 is an anti-inflammatory cytokine that inhibits the host's response to viral infection [5]. Moreover, recent studies have shown that higher levels of IL-6 and also interleukin 10 (IL-10) were more significant than other cytokines in COVID-19 patients experiencing cytokine storm, which is an interesting issue in COVID-19 patients [5]. The reasons for the increase in inflammatory cytokines are not clear, but they could play a crucial role in cell-associated organ damage. The significant rise of IL-10 in COVID-19 is a unique characteristic of the cytokine storm. This was assumed to be a negative anti-inflammatory mechanism [7]. As this is a hospitalized-based study monitoring the clinical role of pro and anti-inflammatory factors in COVID-19 infection can contribute to regulating the infection. This study aims to identify and characterize potential biomarkers of COVID-19 in serum samples and to detect the negative association of anti- and pro-inflammatory factors. The primary objective of this study is to identify potential biomarkers in serum samples of COVID-19 patients and to detect the expression of IL-6 and IL-10. While the second objective includes in silicon molecular docking of IL6 and IL10 and to develop a database of hospitalized COVID-19 patients with respect to identified markers. All these approaches will help in knowing the prognosis, pathogenesis, and, treatment of the disease.

## 2. EXPERIMENTAL DESIGN:

The purposed study is a hospital-based case-control study. The priority of recruiting all the study subjects is that they should have a COVID-19 positive diagnosis. All the study participants included in this study would follow the inclusion and exclusion criteria [fig 1]. The inclusion criteria for the recruitment of the study participants are that all the samples collected from the patients should be COVID-19 RT-PCR positive and/ or COVID-19 antigen-positive and the HR-CT score of the patients should be suggesting out COVID-19 related pathology. Study participants for healthy controls not having co-morbidities or pre-existing diseased conditions will be included in the study. The exclusion criterion will be that the samples would be positive for other similar COVID-19 related symptoms which can be by any other virus or bacteria except for the severe acute respiratory syndrome corona virus 2. Individuals with an immunosuppressive or immunodeficient state, such as HIV infection or other recurrent serious infections, as well as children under the age of 18 and pregnant or breastfeeding women will be excluded from this study.

The study work will be divided into three phases and based on the ICMR clinical management protocol for COVID-19 (2021) the study participants are divided into two cohort groups mild and severe groups. The first phase involves the characterization of COVID-19 biomarkers using proteomics techniques, followed by the second phase, which involves detecting the levels of IL6 and IL10 in COVID-19 patients serum samples, and the third phase, which involves in-silico docking of IL6 and IL10 [fig 2].

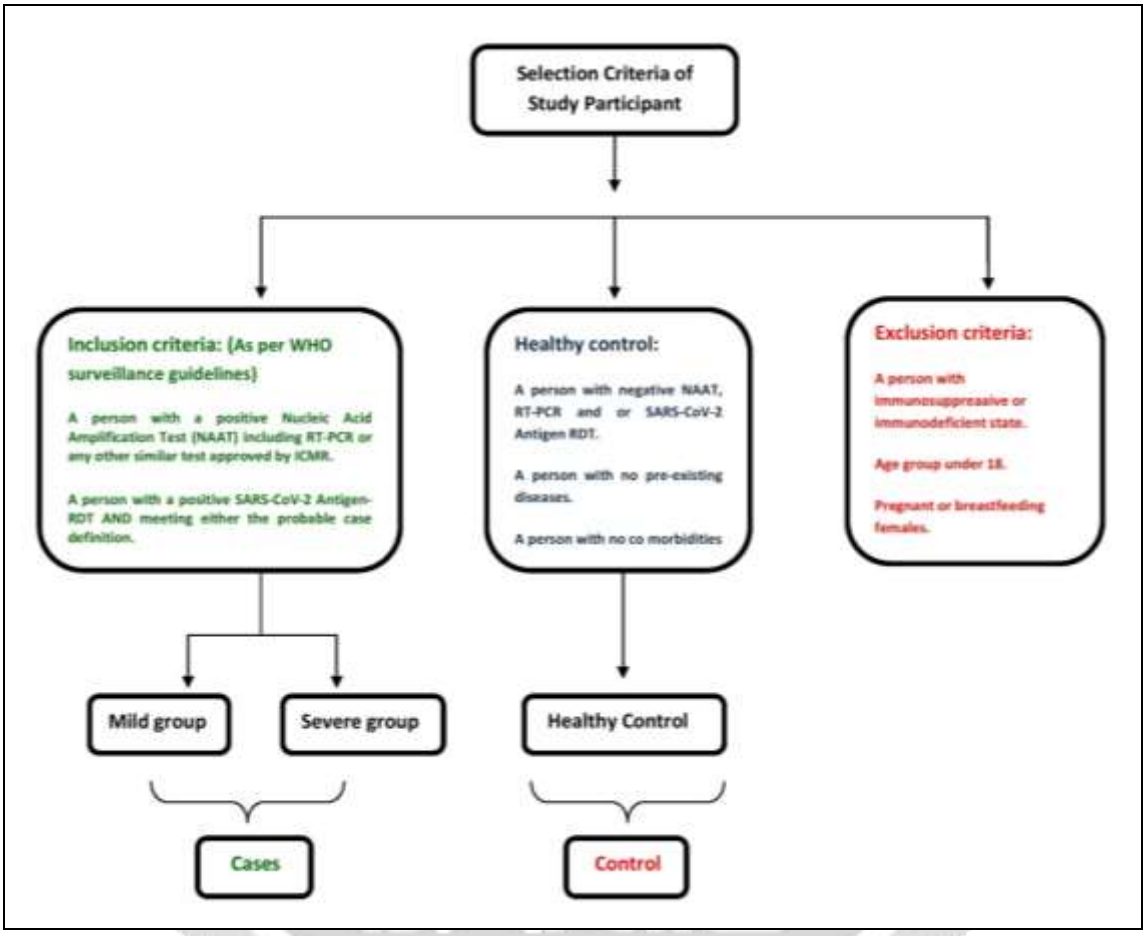


**Figure 1:** Inclusion and exclusion criteria for recruitment of study participants.

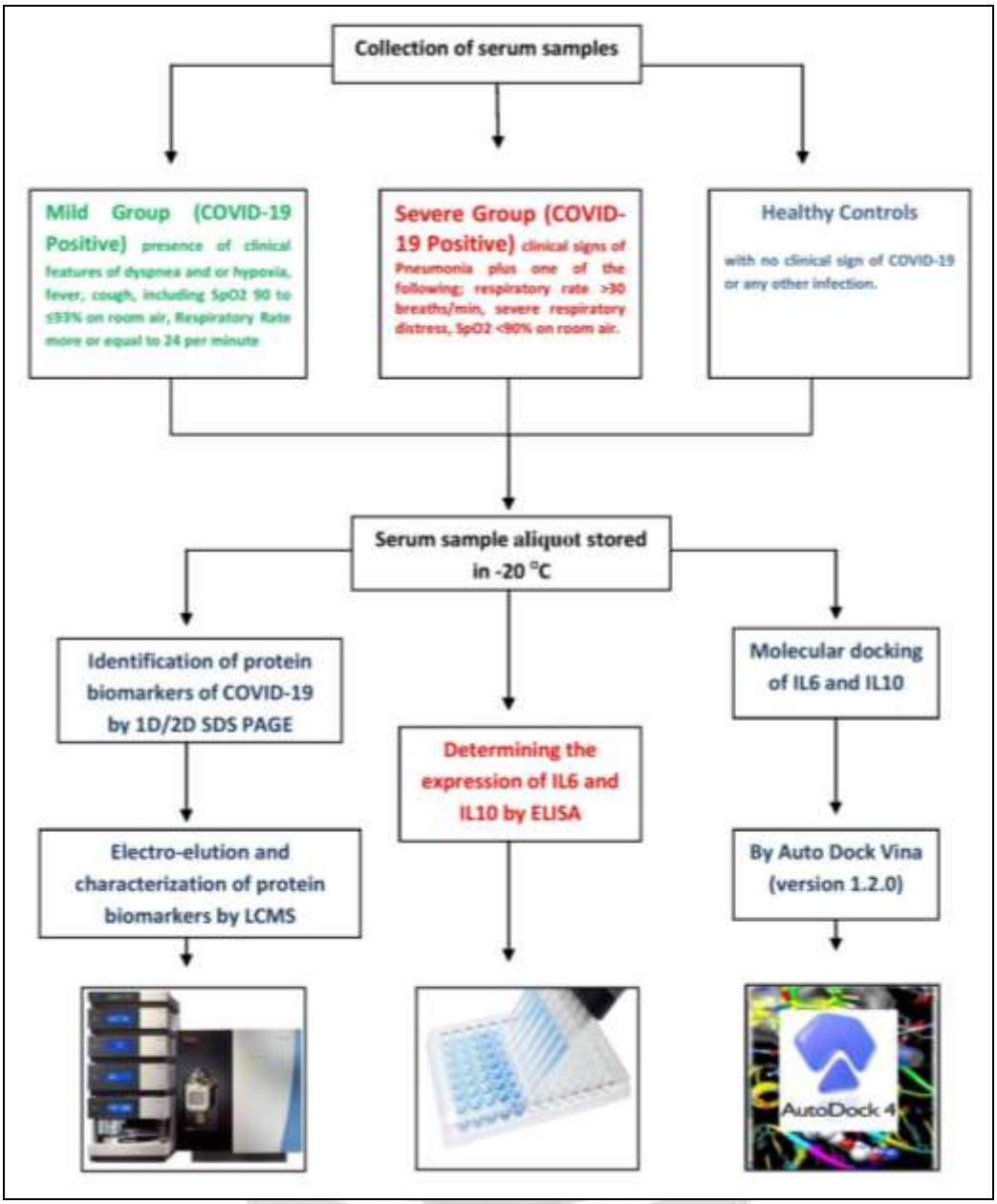


**Figure 2:** Schematic study work flow.

**2.1 Sample collection and processing:**

The recruitment of all the blood samples of COVID-19 patients will be performed based on the patients enrolled in CIIMS Hospital, Nagpur and whose COVID-19 RT-PCR and or COVID-19 rapid antigen was positive. The control samples are also collected from healthy patients enrolled in this study. All the protocols for recruitment and blood collection were approved by the Institutional Ethical Committee of Central India Institute of Medical Sciences (CIIMS), Nagpur. 2-3 ml of venous blood was collected from consenting participants in plan vaccutainers by trained phlebotomists. Serum was separated by centrifugation at 1200 rpm for 10 min and immediately frozen at -20$^{o}$C until use.

**2.2 Materials and Methods:**

**2.2.1. 1D SDS-PAGE (one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis):**

1. Acrylamide
2. Bisacrylamide
3. TRIS Buffer
4. APS (ammonium persulfate)
5. Bromophenol Blue
6. Glycerol
7. Glycine
8. SDS (sodium dodecyl sulfate)
9. Methanol
10. Glacial acetic acid
11. b mercaptoethanol
12. Commassie R250
13. Mili Q H2O

Equipments: Vertical slab gel system, power pack, Micropipettes, Micropipettes tips, syringe, Gel rocker, Gel Dock system, pH meter.

**2.2.2 2D SDS-PAGE (two dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis):**

1. Urea
2. (3-cholamidopropyl)dimethylammonio]-1 propanesulfonate (CHAPS)
3. Dithiothreitol (DTT)
4. Bromphenol Blue
5. Nanopure water
6. Tris HCL
7. Glycerol
8. All the above materials mentioned in 1D SDS-PAGE

Equipment: PROTEAN IEF cell Ready Strip IPG strips, pH4-7(7cm, 11cm, 17cm), IEF Focusing tray with lid (same size as IPG strips), Electrode wicks, precut, Blotting filter papers, Mineral oil, Forceps pipette for volume ranging from 4-1000µl, Strip plate, Plastic (Saran) wrap, 8-16%SDS-PAGE gels, SDS-PAGE electrophoresis cell Power supply appropriate for SDS-PAGE system Tris/Glycine/SDS running buffer, SDS-PAGE protein stain, Distain solution (40% methanol,10% acetic acid), Disposable rehydration /equilibration trays with lid (same size as IPG strips), 100ml graduated cylinders, High purity water.

**2.2.3. Nanodrop technique:**

1. Distilled water.
2. Sample for quantification of 1D equivalent to 40µg of protein.

Equipment: Nanodrop (thermofisher).

**2.2.4 Identification of protein biomarker by one and two-dimensional SDS PAGE (sodium dodecyl sulphate polyacrylamide electropgoresis):**

Quantification of the protein concentration of the serum sample will be performed by Nanodrop technology which is to be equivalent to 40µg volume of proteins and then 10µl SDS dye is to be added to the sample preparation. Then the procedure for the separation of proteins is performed by assembling the glass plate /gel cassettes in the electrophoresis equipment and by pouring the running (10%) and stacking gels (4%) [Table 1]. After pouring the stacking gel solution, the comb is inserted immediately and allows the gel to polymerize for about 15-30 minutes. Wells are washed with tank buffer to remove the air bubbles and gel particles and it will be run at 125V. Then the coomassie staining is performed followed by de-staining of the gel. The proteins are detected as blue bands on a clear background after distaining.

The sample preparation for two-dimensional SDS-PAGE will be performed with the IPG strips [table 2], the whole process is divided into a few steps first is the rehydration step in which the sample is diluted in the rehydration buffer and put on the IPG strips. The next is the equilibration step in which the transfer of the IPG strips into a clean equilibration buffer is done and the last step is the assembling of the IPG strips on top of the acrylamide gel and then

carrying out the one-dimension electrophoresis. After the electrophoresis is over proceed for Coomassie staining which is the same as for the one dimensional. Analysis of the gel images will be performed by using the gel documentation system (Bio-Rad) will be imported into a 2D gel analysis software package, PD Quest (Bio-Rad) and then the gel would be analyzed for spotting of proteins, which are related to the COVID-19 prognosis.

**Table 1:** Resolving and stacking gel protocol of SDS PAGE.

| Sr. No | Reagent for SDS PAGE (Resolving Gel 10%) | Volume (ml) |
|---|---|---|
| 1 | Distilled Water | 11.9 |
| 2 | 30% Acrylamide | 10.0 |
| 3 | 1.5 M Trish (Ph 8.8) | 7.50 |
| 4 | 10% SDS | 0.30 |
| 5 | 10% APS | 0.30 |
| 6 | TEMED | 0.012 |

| Sr. No | Reagent for SDS PAGE (Stacking Gel) | Volume (ml) |
|---|---|---|
| 1 | Distilled Water | 6.80 |
| 2 | 30% Acrylamide | 1.70 |
| 3 | 1.5 M Trish (Ph 6.8) | 1.25 |
| 4 | 10% SDS | 0.10 |
| 5 | 10% APS | 0.10 |
| 6 | TEMED | 0.01 |

**Table 2:** Sample preparation of 2D SDS PAGE.

| Strip Length | 7cm | 11cm | 17cm |
|---|---|---|---|
| Sample volume(max) | 125µl | 185µl | 300µl |
| Protein loaded (Max) | 169µg | 250µg | 405µg |

**2.2.5. LC-MS (liquid chromatography mass spectrometry):**

1. Acetonitrile ( 2% ACN )
2. Formic acid 0.1%
3. H2O 9.5%
4. Trifluoroacetic acid
5. Methanol
6. Ethyl acetate
7. Hexane
8. Heptane
9. 2-Propanol
10. Ammonium hydrogen carbonate

11. Sodium formate solution
12. Difluoroacetic acid
13. Trifluoroacetic acid
14. 2,2,2-Trifluoroethano
15. 1,1,1,3,3,3- Hexafluoro-2-propanol

Equipment: Thermo finnigan LCQ ion trap LC-MS/MS.

**2.2.6. Characterization of protein band by electro-elution followed by liquid chromatography mass spectrometry (LC-MS) analysis:**

The identified protein biomarkers by SDS PAGE will then subsequently excised and be electro eluted by the electro-elution method. The electro-eluted protein will then be characterized using the advanced proteomic technique LC-MS/MS. A Finnigan LCQ ion trap MS coupled in line with a high-pressure liquid chromatography (HPLC) system will be used for LC-MS. A 75 um (ID) x 10 cm length, 3um packing C18 capillary column, packed in-house, will be connected to a specially designed nanoSpray device that is capable of delivering stable electrospray at flow rates of 100 nl/min to 1500 nl/min. Mobile phases are as follows: Solvent A (2% ACN, 97.5% H2O, 0.1% formic acid) and Solvent B (90% ACN, 9.5% H2O, 0.1% formic acid). Analysis of the LC-MS/MS data performed by the ion trap MS will be set to operate in a data-dependent mode with Automatic Gain Control (AGC). The MS/MS data will be first evaluated against several internal quality control (QC) standards. After passing the QC standards, the MS/MS data will be loaded into the proprietary ProtQuest search engine to search the most recent non-redundant protein database. The results from the ProtQuest search will be then manually analyzed.

**2.2.7. ELISA (enzyme-linked immunosorbent assay):**

1. Human IL-6 ELISA kit 96 well plate (Diaclone)
2. Human IL-10 ELISA kit 96 well plate (Diaclone)

**2.2.8. Detection of IL-6 and IL-10 level in serum samples of COVID-19 patients:**

Human IL6 high-sensitivity ELISA kit (DIACLONE) will be used for detection of IL6 levels [fig 3] and Human IL-10 ELISA kit (DIACLON) will be used for detection of IL10 in serum samples of COVID-19 positive patients [fig 4] as per manufacturer's instruction.

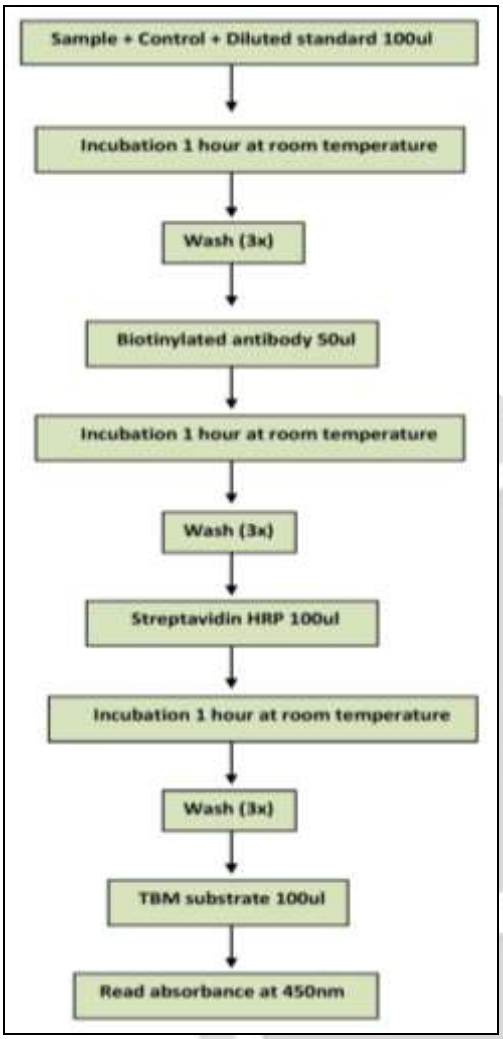


**Figure 3:** Flow chart of IL10 protocol.

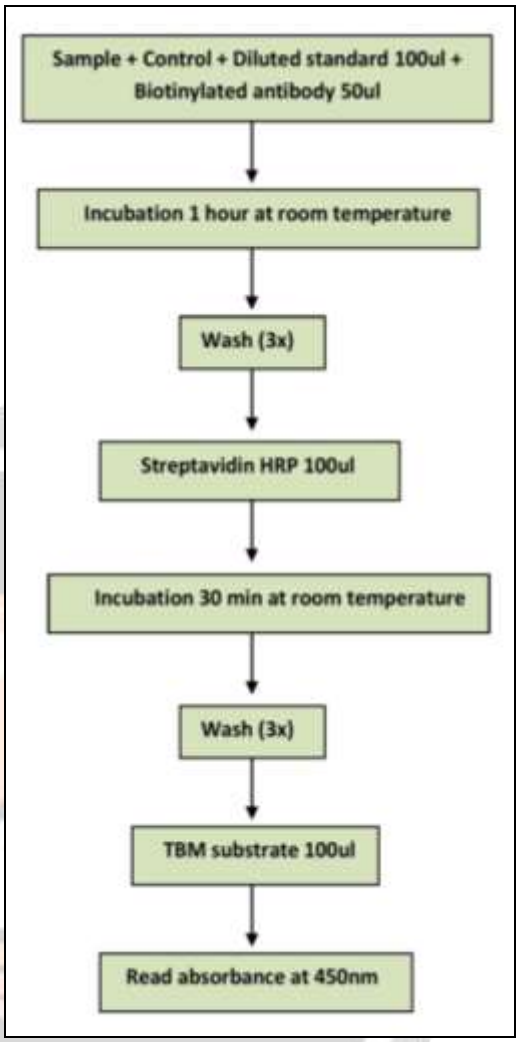


**Figure 4:** Flow chart of IL16 protocol

**2.2.9. Standardization of IL6 and IL10 kit ELISA assay:**

The standardization of both IL6 and IL10 ELISA kit assay will be performed by checkerboard method to know the exact concentration of antigen and antibody. The standard vial provided in the IL6 and IL10 kit individually is to be reconstituted with standard diluents (10X). This reconstitution provides a stock of 400 pg/ml. Then serial dilution is made directly in the assay plate provided in the kit. The concentration range will be from 400 to 12.5pg/ml from well A1 to F1. G1 well concentration will be zero and H1 well will consist of control which is provided in the kit [fig 5].

The capture antibody highly specific for IL-6 and IL10 has been coated to the wells of the microtiter strip plate provided in the kit. Sample, control, and diluted standard are to be added into the wells with the Biotinylated antibody provided. Binding of IL-6 samples and known standards to the capture antibodies and subsequent binding of the Biotinylated anti-IL-6 and IL10 separately to the secondary antibody and analyte is completed during the same incubation period. Any excess unbound analyte and secondary antibody are removed by washing. Then the streptavidin-HRP conjugate solution is added to every well, following incubation excess conjugate is removed by careful washing. A chromogen substrate will be added to the wells resulting in the progressive development of a blue-colored complex with the conjugate. The color development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced colored complex is directly proportional to the concentration of IL-6 and IL10 present in the samples and standards. Reading absorbance is to be at 450 nm. The amount of IL-6 and IL-10 in each sample is determined by extrapolating OD values against IL-6 and IL-10 standard concentrations using the standard curve.

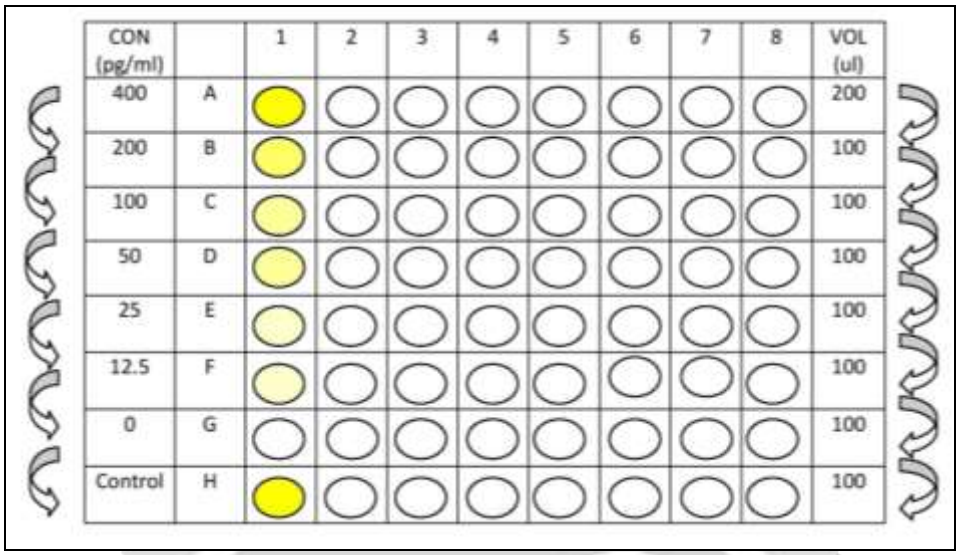


**Figure 5:** Standardization of IL6 and IL10 kit ELISA assay.

**2.2.10. In silico molecular docking of IL6 and IL10:**

To demonstrate the binding of pro (IL6) and anti (IL10) inflammatory factors and to reveal that IL10 can be a potential drug candidate for circumventing the COVID-19 infection in silico molecular docking approach will be used. IL6 will be the receptor and IL10 will be the ligand and the type of docking will be protein-protein docking. The three-dimensional protein structures are to be downloaded in the form of PDB format from RCSB PDB software. Further optimization and viewing of this protein structure are done in the Swiss PDB viewer. The final receptor and ligand docking are performed in AutoDock Vina (version 1.2.0) [fig 6].

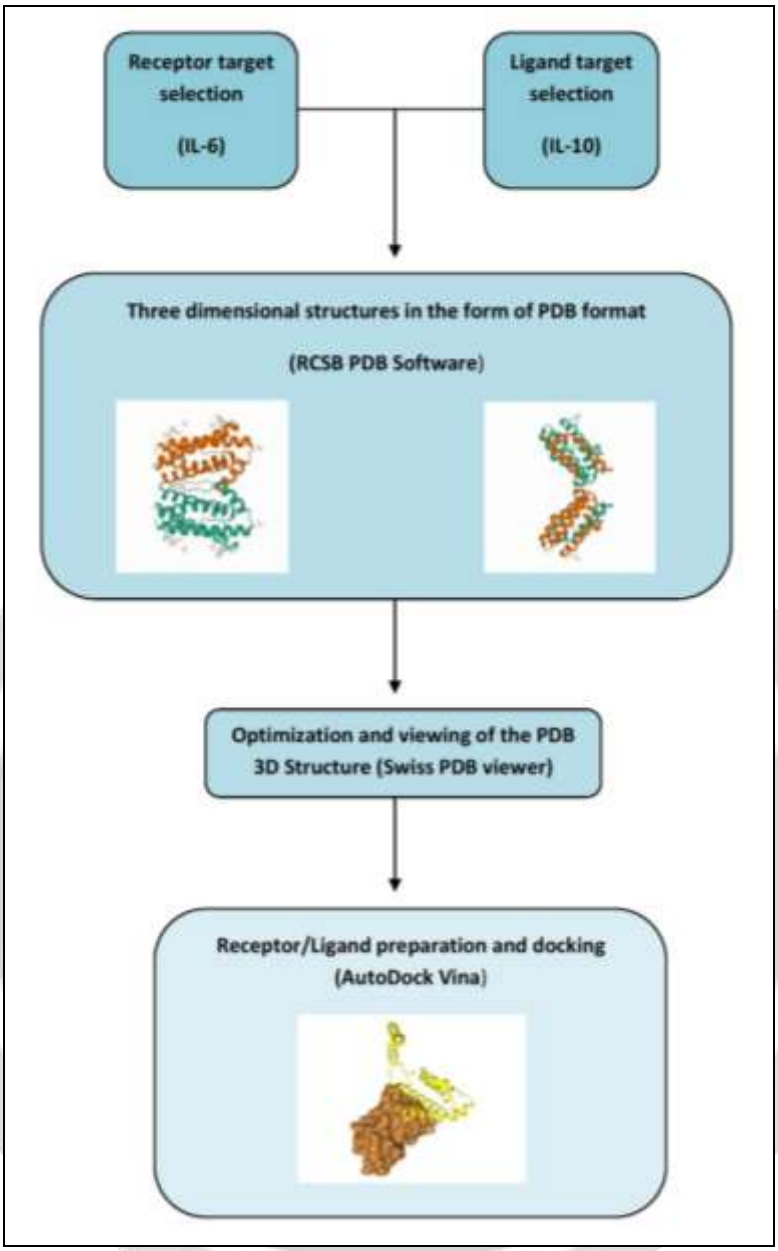


**Figure 6:** Schematic representation of in-silico molecular docking of IL6 and IL10.

**2.2.11. Data Collection:**

All the patients included in this study were further screened for collection of their demographic details which includes age, and gender and medical condition as co-morbidities, infection stage, HR CT score of severity, symptoms, days of hospitalization, and condition on the discharge of hospitalization as improved or deteriorate [fig 7].

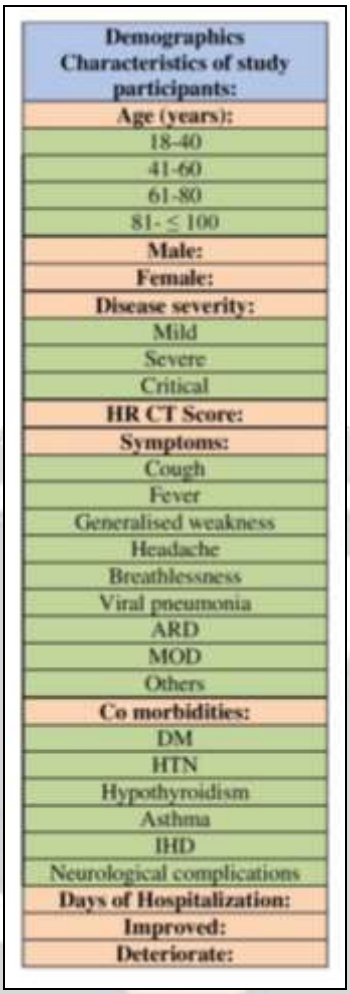


**Figure 7:** Demographic characteristic of study participants.

**2.2.12. Statistical analysis:**

On a nominal scale, the frequencies (percentage) of demographic, clinical, and risk factors would be calculated. Comparison between biomarkers of different symptomatic groups mild and severe will be performed using the Chi-square test and ANOVA in MedCalc statistical software (version 10.1.2). Figures and graphs will be prepared using Graph Pad Prism 5 and Sigma plot updated version. Molecular docking will be performed with the help of Auto Dock Vina software (version 1.2.0).

**2.2.13. Troubleshooting:**

| Sr. No | Techniques | Problems | Reason | Solution |
|---|---|---|---|---|
| 1 | 1 D SDS PAGE | Low intensity of the bands on gel | voltage problem | Check the power pack supply and decrease the voltage by 20-25% |
| | | Bands are distorted | The polymerization around the sample wells is poor | Increase the amount of APS and TEMED. |
| | | Gel casting issue | Time that the gel polymerizes | Use freshly prepared APS and TEMED. Quality of the acrylamide or bisacrylamide is poor. |

| | | | | |
|---|---|---|---|---|
| | | | is too long | |
| 2 | ELISA | High background | Longer incubation time than required | Follow manufacture kit optimize protocol carefully. |
| | | Uneven color development | Incorrect washing | Ensure all wells are filling with wash buffer. At the end of each washing step, invert plate on absorbent tissue and allow to completely draining, tapping forcefully if necessary to remove any residual fluid. |
| | | Unexpected results | Dilution error and technique problem | Check pipetting technique and calculations. Proper mixing of reagents and wash steps are critical. |

**Table 3:** Troubleshooting in respect of 1D SDS PAGE and ELISA.

**3. EXPECTED OUTCOME:**

Dtection of potential biomarkers of COVID-19: In this proposed study identification of protein in serum samples hat will be upregulated in COVID-19 infection and absent in healthy controls will be determined by 1D SDS PAGE and 2D SDS PAGE. These identified proteins will be further analyzed by the advanced proteomics technique LC-MS. Then the potential biomarkers will be reported for prognosis, pathogenesis, and treatment of COVID-19 infection.

Detection of negative association of IL6 and IL10: The detection of pro (IL6) and anti (IL10) inflammatory response in COVID-19 positive serum samples is to be done by ELISA technique. Comparing the levels of both cytokines will help in the association with the severity of the disease.

IL-10 as a potential drug candidate COVID-19 infection: With the help of in silico molecular docking of IL6 and IL10 the demonstration of the binding of IL6 and IL10 will be performed. This will be beneficial in revealing that IL10 can play a potential drug candidate for overcoming the COVID-19 infection severity.

Development of hospital based database of COVID-19 patients: A Complete database from all the recruited COVID-19 hospitalized patients will be developed using statistical software which can be further used to analyze the infection.

| Sr. No | Methods | Intentions | Outcome |
|---|---|---|---|
| 1 | 1D/2D SDS PAGE | To separate proteins by molecular weight from the serum samples this can be identified as biomarkers of COVID-19. | To report up regulated proteins in COVID-19 infection which are absent in healthy controls. |
| 2 | LC-MS | To indentify, characterize and known | To report the expression of proteins |

| | | | |
|---|---|---|---|
| | | the structure of proteins. | which are specific to COVID-19. |
| 3 | ELISA | To determine the levels of inflammatory cytokines in serum sample. | To report the levels of IL6 and IL10 in different stages of COVID-19 infection. |
| 4 | MOLECULAR DOCKING | To identify novel therapeutic relevant target for COVID-19. | To reveal that IL-10 can play a potential drug target for COVID-19 infection. |

**Table 4:** Methods inventory and expected outcome of the study.

## 4. DISCUSSION AND CONCLUSIONS:

This study was designed to find novel biomarkers for COVID-19 in serum samples of patients admitted to the hospital. Proteomics profiling of COVID-19 serum biomarkers by LC-MS can help in monitoring and predicting the development of the infection and understanding the prognosis and pathogenesis of this disease. These identified novel biomarkers can also help in predicting the severity and outcome of COVID-19. The association of inflammatory factors and immune response in COVID-19 is also targeted in this study by focusing on the levels of IL6 and IL10. This study can provide evidence that inflammation reflected by the cytokine storms can be controlled by the anti-inflammatory cytokine IL10 which can reduce the inflammation and severity of the infection. IL10 evaluation in SARS-CoV-2 infection can also help in revealing the potential to play a role as a drug candidate in reducing the severity of the infection. Furthermore, this research will aid in the development of new approaches for regulating the transmission and treatment of COVID-19 disease.

## 5. CONFLICT OF INTEREST:

The authors declare no conflict of interest.

## 6. ETHICAL APPROVAL:

The study was approved by the Institutional Ethical committees of Central India Institute of Medical Sciences (CIIMS), Nagpur and Datta Meghe Institute of Medical Sciences (DMIMS), Sawangi, Wardha.

## 5. ACKNOWLEDGEMENT:

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