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REVIEW ARTICLE

Immunologic aspect in diagnosis and treatment of SARS-COV-2 patients

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A B S T R A C T

Recent worldwide outbreak of novel coronavirus disease (CoVID-19) has affected massive human population including Pakistan, and has caused a huge number of mortalities in few months. CoVID-19 is an infectious disease caused by a virus, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) which is single stranded RNA enveloped beta coronavirus and affects lower respiratory tract. It transmits from human to human through respiratory droplets. It uses its S-protein to recognize ACE2 (Angiotensin Converting Enzyme-2) receptors in lung epithelial cells where it attaches and causes infection. The incubation period is 2-14 days. In pre-symptomatic phase, body's immune system starts antibodies production. Significant antibodies are IgM and IgG that produces within 03-06 days and 8-12 days respectively. This review provides the available information about immunological aspects in terms of diagnosis and screening of CoVID-19 and potential therapeutic targets for combating SARS-CoV-2 infection. Immunologic techniques to detect these antibodies are ELISA (Enzyme-linked Immunosorbent Assay), CMIA (Chemiluminescent Micro particle Immunoassay) and ICT (Immunochromatographic Test). Among these, ELISA and CMIA are found to be highly specific and sensitive in convalescent phase of infection. While the fundamental confirmatory test for SARS-CoV-2 infection is RT-PCR (Reverse Transcription Polymerase Chain Reaction) which detects the viral RNA in respiratory samples preferably nasopharyngeal swab. Serological assays are essential to find out rate of infection, and most importantly antibody titers in recovered patients to be used for therapeutic purpose. After some successful studies Convalescent Plasma is considered as a good therapeutic option in the absence of specific antiviral therapy.

Keywords: *Antibodies, ELISA, CMIA, convalescent plasma, COVID-19*

Introduction

The current rapid worldwide outbreak of a novel flu-like Coronavirus disease 2019 (CoVID 19) began in the city of Wuhan in China. Initially the causative agent was unknown and it was treated like pneumonia because of similar clinical characteristics.[1] Several such cases were reported by the Government of China on December 31st 2019.[2] Experts from Centers for Disease Control (CDC) performed analysis on respiratory samples and declared that this pneumonia is caused by the family of Coronavirus that belongs to the genus betacoronavirus.[3,4] Further, next generation sequencing revealed that this novel virus is RNA enveloped beta coronavirus having phylogenetic similarity to previously known beta coronaviruses that infected a massive human population in 2002-04 during SARS outbreak caused by SARS-CoV (Severe Acute Respiratory Syndrome Coronavirus) and in

2012 during MERS outbreak caused by MERS-CoV (Middle East Respiratory Syndrome Coronavirus).[5] So, it was named as SARS-CoV-2 by international Committee on Taxonomy of viruses. Subsequently the disease was named as CoVID-19 by World Health Organization (WHO).[6, 7]

Within a month, cases reached up to 6065 globally in which 99% cases were from China but the virus spread to 15 other countries quickly. So on 30th January 2020, WHO declared the outbreak of CoVID-19 a "public health emergency of international concern".[6, 1] the number of cases were increasing exponentially worldwide. As of 11th February 2020, WHO data report has shown around 43000 confirmed cases from more than 28 countries. Hence, the CoVID-19 outbreak was declared a "Global pandemic" by WHO on 11th March 2020.[6] By June 18, 2020 CoVID-19 outbreak has affected almost 213 countries including Pakistan. According to WHO, total of 8,417,100 confirmed cases with 451,661 mortalities worldwide. While number of confirmed cases in Pakistan has reached up to 160,118 with 3,093 mortalities, currently these cases are increasing at an alarming rate and the situation is worst.8

Origin:

Novel coronavirus SARS-CoV-2 is of zoonotic origin like other betacoronaviruses.[6,9] Full genome sequencing of SARS-CoV-2 extracted from Broncho alveolar fluid sample that was taken from a patient who worked in seafood market revealed that there was 96.2% nucleotide sequence similarity to bat SARS-related-CoV (bat-SL-CoVZC45) and a low similarity of 79% and 50% to SARS-CoV and MERS-CoV respectively.[3] It is also suggested that in addition to bats, pangolin species are also natural reservoir of SARS-CoV-2.[7]

Bat (Rhinolophus affinis) is still the most probable species for the origin of novel SARS-CoV-2.[7] Tang, Xiaolu, et al. suggested that further research analysis is required to find out the intermediate animal host which is responsible for the transmission of SARS-CoV-2 from its original host to humans because the relative coronaviruses MERS-CoV and SARS-CoV do pass to their intermediate hosts before invading into human body such as civets or camels.[4]

Pathogenesis and genetic structure:

Single stranded RNA Coronavirus (CoV) is spherical to pleomorphic particle with diameter of 80-120nm. There are four different types of Coronavirus that are Alpha (α-CoV), Beta (β-CoV), Delta (δ-CoV), and Gamma Coronavirus (γ-CoV).[1, 7]

Six types of coronaviruses have previously infected human beings and have caused respiratory diseases. Two of them belong to the class of Alpha Coronavirus which includes 229E and NL63 while the rest belongs to the class of Beta Coronavirus named OC43, HKU1, SARS-CoV and MERS-CoV. SARS-CoV-2 is the seventh known Beta Coronavirus that has infected human population.[1, 7] SARS-CoV-2 uses ACE2 as a receptor for binding to enter into lung epithelial cells. S-Protein on the surface of SARS-CoV-2 helps recognize the corresponding receptor to get entry into the cell and cause infection. Structural analysis reveals that SARS-CoV-2 binds with ACE2 receptor with 10 time's higher affinity as compared to SARS-CoV.[1, 10]

There are four structural proteins in the virus, Spike (S-protein), Membrane (M-protein), Envelope (E-protein) and Nucleopsid (N-protein). Envelop of the virus is made up of Spike, Membrane and Envelop protein that gives shape to the virus.[10]

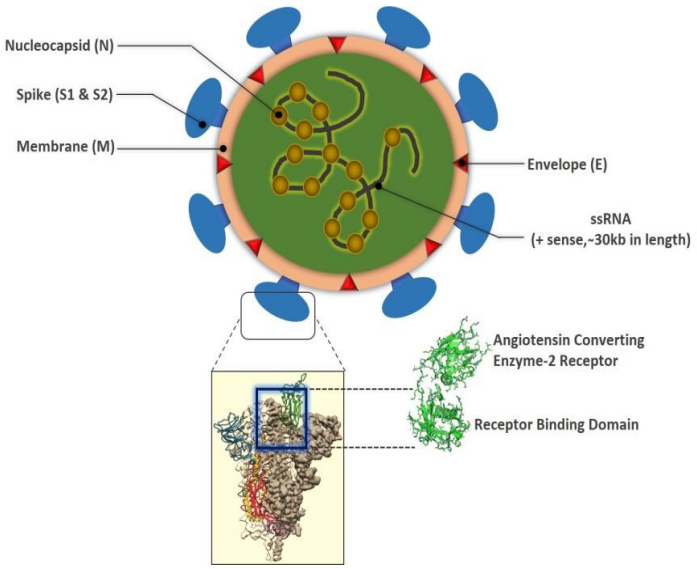

Figure 1. Genetic structure of SARS-CoV-2

The unique crown like appearance of the virus is due to the embedded polymers of S-protein in its envelop.[1] Spike glycoproteins in the viruses target the respiratory tract cells by binding to human ACE2 receptors having two subunits. The S1 subunit comprised of single peptide, an N-terminal domain and RBD (Receptor binding domain) that causes the direct interaction of Spike protein to the host receptor.[3, 7]

S2 subunit contains fusion peptides that makes it highly conserved. After the binding of Spike protein with target cells receptors, envelop of viruses fuses with the cell membrane and releases viral RNA into the target cell.[1,3,7] Mutations are observed in spike proteins that plays important role in differentiation mechanism and infectious capability of SARS-CoV-2.[5,10]

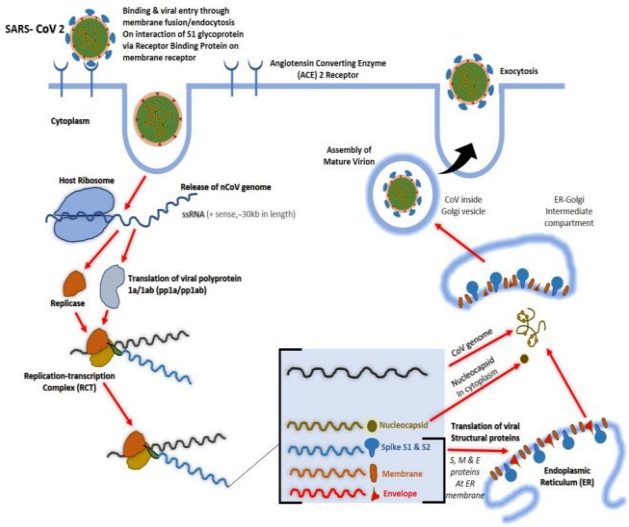

Figure 2. Entry and replication of SARS-CoV-2 inside the cell

Viral genome contains 12 functional open reading frames (ORFs), first ORF translates pp1a and pp1ab polypeptides that encodes for NSP (Non-structural proteins) namely NSP3 (papain-like protease), NSP5 (main protease), NSP12 (RNA dependent RNA polymerase), NSP13 (helicase) and other NSPs while remaining ORFs encodes for structural proteins.[3]

Mutations are observed in these NSPs, the NSP2 and NSP3 as well as in spike proteins that plays important role in differentiation mechanism and infectious capability of SARS-CoV-2.[5, 10]

Viral spread and transmission:

The respiratory droplets that come out of coughing and sneezing of an infected person are the source of the viral spread. When enter through inhaled air into another person's body, they stick to the mucosa of nasal cavity, mouth, eyes or in lungs where the viruses get the chance to invade into the cells.[2] in this context, WHO regarded CoVID-19 as airborne. Evidences have proved that its human to human transmission is not only from symptomatic patients but also occurs during asymptomatic phase when patient does not even know about his illness. Incubation period of SARS-CoV-2 is estimated to be 2-14 days.[11]

Humoral and cellular immunity:

ACE2 is expressed highly on apical side of lung epithelial cells in alveolar space, so this virus can likely enter those cells. It is also evident from the fact that early injury of lung was often seen in distal airway. Main components of innate immunity in airways are epithelial cells, alveolar macrophages and dendritic cells (DCs). DCs are located underneath the epithelium while macrophages are located at the apical side of epithelium.[4] Upon entry of the viruses, humoral and cellular immunity is stimulated when these viral antigens are presented to CD4+ helper T cells or CD8 cytotoxic T cells by macrophages and DCs in association with MHC class-2 and MHC class-1 molecules respectively.[12]

Two types of antibodies that have diagnostic significance are produced by the immune system named as IgM and IgG antibodies. IgM antibodies are produced earlier than IgG antibodies.[13] it takes about 03 to 06 days for IgM antibodies to be produced after the viral attack, giving seropositive results in current or recent infection. While IgG antibodies provide long term immunity having high affinity responses and are important for immunological memory. IgG takes longer to produce. It is detected after 8 days of infection.[13]

IgG antibodies are specific to Spike and Nucleopsid proteins and play a protective role against infections because they last longer while SARS-specific IgM remain in the blood for about 12 weeks then they degrade.[12]

The virus usually escapes the innate immune response and gets entry into the cell. Immune system first recognizes the unique molecular patterns of the

pathogens by PRRs (Pattern Recognition Receptors). The PRRs for respiratory viruses are TLRs (Toll-like Receptors) [3, 7, 8] present throughout the airway on different cells for defense mechanism.[4, 12] this recognition is led to signaling and to the production of pro-inflammatory cytokines. Unfortunately, by now, there is a little knowledge about antigen presentation of SARS-CoV-2. Studies described that the virus forms a double membrane vesicles during replicating in the cell that prevents it to be recognized by the host innate immunity. Additionally, activation of IFN (interferon) beta promoter and inhibition of nuclear transport of IFN regulating factor 3 by ORF4a, ORF4b, ORF5, and M-proteins also helps the virus to escape from innate immune system.[12]

Cytokine storm and viral sepsis:

Infection of SARS-CoV-2 in critical patients with symptoms of severe pneumonia, sometimes leads to overproduction of inflammatory cytokines, also termed as cytokine storm, which is uncontrollable. This leads to progression of disease, and to acute lung damage and acute respiratory distress syndrome (ARDS) that is a major contributing factor to mortality due to CoVID-19. The cytokines includes IFN-γ, IL (Interleukin)-1, IL-6, IL-12, and TGFβ (Transforming growth factor beta).[14,15,16] Cytokine storm may also lead to hyper activation of T cells, natural killer cells, macrophages, and different chemical mediators from immune cells in addition to excessive production of many cytokines. This impairs lung microvascular and alveolar barrier and it brings off vascular leakage, edema, and the conditions of hypoxia. Such conditions are potentially fatal.[14]

A very little published data has revealed that autopsy samples which included lungs, liver, kidney, and heart of elderly died patients had huge amount of inflammatory cell infiltrate, fibrosis, necrosis, and bleeding. Lymphatic organs and spleen also showed atrophy and other conditions of bacterial sepsis like disseminated intravascular coagulopathy (DIC). That's why a term 'viral sepsis' is coined in such condition. This undesired immune response and organ failure contributes to the mortality rate of the disease.[16]

Laboratory Diagnosis:

Two basic technologies for detection of the infection are molecular and serological methods of testing.

1. Molecular Methods:

A molecular method of testing is based upon isolation and detection of viral nucleic acid in the given specimen. Currently the most effective front line method for detection of viral load is RT-PCR (Reverse Transcription Polymerase Chain Reaction).[17]

Sample requirement:

RT-PCR detects the virus using respiratory samples. Broadly recommended samples are upper respiratory samples that include nasopharyngeal swabs (NPS), oropharyngeal swab and nasal aspirates while sputum, Broncho alveolar lavage (BAL) and aspirates of trachea are included in lower respiratory samples that are less likely to be collected. Viral loads detection in upper and lower respiratory samples depend upon the days after onset of illness. Low viral loads in the area sampled may lead to a false negative result.[18] Study showed that in the first week of onset of illness, high viral loads were observed that declined with time.[18]

i. RT-PCR

Viral RNA is detected by reverse transcription followed by amplification of targeted portion of cDNA using polymerase chain reaction (PCR). Following extraction of RNA by phenol-chloroform method, viral RNA is converted into cDNA through 'reverse transcription 'and then targeted portions of cDNA are amplified up to a detectable range using 'polymerase chain reaction'.[18]

Finding and analyzing SARS-CoV-2 related viral genome sequences for designing a set of primers was also a challenging task.[18] Those molecular targets include the genes that translate structural proteins like S (spike), E (Envelope), N (Nucleopsid), M (transmembrane) and Hel (helicase). Corman et al. discovered 3 regions for primer design. First one was RNA-dependent RNA polymerase gene or RdRp gene in the open reading frame (ORF). Second one is 'Envelope protein' gene or E gene and third one is 'Nucleopsid protein' or N gene.[18] To avoid cross-reaction with SARS-COV, different molecular targets are used for RT-PCR by different researchers in different countries.[19] Some of the viral genome sequence may not be constantly expressing and therefore the primers specific to that portion may not provide the real viral load information, although it may detect the presence of the virus. RT-PCR technology is high in specificity and

sensitivity but it is highly essential to collect the respiratory specimen at the right time from the right site for the accurate diagnosis because samples taken inappropriately or taken from the site with low viral load while missing the time window may lead to false negative results.[18] Many researchers found low sensitivity of RT-PCR because of this reason.[1,13,19] 83.3% sensitivity of RT-PCR was found in a study due to poor quality of nucleic acid in the specimen which led to false negative results.[20] However RT-PCR method remain highly specific and sensitive in early phase (within 2 weeks) of infection.[21]

2. Serological methods:

Serological methods are best to use not only for diagnosis purpose but also for analysis of epidemiological variables and prognosis of the disease by analyzing humoral response especially the detection and quantification of antibodies which are produced rapidly after infection.[22] Serological analysis against SARS-CoV-2 involves ELISA (Enzyme Linked Immunosorbent Assay), CMIA (Chemiluminescent Micro particle Immuno Assay), and ICT (Immunochromatographic Technique) with variations in their sensitivity, specificity, positive predictive value and negative predictive value.[21]

i. ELISA

Most important antibodies that can provide useful information about the course of infection are immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies. ELISA is a plate-based assay technique which is used for detection and quantification of proteins, peptides, antibodies, and hormones. In this technique, an immobilized antigen on a solid surface is attached with enzyme linked antibody. By adding conjugated enzyme, a measurable product is produced.[23]

In case of SARS-CoV-2, purified recombinant viral Nucleopsid proteins (rNPs) are used as coating antigens for the detection of IgM and IgG antibodies.[23] Mainly ELISA checks whether body's immune system has developed antibodies against SARS-CoV-2. It is also helpful after the recovery of the patients or in convalescent phase to check his immune status.[18] High levels of sensitivity and specificity were reported in studies but they didn't recommend ELISA as a backup of RT-PCR because antibodies might not be detected in asymptomatic phase and very early phase of disease onset.[17,18,23]

However, Guo L et al reported a higher detection rate (98.6%) of IgM ELISA as compared to PCR (51.9%) after 5 days of the disease onset.23 Study shows the median duration for IgM is 5 (03-06) days while for IgG, it is 14 (10-18) days. This study concludes that humoral response can be a reliable tool for the diagnosis of CoVID-19.[23]

ii. CMIA

Chemiluminescent Micro particle Immuno Assay (CMIA) is a quantitative measurement of immunoglobulins in patent's serum sample. It is intended to detect IgG as well as IgM antibodies production in a patient's serum against SARS-CoV-2.[24]

This test uses protein-coated micro particles. CMIA relies on mixing patient samples with a known viral protein, buffer reagents and specific enzyme labeled antibodies that allow a light based, luminescent read out. Antibodies present in the sample react to viral proteins followed by addition of enzyme labeled antibodies. A chemical reaction takes place that produces light, which is the measure of antibodies in the sample. This test can measure multiple types of antibodies including IgG, IgM and IgA.[24, 25]

There are few studies evident which use CMIA method of testing one of them was done by Mathur et al. Average sensitivity and specificity of general serological assays they reported were 84.90% and 98.63% respectively while CMIA turned out to be 89.36% sensitive and 99.63% specific in Convalescent phase.[25]

iii. ICT

Immunochromatographic Technique (ICT) is another serological method, also known as rapid chromatographic technique.[26] SARS-CoV-2 test device is newly developed colloidal gold based kit on which Nucleopsid protein (N-protein) of SARS-CoV-2 is used as an antigen. ICT contains a detection zone on top of a nitrocellulose strip. In this zone, IgG antibodies, IgM antibodies and SARS-CoV-2 N-protein have been immobilized onto a test line and control line separately. Colloidal gold particles are used where IgG/IgM antibodies and N-protein are to be coupled, it serves as a detector. Device contains two wells at the bottom for serum sample and buffer respectively.[26]

Shen et al. in their study evaluated the performance of colloidal gold immunochromatography assay for SARS-CoV-2 combined IgG/IgM. The method turned out to be sensitive and specific for CoVID-19 with noticeable increase in sensitivity of antibody assays with the course of disease.[26] in another experimental study researchers utilized antibody test using lateral flow immunoassay technique which takes less than 15 minutes to generate results. Results showed 88.66% and 90.62% sensitivity and specificity respectively. However, false negative and false positive results were also reported.13 ICT technique is still in use by many researchers and in hospital laboratories for rapid diagnosis but reliability of this technique is still is still questionable.[13]

Importance of serological data:

IgM antibodies specific to SARS-CoV-2 start to appear in the blood within 03 days of infection while stays in the body for approximately 42 days.[17] While IgG antibodies are detectable from day 05 and start increasing onwards. Studies showed that median time for seroconversion is 20 days. By that time about 60-75% of patients became immune to the infection and develops IgG antibodies against the virus.[17] following statements can be given using immune response timing.

1. Serological data gives some epidemiologically important variables such as rate of viral attack and spread essential for the assessment of community transmission.[22]
2. It has the prognostic importance by monitoring the effect of pharmacological and non-pharmacological treatment population wide. [22]
3. To detect immunologic response of recovered individuals and to make decision whether their antibody isolates can be used for the treatment purpose via Convalescent plasma therapy.[22]

Treatment and Management Strategies:

Corticosteroids:

Corticosteroid is a most commonly and traditionally used drug for its anti-inflammatory response. But it is also known for its immunosuppressive response. This two sided characteristic makes it controversial in its use.[14, 16] although it has been successfully used during SARS epidemic in resolving fever, and lung infiltrate but recent WHO guidelines do not recommend its use for SARS-CoV-2 patients for not showing significant results in patient's recovery.[14]

Interferon:

Use of interferon (IFN) in animal and human model showed mixed results. Early administration of IFN and ribavirin showed some beneficial results in lowering the viral load and improving patient's health. However, late application showed no beneficial results. [14]

Monoclonal antibodies:

Another potentially useful treatment strategy involves the utilization of anti-IL-6 monoclonal antibodies. It has been used in patients with Cytokine Release Syndrome (CRS) and found beneficial in the control of cytokine storm.[14] Studies have shown that besides lowering the cytokine storm, use of anti-IL-6 monoclonal antibodies results in increased expression of CD4+ and CD8+ T cells hence, improving the overall immune system for the wellbeing of the patient.[14,15]

Convalescent Plasma Therapy (CP therapy):

Convalescent Plasma Therapy is an adaptive immunotherapy. In this therapy, plasma of recovered patients of CoVID-19 is used because it carries enough titer of anti-SARS-CoV-2 antibodies and may improve clinical condition and survival of CoVID-19 patients.[27] Major problem whole world is facing in controlling CoVID-19 pandemic is not having approved specific antiviral agents for novel Coronavirus disease.[28] However, in addition to some pharmacological treatment options, an effective non-pharmacological treatment to improve the rate of survival is Convalescent Plasma Therapy.[28,29]

Donor Selection Criteria

Criteria for valuable donors for Convalescent plasma therapy recommended by 'New coronavirus pneumonia diagnosis and treatment program' (6th edition) published by National Health commission of China which is also followed by various successful experimental case studies. Recovered patients selected for plasma collection will be according to the following recovery criteria: [27, 28, and 29]

1. Body temperature normality for more than 3days.
2. Respiratory symptoms resolution.

3. Two consecutive RT-PCR assays of SARS-CoV-2 should be negative.
4. Blood should be collected after 3 weeks of onset of illness and 4days after discharge from the hospital.
5. Serology tests need to be performed for HIV, anti-HBV, HCV, donor need to be seronegative for all of them.
6. Enough anti-SARS-COV-2 antibody titer should be present in plasma (at least >1:640)
7. ABO-compatibility should be highly observed.
8. Written consent form of the donor.

Successful plasma therapy: evidences from case studies:

A study performed by Duan et al. in April, 2020 carried out in hospitals of China. Severely ill 10 patients were given Convalescent Plasma Therapy.[27] Results showed that a dose of convalescent plasma has significantly increased neutralizing antibodies in the patients. After 03 days of convalescent plasma therapy, patient's clinical symptoms were improved leading to virus elimination from the blood within 07 days. Decrease in lung lesions were also reported through radiological examination.[27]

In another study by Shen et al.[5] severely ill patients were administered convalescent plasma containing neutralizing antibodies.[28] Results turned out beneficial. In 04 out of 05 patients, body temperature normalized within 03 days. SOFA (sequential organ failure assessment) score (high SOFA score indicates severe illness, range 0-24) was successfully decreased along with decreasing viral loads. Within 12 days of Convalescent plasma transfusion viral loads of the patients became negative.[28] Moreover, above mentioned studies did not report any adverse event of convalescent plasma therapy. However, a donor selection criterion is recommended to be followed properly.[28]

Discussion

Various methods of diagnosis and their importance are already discussed in the article. Molecular testing and serological assays both are important. As CoVID-19 has strong infectivity, so a rapid and accurate diagnostic technique is required for identification, isolation and treatment of patients. Currently RT-PCR is acting as the fundamental test to detect exposure and infection. It has more specificity and accuracy than other serological testing techniques. However, expertise of the technologist performing the test is also really crucial, so a false negative test could not be reported.[20, 21, 23] Secondly, RT-PCR lacks the ability to find out who has developed the immunity before and after the onset of symptoms, here ELISA and/or CMIA comes into play and has the ability to quantify the amount of antibodies produced in the form of titers. Studies revealed that in very early days of infection the detection rate of the disease of PCR was 51.9% while that of ELISA and CMIA was 98.6%. For screening purpose among different serological techniques CMIA has found to be more reliable and more appropriate. ICT has comparatively low sensitivity and specificity than ELISA and CMIA. Average sensitivity and specificity of ELISA has been reported as 84.90% and 98.63% respectively while CMIA turned out to be 89.36% sensitive and 99.63% specific in Convalescent as well as asymptomatic phase.

Among different management strategies for CoVID-19 mentioned above, use of monoclonal antibodies and CP administration found out to be more suitable as compared to different immunomodulatory therapies i.e. use of corticosteroid and interferon. As mentioned above use of these immunomodulatory agents has not been approved by WHO as treatment agents as these are only useful if use in early stage of the infection. But compared to these, use of monoclonal antibodies and CP therapy is useful. Monoclonal antibodies are found out to be beneficial especially in controlling and minimizing the cytokine storm and lowering the adverse effects of over exaggerated immune response. Hence, cytokine storm and viral sepsis that tend to increase mortality rate of the disease are better to be treated with monoclonal antibodies than immunomodulatory therapies. Besides monoclonal antibodies, administrating convalescent plasma is also a very useful way in neutralizing viral antigen. Many successful studies revealed it to be one of the best ways in recovery of the patients.

For the purpose of administrating convalescent plasma to cure patients, CMIA and ELISA can be considered best techniques to detect immunity development in recovered individuals due to their higher specificity and sensitivity. The data helps doctors in finding suitable sources of convalescent plasma for

therapeutic purpose which is comparatively a better option in the absence of approved and specific antiviral therapy as of now.[28] The optimal dosage of convalescent plasma and criteria of donor selection were different in different studies which further needs to be clarified if this treatment strategy is to use in future.

Conclusion

Immunologic testing especially 'CMIA' and 'ELISA' are better options for screening individuals for CoVID-19 for being relatively cost effective and having more specificity and sensitivity, and it does not require any special skills. However, RT-PCR is still the gold standard in diagnosing SARS-CoV-2. The techniques are also useful in checking the level of antibodies in recovered patients so their plasma can be used for CP therapy. CP therapy and anti-IL-6 monoclonal antibodies can, so far, be considered better treatment options for CoVID-19 especially if there is no approved anti-viral drug is available.

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