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Review Article

Diagnostic and Prognostic roles of Biochemical Investigations in Covid-19

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A R T I C L E I N F O

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A B S T R A C T

Corona virus disease-19 or Covid-19 pandemic is a global crisis at this point of time. The spread of this infection is so rapid and uncontrollable that 22,878,350 people have got infected within this period of time worldwide. In India the situation is worsening day by day. Fortunately, the mortality rate is lower in India compared to other countries as per the statistics. Wearing mask, frequent hand sanitization and social distancing are the key practices to prevent the transmission of the infection. Strategic lockdown, tracing out positive cases, mandatory quarantine for who get exposed and many other measures have been implemented to control the situation. But it has become inevitable to confront the disease now as vaccine is under trial. RTPCR as a diagnostic test is the gold standard for Covid-19, but it has some limitations also. From sample collection to result interpretation, it requires expertise and specific training. On the other hand, serological tests like assays of IgM, IgG or total antibodies against Covid-19 are easier to perform from patient's blood sample. These procedures are simple like other routine blood tests. But these tests are not suitable for early diagnosis of Covid-19 since the antibodies against this virus appear in the blood later, in the middle and last stages of the disease. These are effective for serological survey of the community. In the treatment strategy of Covid-19, biochemical investigations have important role as prognostic markers. Hospitalization, severity assessment and decision making for ICU admission can be possible effectively by reviewing these biochemical markers. Hyper stimulation of pro-inflammatory cytokines or Cytokine storm is the underlying immunological event that causes the severity in Covid-19 cases. Disseminated intravascular coagulation (DIC) and Acute respiratory distress syndrome (ARDS) are the after effects of such events. Various studies and surveys say that pro-inflammatory marker IL6 along with CRP is a good indicator of disease severity. Serum LDH level is a good predictor of hospital stay of the patient. D-Dimer is another biomarker which rises in blood due to hyper coagulable condition of the body caused by Covid-19. High sensitive Troponin I level in the blood is found high in positive Covid-19 patients having cardiovascular complications. There are many other parameters like serum albumin, ferritin, aminotransferase, creatinine, platelet count etc. whose blood levels are seen to be associated with the disease severity. These markers can be used adjunct to the clinical and radiological examinations in the management of Covid-19 patients.

Introduction:

The world is witnessing the threat to mankind as Covid-19 pandemic which began in Wuhan of China in December 2019.Corona virus is a group of viruses of 7 different strains which can infect human body, but common strains are 229E, NL63, OC43 and HKU1.WHO has announced Corona Virus Disease 2019 as Covid-19 in February 2020 [1] and it has been declared as pandemic officially.Covid-19 is unique for its rapidity to spread the infection. Clinical features vary from asymptomatic cases to cases lead to death. The incubation period is 2-14 days. A person gets infected from another infected person mainly from respiratory droplets.[2] It also spreads via infected fomites. The common symptoms are fever, cough with sneezing and breathlessness and acute viral pneumonia in moderate to severe cases. Atypical symptoms include anosmia, tastelessness, headache, bodyache, Gastroenteritis etc. Now a day most positive cases are found asymptomatic. According to the scientists this RNA virus of Covid-19 is undergoing self mutation very rapidly. So currently no vaccine or specific treatment is available to cure the disease. Only supportive treatment is the option for patient management. Frequent hand sanitization, wearing mask and social distancing of at least1 meter are the recommended guidelines for prevention of the spread. But still the infection rate is rising day by day. Currently the infection rate is 87.84 per 1 lakh in the world and 17.32 per 1 lakh in India.The low infectivity rate in India may be due to lower testing rate or may be due to natural immunity. The death rate is also lower in India0.49 per lakh compared to the world which is 5.17 per lakh. Death rate is highest in Great Britain 59.62/lakh. It is may be due to the percentage of aged people is higher in those countries having higher mortality rate. So it has become much

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essential to formulate some ways to diagnose the disease at the earliest of the infection as well as monitor the prognosis of the infected patients by biochemical investigations. In India with limited resources, reliable laboratory diagnosis and patient monitoring is a big challenge to the battle against Covid-19. The comparative aspects of various diagnostic tests to detect Covid-19 and the role of many biochemical parameters to assess and monitor patients suffering from this disease are discussed in this article.

Laboratory investigations for diagnosis

Real time Reverse Transcription PCR :

Real time Reverse TranscriptionPCR or rRTPCR is the most sensitive and specific method till date to identify Covid-19 from naso-oropharyngeal swab of a patient. Sputum and BAL fluid can also be used as specimen for testing by rRTPCR method. This method confirms the diagnosis in a qualitative way as well as can quantify the viral load. The sample collection is very critical in this case. First of all the sample collectors should wear Personal Protective Equipment (PPE) with proper training of using it otherwise the virus will spread to the collector. If the swab is not taken properly or if the virus shedding remains low at the time of collection, the RRTPCRmay give false negative result. rRTPCR detects various genes of Covid-19 for detection. Open reading frame 1ab gene (ORF 1ab), Nucleocapsid protein gene (NP), E gene, S gene, Nsp 14 gene, RdRp gene etc. (Fig 1)

Limitations of rRTPCR :

In spite of being the gold standard method for diagnosing Covid-19, rRTPCR has some limitations. The whole process has two main steps. First is, RNAextraction from the sample. That can be done manually or through automated system. But one should be very cautious to perform the extraction process in a RNAsefree environment and to keep in mind that RNAitself is very labile unlike DNA. The second step is RRTPCR proper. Both the processes demand expertise and efficiency. Everylab has to standardise its own PCR system on the basis of which particular gene is to be amplified and to make a stringent Standard Operating Procedure(SOP)of that. WHO has published some protocols in their website (Fig 3). Another thing is, sample transportation which is to be carried out with all safety measures to prevent contamination as the respiratory samples are highly infectious. The time required for all these, increases the TATof the RRTPCR test which itself takes 3 to 4 hrs to be completed. So the overall TATmay goes up to 2 days when there is huge number of samples to be tested in batches. Sample storage is also crucial. Specimen can be stored at 2-8°C up to 72 hrs and if delay in testing or shipping is expected the sample should be stored below -70°C. The cycle threshold (Ct) value is the determinant for result

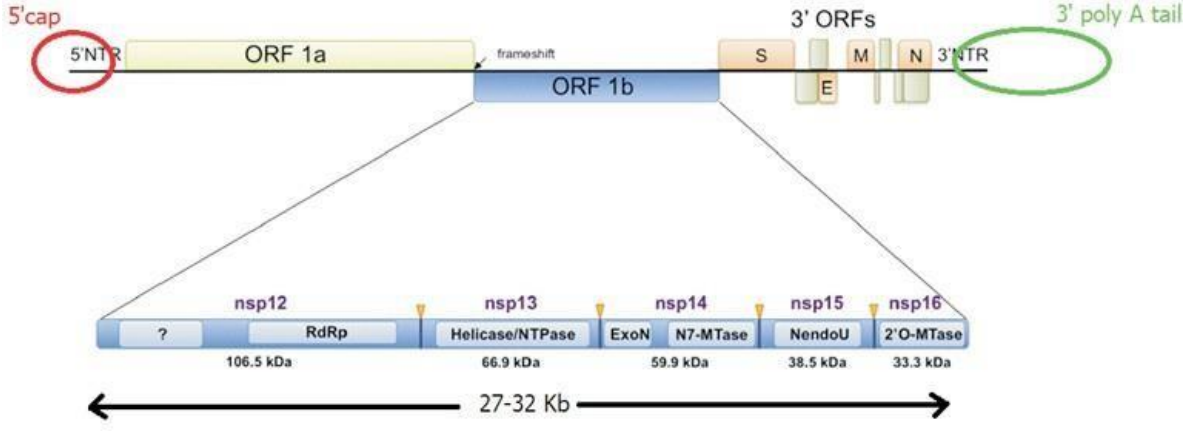

Figure 1 : Corona virus genome

A study had been conducted by R. Liu et al to see the positivity rate among the different types of sample for testing along with which gene was opted for Covid-19 detection. The positivity rate is highest when detection is done by identifying ORF 1ab gene as per their study. It can also be noticed in that study that maximum positive cases are seen from BAL fluid then from sputum and then from nasopharyngeal swab. So it is clear that the chance of false negativity is higher when nasopharyngeal swab is taken for testing. (Fig 2)

interpretation. A Ct value less than 37 is considered to be negative and value more than 40 is declared as positive, but the ct value varies from lab to lab. Point of care PCR technology also has come in the scene. This PCR gives result within 1 hr and the test can be performed on bedside. Isothermal PCR reaction takes place here, so no need of temperature to rise to perform the polymerization. This technique uses Loop mediated

Nuclear Acid	Sputum (n=57)		Bronchoalveolar Lavage Fluid (n=5)		Nasal and Pharyngeal Swabs (n-4818)		Total	TotalPositiveRate
	n	Positive Rate	n	Positive Rate	n	Positive Rate		
NP	28	49.12%	4	80.00%	1910	39.64%	1942	39.80%
ORFLAB	29	50.88%	5	100.00%	1066	40.81%	2000	40.98%
DOUBLE POSITIVE	28	49.12%	4	80.00%	1843	38.25%	1875	38.42%

Figure 2 : -CoV-2 NAT positive rate of 4880 cases with their respiratory specimens by RT-PCR. [17]

amplification (LAMP) and the detection by photometry or fluorometry or by lateral flow detection[12,13].

Institute	Gene Targets	Amplicon Size (bp)	Sensitivity	Specificity	Concentration/Volume of Reagents	Does the Protocol Recommend Specific Kits?
China CDC	ORF1ab gene	NR	NR	NR	NR	NR
	N gene	NR	NR	NR	NR	NR
Institute Pasteur, Paris, France	RdRp: nCoV_IP2 gene	108 bp	95% hit rate for approx. 100 copies of RNA GE. LOD for $1x10^5$ RNA copies is ~21 cycles LOD for $1x10^4$ RNA copies is ~30 cycles	No cross reactivity	Final concentration of 0.4 µM of each primer and 0.2 µM of probe	RNA extraction via NucleoSpin Dx Virus and Invitrogen SuperscriptTM III Platinum®
	RdRp: nCoV_IP4 gene	107 bp				
	E gene	125 bp				
US CDC, USA	N1 gene	71 bp	LOD: $1x10^{0.5}$ RNA copies/µL and 10 RNA copies/ µL for Qiagen EZ1 and Qiagen respectively.	Probe showed high sequence homology with SARS coronavirus and Bat Sars-like coronavirus	20 µM primers, 5 µM probe; 15 µL total volume	For the RT-qPCR TaqPathTM 1- Step RT-qPCR Master Mix. For extraction, they recommend bioMérieux NucliSens® systems, QIAamp® kits, QIAGEN kits, Roche Kits and Invitrogen kits
	N2 gene	67 bp				
	N3 gene (removed from diagnostic panel 3/15/20)	72 bp				
National Institute of Infectious Diseases, Japan	N gene	NR	Average Cq value of specimen was 36.7 and 35.0 for the positive control (500 copies of RNA transcript)	NR	1 µL of 20 xprimer and probe mix in a 20 µL reaction with 5 µL of RNA. F primer at 500 nM, R primer at 700 nM, probe at 200 nM.	RNA extracted using QIAamp viral RNA mini kit (Qiagen). Reverse transcription via Super Script IV Reverse Transcriptase (Thermo). RT-PCR via QuantiTect Probe RT-PCR Kit (Qiagen)
Charité, Germany	RdRp gene	NR	LOD: 3.8 RNA copies/ reaction, 95% hit rate; 95% CI: 2.7-7.6 RNA copies/reaction	No reactivity with other human respiratory viruses	RdRP: F-600 nM/reaction, R-800 nM/rxn, P-100 nM each/ reaction.	RNA extracted using MagNA Pure 96 system (Roche), RT-PCR via Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen).
	E gene	NR	LOD: 5.2 RNA copies/reaction, at 95% hit rate; CI: 3.7-9.6 RNA copies/reaction		E gene: F-400 nM/r reaction, R-400 nM/ reaction, P-200 nM/ reaction	
HKU, Hong Kong SAR	ORF1b-nsp14 gene	132 bp	NR	No reactivity with respiratory cultured viruses and clinical samples.	10 µM primers, 10 µM probes	QIAamp Viral RNA Mini Kit or equivalent and TaqMan Fast Virus Master mix.
	N gene	110 bp				
National Institute of Health, Thailand	N gene	NR	Positive control detected at less than 38 cycles.	NR	40 µM primers, 10 µM probe	Macherey-Nagel Nucleospin RNA virus and Invitrogen superscriptTM III Platinum One-Step Quantitative

Figure 3 : Summary table of available protocols posted to the WHO's website. [18]

Serological assays in Covid-19 detection:

Serological test comprises of IgM and IgG detection from blood. Some companies have brought total antibodies or anti Covid-19 antibody in the market. Serological tests are much more convenient to perform than rRTPCR. First of all these tests are done from blood. So sample collection is easier and also comfortable for patients than swab collection. The chance of exposure for the phlebotomists is little bit less when blood sample is collected just like other routine tests. The transportation is also less crucial as the antibodies in the blood are much more stable than RNA in the sample. The antibodies remain distributed in the blood homogenously, so chance of detection is higher. In rRTPCR the detection probability depends on viral particle shedding during collection and the rate of virus shedding is not uniform. rRTPCR cannot detect those cases where the viral load is very low below the detection level. But antibodies will be produced sufficiently in the body against even low viral infection and so serological tests can give positive results. Basically for asymptomatic cases serological tests can yield better outcome to assess the immunity of the population [15]. The sensitivity and specificity are higher in case of IgM assay (73% and 100% respectively) than IgG (70.8% and 96.6% respectively).Positive predictive value is also higher in case of IgM but negative predictive value is better in IgG assay. Antibodies come later in the blood so it is not very helpful for early diagnosis of Covid-19. IgM and IgG antibodies start rising after 4-5 days after development of the symptoms. Some data have shown that IgM antibody increases considerably after 9 days post symptomatic and stays for more than a month. The IgG Covid-19 antibody becomes evident in the blood after 12 days of manifestation of symptom[14].So it is obvious that the antibodies of Covid-19 appear in the blood mostly in the middle and later stage of the disease (Fig 4).The rise of antibodyresponse is also veryslow in humans.PMCID published a data on 19th April 2020 which showed that66 Covid-19 pneumonia patients confirmed by nucleic acid test had been evaluated for IgM and IgG assays in the disease course of 13 to 29 days post symptomatic. They found IgM positivity rate 77% and IgG positivity rate 83 %. The Nucleocapsid protein of SARS Cov-2is generally used for coating antigen for ELISA or for other immunoassay platforms. This protein shows cross reactivity with other non cov-2 viruses[16]. So as a

diagnostic test the serological assays are not very beneficial. But combination of rRTPCR and serological assays can give better diagnostic approach (Fig 5).

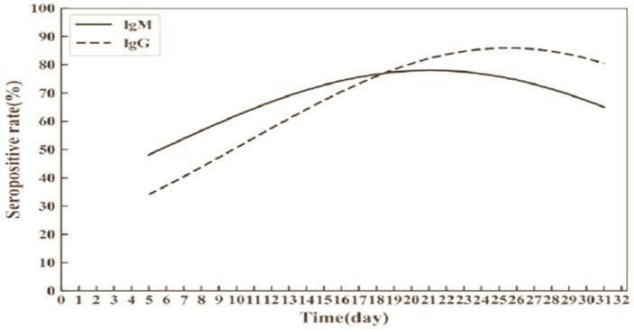

Figure 4: Detection of IgM and IgG antibodies in different periods

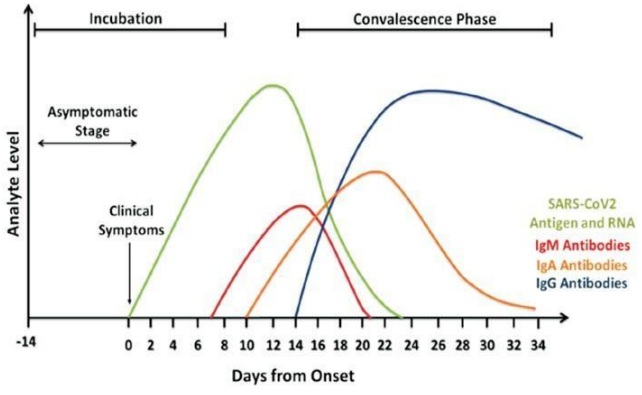

Figure 5: Serological assays during Covid-19 infection

Role of biochemical investigations in patient monitoring

After diagnosis, the biochemical assays can predict the clinical course of the disease which helps in patient management. In fact the role of biochemical assays start after diagnosis of Covid-19 for patient monitoring and prognosis and also for therapeuticinterventions.

Cytokine storm and Covid-19

In severe form of the disease the pathogenecity occurs due to excessive release of pro-inflammatory cytokines which is called Cytokine Release Syndrome (CRS) or cytokine storm.This initiates various serious immunological events like Disseminated Intravascular Coagulation (DIC) that leads to cardiac and renal shutdown, hepatic failure and severe hypoxic injury to every cell of the body. The patient succumbs due to multi organ failure. Patients with moderate to severe Covid-19 infection show DIC because of this cytokine storm. Proinflammatory cytokines like IL 1 IL 2 IL 6 and TNF alpha induce expression of tissue factor on lymphocytes that triggers the increased stimulation of the clotting cascade. These pro-inflammatory cytokines suppress anti coagulant system also by inhibiting the activity of protein c protein s and thrombomodulin system. Increased expression and release of PAI-1 protein has also been observed in this hyper cytokine situation and it interferes with dissolution of clot formed in the body. Another devastating event develops in those patients is the accumulation of exudative fluids in various organs mainly in lungs causing Acute Respiratory Distress Syndrome (ARDS). They are responsible for most of the deaths worldwide in this pandemic. This hyper-inflammatory event is a severe autoimmune condition observed in severe cases of Rheumatoid arthritis, Tuberculosis and in someviral infections like HIV, SARS and Covid-19 etc. This phenomenon is known as Hemophagocytotic Lymphocytohistiocytosis (HLH). Cytokine storm and Macrophage Activation Syndrome (MAS) are secondary manifestation of HLH, known as sHLH. There are many pro inflammatory and inflammatory cytokines present in the immunological system e.g IL 1B, IL 2, IL 6, IL 8, IL 17, TNF alpha etc. Most are seen to remain high in Covid-19 pneumonia and has been observed to become low after successful treatment. So they can predict the disease prognosis very well. Out of those cytokines, only IL 6 is found tobe mostlyused prognostic marker.

T cell function in Covid-19

Another important immunological adversity happens in Covid-19 is decrease in T cell count. Main reason is again excessive release of pro-inflammatory cytokines. IL 6 and TNF alpha causes increased apoptosis of CD4 and CD8 T cells. IL 10 induces pronounced expression of some receptors like programmed cell death 1 (PD 1) and T cell immunoglobulin and mucin domain containing 3 (TIM-3) on T cell and all these causes inhibitory effect on T cell functions. Another phenomenon is seen here T cell exhaustion which is a state of non functional and ineffective T cell undergoing rapid destruction.

Haematological and Biochemical markers

Apart from the cytokines, there are some haematological and biochemical markers also to monitor the disease outcome. It has been observed that thesepatients are having leucocytosis with Neutrophilia and Lymphocytopenia. Increased Neutrophil Lymphocyte ratio (NLR) isa good indicator of severity[5]. Platelet count shows decreasingtrend. Among the biochemical markers CRP, LDH, Ferritin, D-Dimer and IL 6 are observed to increasealong with disease severity as like Cardiac troponins, serum creatinine. Serum albumin level falls in Covid-19 infected patients. Some more markers are there like liver aminotransferases, Procalcitonin, Prothrombin time etc. which are not so good indictor but help in monitoring disease progression.

C-reactive protein and Interleukin 6:

C-reactive protein (CRP) is produced in the liver in response to any inflammation. It is a direct product of IL 6.In Covid-19 pneumonia it is observed that CRP values can delineate disease severitybetter than radiological findings like CT scan of lung [3]. Two studies have shown the severity stratification in the same manner only the ranges of values are little bit different. Li H. et al. and Wang et al. both have categorized the disease into mild to moderate, severe and critically severe stages according to the serum CRP levels (Fig.6). Similarly Chen et al. and Diao et al. have worked on blood IL 6 levels to assess the risk of disease severity. Chen et al. also studied on serum IL 2R level. These studies have highlighted the role of these biochemical assays in the decision making of a Covid-19 infected patient to be admitted in the ICU or not. These are the severity markers for Covid-19 patients. In one hand these tests are saving the life indirectly and on the other hand they can curtail the burden of unnecessary hospitalisation (Fig 7). Serum Amyloid A is also found to be raised in Covid-19 but its specificityis doubtful[4].

Mild to moderate	Severe	Critically severe	References
33.22 ± 32.2	66.04 ± 44.89	97.44 ± 58.60	Li H, et al.
2020 1.52 ± 1.56 (mild) 16.76 ± 18.38 (moderate)	54.15 ± 1.06 (severe)	105.00 ± 12.73 (critical)	Wang et al. 2020

Figure 6: CRP levels in mg/L in Covid-19 patients.

IIncreased expression of IL-2R and IL-6 in serum is expected to predict the severity of COVID-19	34 ± 7 (less severe)	72 ± 12 (More severe)	Chen et al (2020)
Significantly higher baseline levels of IL-6 in those requiring ICU compared to those who do not	51 ± 7.4 (not requiring ICU admission)	186 ± 28.3 (Had to be admitted to ICU)	Diao et al (2020)

Figure 7: IL6 levels in Covid-19 patients.

LDH and disease severity:

LDH is generally increased in the blood after tissue damage. So in severe Covid-19 cases serum LDH level rises significantly[6]. Study has shown that the duration of ICU stay is positively associated with LDH value more than 250 U/L. Excessive tissue damage caused by sars cov-2 infection delays the recovery, so monitoring the LDH level patient can be shifted from ICU to HDU or general ward. Xiong et al have shown that there is positive correlation between LDH level and early lung damage which are evidenced by the findings in CT scan of chest in Covid-19 positive patients.

D-Dimer:

D-Dimer or Fibrin degradation product is generated due to blood clot lysis after formation of blood clot in the body[7]. The fibrin cross links come in the circulation after clot lysis when the body remains in hypercoagulable state. Studies state that D Dimer value more than 2 microgram/ml has very critical outcome[8]. Those patients may deteriorate anytime and should be transferred to ICU immediately. It is now well known fact that moderates to severe form of Covid-19 cases cause microclot formation in most of the organs particularly in lungs so high D-Dimer value is associated with verypoor outcome.

Platelet count:

A low platelet count along with decreased serum albumin level and high crp value is associated with increased mortality. Platelet count below 1,40000 / cu mm is a additive indicator for disease severityin sars cov-2.

Cardiac Troponins:

Death due to cardiovascular diseases is much high in Covid-19 positive patients[9,10,11]. Many patients have died in cardiac arrest after being infected by covid-19. It is happening due to hypercoagulable state of the body causing micro infarcts in every tissue of the body including heart tissue. So cardiac troponins have become a potent marker for disease severity caused by Covid-19. High sensitive (hs) Troponin I has shown significant association with disease mortality. Shi et al have shown that hs Troponin I value less than 0.006 microgram/lit is positively linked to the survivors where value between 0.08 to 1.12 microgram/lit is associated with non survivors in Covid-19. hs Troponin I monitoring also helps in decision making to admit the patient in ICU and to start inotropes and vasopressor drugs as soon as possible.

Serum creatinine:

We have seen patients with severe covid infection manfest multiorgan failure. So the kidneys get damaged also due to micoclot formation, infarction and ultimately necrosis. So serum creatinine rises in covid-19 patients. Data from various cases conclude that serum creatinine value more than 7 mg/dl is associated with fatal outcome in covid-19 infected patients.

Discussion

This novel Corona virus has been discovered since 7 months only. So we are very new to deal with it. The discovery of the vaccine against this virus will be a milestone for mankind, but it will take time to be administered in mass. In between the time, we have to fight against the infection with our conventional medical knowledge and these laboratory biomarkers are the weapons in this battle. If we do right diagnostic test in the right time, we can identifypositive cases early. The clinical judgments can be reinforced with the help of biochemical assays to decide the treatment modalities.

Conclusion

We can save many lives if we monitor biochemical and haematological markers systematically along with radiological investigations. We can utilize our resources like hospital beds, quarantine beds, safe homes effectively if we monitor and manage patients in this way. We can categorize positive patients who require hospitalisation and who will be cured in home treatment only. Unnecessary bed occupancy can be avoided. This is the time for all medical branches to work hand by hand to get over this crisis period.

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