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COLLECTION, HANDLING AND TESTING OF CLINICAL SPECIMENS FROM PERSONS WITH COVID-19

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ABSTRACT

Corona viruses are a large family of viruses which may cause illness in animals or humans. In humans, several coronaviruses are known to cause respiratory infections ranging from the common cold to more severe diseases such as Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome(SARS) thathave a higher case fatality rate. In December 2019, a cluster of patients with a novel coronavirus was identified in Wuhan, China[1]. Initially tentatively named 2019 novel coronavirus (2019-nCoV), the virus has now been named SARS-CoV-2 by the International Committee of Taxonomy of Viruses (ICTV)[2]. This virus can cause the disease named coronavirus disease 2019 (COVID-19). The SARS-CoV-2 has demonstrated the capability to spread rapidly, posed potential public health threat and leading to significant impacts on healthcare systems globally.The COVID-19 pandemic, caused by the SARS-CoV-2 virus, has led to more than four million confirmed cases, with over 283,868 deaths globally, as of May10, 2020. In India, the first laboratory-confirmed case of COVID-19 was reported from Kerala on January 30, 2020.As of 10 May 2020, the Ministry of Health and Family Welfare have confirmed a total of 62,939 cases and 2,109 deaths in the country[3].The living and working conditions of billions of people worldwide have been significantly disrupted due to different forms of social distancing and lockdowns in many cities. The most common symptoms at illness onset are fever (99%), fatigue (70%), dry cough (60%), myalgia (44%) and dyspnoea[4,5]. Less common symptoms are headache, dizziness, diarrhoea, nausea and vomiting[6]. Symptoms such as pharyngeal pain, dyspnoea, dizziness, abdominal pain and anorexia are more likely to be present in patients with severe illnesses[5]. In addition, patients who are elderly, have underlying co-morbidities including hypertension, diabetes, cardiovascular disease and cerebrovascular disease are more likely to have adverse outcomes.

Though several trials for candidate vaccines and potential therapies are underway, there is currently no cure, and in the absence of either proven effective therapy or a vaccine, diagnostic testing becomes a valuable tool. The diagnostic testing for COVID-19 is critical to tracking the virus, understanding epidemiology, informing case management, and to suppressing transmission.

Virus isolate is the gold standard for establishment and standardization of assay performance. Since SARSCoV-2 virus isolate was not available earlier, based on the genetic sequence of SARS-CoV-2 and closely related SARS-CoV (2002-2003), the WHO shared protocols (*E, N, RdRp* and *S* genes) for screening and confirmation of probable cases[7]. Since the virus sequences were made available in public domain and source of positive controls and probes could be identified, India immediately established a network of testing laboratories for the new SARS-CoV-2 virus very swiftly. Starting with availability of validated diagnosis at the ICMRNIV, Pune, testing capacity was further upscaled to another 13 DHR/ICMR VRDLs[8]. The apex laboratory standardized the testing protocols within one week, and the VRDLs initiated testing

within two weeks of release of laboratory protocols by the WHO. Presently 332 government and 121 private laboratories working to test the COVID-19 in India.Though still limited in many areas, testing for SARS-CoV-2 is becoming increasingly available. There are currently two broad categories of testing for the virus: those that detect viral RNA and serological tests that detect the host's response to the virus.

COVID-19 SPECIMEN COLLECTION AND SHIPMENT

The clinician should decide necessity for collection of clinical specimens for laboratory testing of 2019-nCoV only after following the case definition as given by the health authorities, Government of India.Appropriate clinical sample need to be collected by laboratory personnel/ health care worker trained in specimen collection in presence of clinician.Proper collection of specimens is the most important step in the laboratory diagnosis of infectious disease. A specimen that is not collected correctly may lead to false negative test results. By following all biosafety precautions and using personal protective equipment's (PPEs), clinical samples need to be sent to designated laboratory by following standard triple packaging[11, 12].Within 5-6 days of the onset of symptoms, patients with COVID-19 infections have demonstrated high viral loads in their upper and lower respiratory tract[13, 14].

The specimen Nasopharyngeal (NP) swab/oropharyngeal (OP) swab collection guidelines follow standard recommended procedures[12]. A nasopharyngeal (NP) swab and/or an oropharyngeal (OP) swab are oftenrecommended for screening or diagnosis early infection[15]. A single NP swab has become the preferred swab, as it is tolerated better by the patient and is safer to the operator. NP swabs have an inherent quality control in that they usually reach the correct area to be tested in the nasal cavity.NP/OP sample collection swabs use only synthetic fiber swabs with plastic shafts. Do not use calcium alginate swabs or swabs with wooden shafts, as they may contain substances that inactivate some viruses and inhibit PCR testing. Place swabs immediately into sterile tubes containing 2-3 mL of viral transport media. In general CDC is now recommending collecting only the NP swab. If both swabs are used, NP and OP specimens should be combined at collection into a single vial. OP swabs remain an acceptable specimen type. **Nasopharyngeal swab*:*** Insert flexible wire shaft minitip swab through the nares parallel to the palate (not upwards) until resistance is encountered or the distance is equivalent to that from the ear to the nostril of the patient, indicating contact with the nasopharynx. Swab should reach depth equal to distance from nostrils to outer opening of the ear. Gently rub and roll the swab. Leave swab in place for several seconds to absorb secretions. Slowly remove swab while rotating it.

Oropharyngeal swab (e.g., throat swab)*:* Insert swab into the posterior pharynx and tonsillar areas. Rub swab over both tonsillar pillars and posterior oropharynx and avoid touching the tongue, teeth, and gums.

Specimens for virus detection should reach the laboratory as soon as possible after collection. Correct handling of specimens during transportation is essential. Specimens which can be delivered promptly to the laboratory can be stored and shipped at 2-8°C. When there is likely to be a delay in specimens reaching the laboratory, the use of viral transport medium is strongly recommended. Specimens may be frozen to - 20°C or ideally -70°C and shipped on dry ice if further delays are expected. It is important to avoid repeated freezing and thawing of specimens[11].

Requirements for Clinical Samples Collection, Packaging and Transport[12]

- Sample vials and Virus Transport Medium (VTM)
- Adsorbent material (cotton, tissue paper), paraffin, seizer, cello tape
- A leak-proof secondary container (e.g., ziplock pouch, cryobox, 50 mL centrifuge tube, plastic container)
- Hard-frozen Gel Packs
- A suitable outer container (e.g., thermocol box, ice-box, hard-board box) (minimum dimensions: 10 x 10 x 10 cm)

Procedure for Specimen Packaging and Transport[12]

1. Use PPE while handling specimen
2. Seal the neck of the sample vials using parafilm
3. Cover the sample vials using absorbent material
4. Arrange primary container (vial) in secondary container
5. Placing the centrifuge tube inside a zip-lock pouch
6. Placing the zip-lock pouch inside a sturdy plastic container and seal the neck of the container (***Note:*** *Sample vials can also be placed inside a zip-lock pouch, covered in absorbent material and secured by heatsealing or rubber bands. Then, the zip- lock pouch should be placedinside another plastic pouch and secured)*
7. Using a thermocol box as an outer container and placing the secondary container within it, surrounded by hardfrozen gel packs
8. Using a hard card-board box as an outer container and placing the secondary container and the gel packs
9. Placing the completed Specimen Referral Form (available on www.niv.co.in) and request letter inside a leak-proof, zip-lock pouch
10. Securing the zip-lock pouch with the Specimen Referral Form on the outer container
11. Attaching the labels: Sender's address, contact number; Consignee's address /contact number; Biological substance Category B; 'UN 3373'; Orientation label, Handle with care.

LABORATORY TESTING FOR COVID-19 VIRUS

Testing on clinical specimens from patients meeting the suspect case definition should be performed in appropriately equipped laboratories by staff trained in the relevant technical and safety procedures. Laboratories undertaking testing forSARS-CoV-2 virus should adhere strictly to appropriate biosafety practices as per National guidelines on laboratory biosafety. There is still limited information on the risk posed by COVID-19, but all procedures should be undertaken based on a risk assessment. Specimen handling for molecular testing would require BSL-2 or equivalent facilities. Attempts to culture the virus require BSL-3 facilities at minimum[16].

A. Viral RNA detection by Nucleic acid amplification tests (NAAT) for COVID-19 virus:

B. The primary test has involved directly detecting SARS-CoV-2 viral RNA through PCR testing. The first quantitative reverse transcriptase PCR tests (RT-PCR) for SARS- CoV-2 were designed and distributed to laboratories around the world in January by the WHO.Despite its shortcomings, RT-PCR remains the backbone of testing for the SARS-CoV-

2virus. It aids the identification of those who are infected, and subsequently the management of patients, and implementation of mitigation strategies for preventing the spread of the disease.

RT-PCR relies on its ability to amplify a tiny amount of viral genetic material in a sample and is considered to be the gold standard for identification of SARS-CoV-2 virus. Currently, RT-PCR tests for COVID-19 generally use samples collected from the upper respiratory system using swabs.The viral genes targeted so far include the N, E, S and RdRP genes. RNA extraction should be done in a biosafety cabinet in a BSL-2 or equivalent facility and heat treatment of samples prior to RNA extraction is not recommended[16].

As illustrated in **Figure 1,** RT-PCR starts with laboratory conversion of viral genomic RNA into DNA by RNA-dependent DNA polymerase (reverse transcriptase). This reaction relies on small DNA sequence primers designed to specifically recognize complementary sequences on the RNA viral genome and the reverse transcriptase to generate a short complementary DNA copy (cDNA) of the viral RNA. In real-time RT-PCR, the amplification of DNA is monitored in real time as the PCR reaction progresses. This is done using a fluorescent dye or a sequence- specific DNA probe labeled with a fluorescent molecule and a quencher molecule, as in the case of TaqMan assays. An automated system then repeats the amplification process for about 40 cycles until the viral cDNA can be detected, usually by a fluorescent or electrical signal[17]. RT-PCR result interpreted by initially if all two targets (nucleocapsid proteins N1 and N2) test positive, a case is considered to be laboratory- confirmed[18]. A cycle threshold value (Ct-value) less than 40 is defined as a positive test, while a Ct-value of 40 or more is defined as a negative test. A Ct-value <40 for only one of the two nucleocapsid protein [N1 and N2] is defined as in determinant and requires confirmation by retesting[18]

The COVID-19 Pandemic is the most defining health care crisis of the present times. It has challenged the health care facilities, overwhelmed the health care personnel and baffled the scientists and researchers. There is no quick fix in a pandemic of this proportion. Rapid collection and testing of appropriate specimens from patients meeting the suspect case definition for COVID-19 is a priority for clinical management and outbreak control and should be guided by a laboratory expert. Suspect cases should be screened for the virus with nucleic acid amplification tests (NAAT), such as RT-PCR. The current recommendations for laboratory diagnosis of COVID-19 infection from the CDC are that clinicians coordinate this testing with local public health authorities and/or the CDC. The preferred testing method is the real-time reverse transcription-polymerase chain reaction (real- time RT-PCR) test method[9, 10].Ensure that adequate Safety procedures during specimen collection are in use and that staff are trained for appropriate specimen collection, storage, packaging and transport[11]. All specimens collected for laboratory investigations should be regarded as potentially infectious.

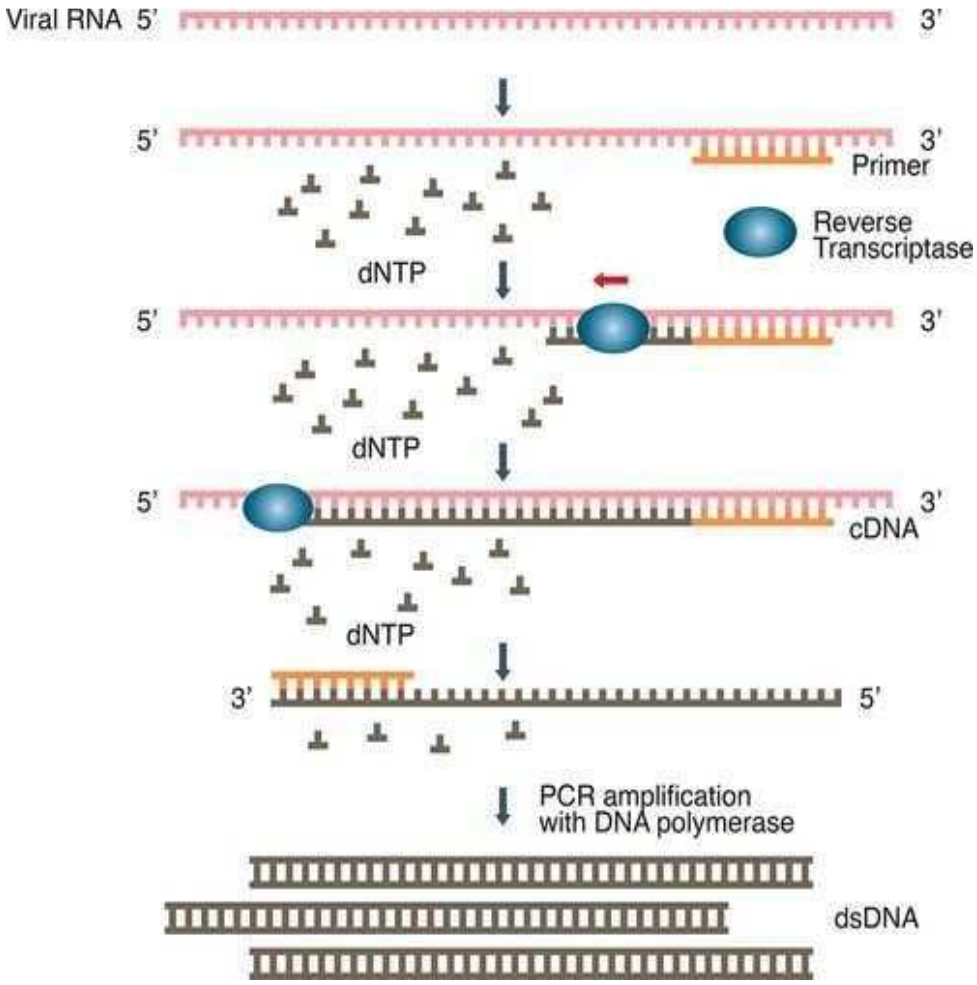

Figure 1.Reverse transcription-polymerase chain reaction (RT-PCR). The RT-PCR creates a cDNA copy of a specific segment of the viral RNA, which is converted to dsDNA that is exponentially amplified.

Laboratory confirmed case by NAATin areas where COVID-19 virus is widely spread a simpler algorithm might be adopted in which for example screening by rRT-PCR of a single discriminatory target is considered sufficient. One or more negative results do not rule out the possibility of COVID-19 virus infection. A number of factors could lead to a negative result in an infected individual, including: - poor quality of the specimen, containing little patient material (as a control, consider determining whether there is adequate human DNA in the sample by including a human target in the PCR testing)

- The specimen was collected late or very early in the infection
- The specimen was not handled and shipped appropriately
- Technical reasons inherent in the test, e.g. Virus mutation or pcr inhibition.

Each NAAT run should include both external and internal controls, and laboratories are encouraged to participate in external quality assessment schemes when they become available. It is also recommended to laboratories who order their own primers and probes to perform entry testing/validation on functionality and potential contaminants.

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SEROLOGICAL TESTING

Serological surveys can aid investigation of an ongoing outbreak and retrospective assessment of the attack rate or extent of an outbreak.Serological testing is defined as an analysis of blood serum or plasma and has been operationally expanded to include testing of saliva, sputum, and other biological fluids for the presence of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies. This test plays an important role in epidemiology and vaccinedevelopment, providing an assessment of both short-term (days to weeks) and long-term (years or permanence) trajectories of antibody response, as well as antibody abundance and diversity. IgM first becomes detectable in serum after a few days and lasts a couple of weeks upon infection and is followed by a switch to IgG.The determination of SARS-CoV-2 exposure relies largely on the detection of either IgM or IgG antibodies that are specific for various viral antigens including, but not exclusively, the spike glycoprotein (S1 and S2 subunits, receptor-binding domain) and nucleocapsid protein. Members of the coronavirus family have four structural proteins: the spike [S],membrane [M], envelope [E], and nucleocapsid [N] proteins. Two of these proteins appear to be important antigenic sites for the development of serological assays to detect COVID-19. Serological methods have focused on detecting serum antibodies against S- proteins from the coronavirus spike[19].

The other proteins that appear to be important antigenic sites for the development of serological assays to detect COVID-19 is the N protein, which is a structural component of the helical nucleocapsid. The N protein plays an important role in viral pathogenesis, replication, and RNA packaging. Antibodies to the N protein are frequently detected in COVID-19 patient[20, 21], suggesting that the N protein may be one of the immunodominant antigens in the early diagnosis of COVID-19[22].The methodology for these determinations includes the traditional enzyme-linked immunosorbent assay (ELISA) (Figure 2),immunochromatographic lateral flow assay (Figure 3).

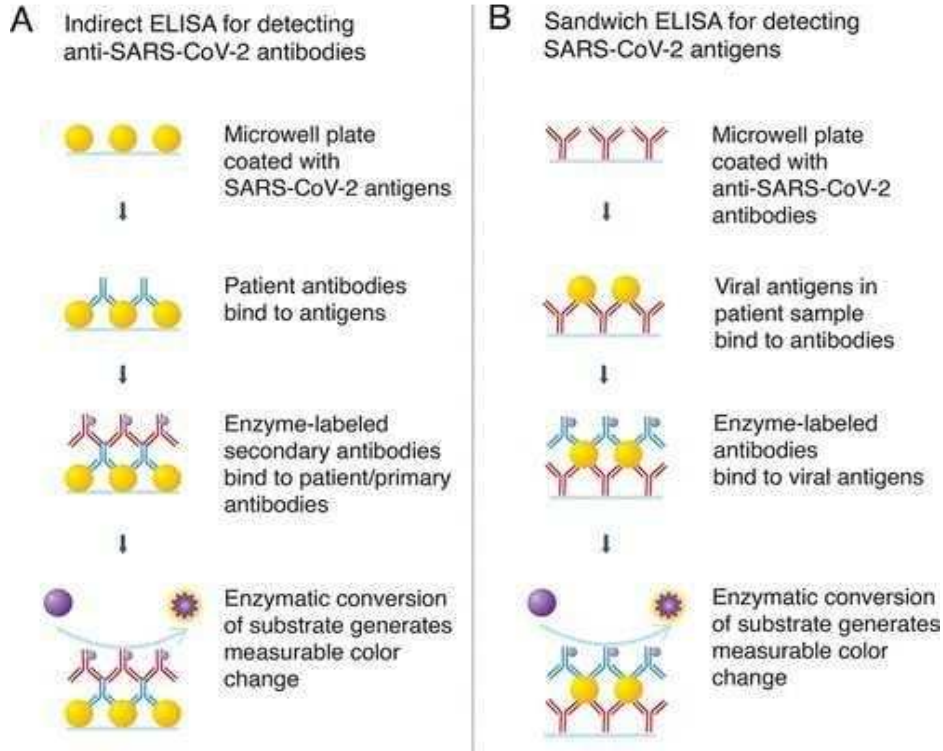

Figure 2: ELISA assays detecting antibodies (A) or antigens (B).

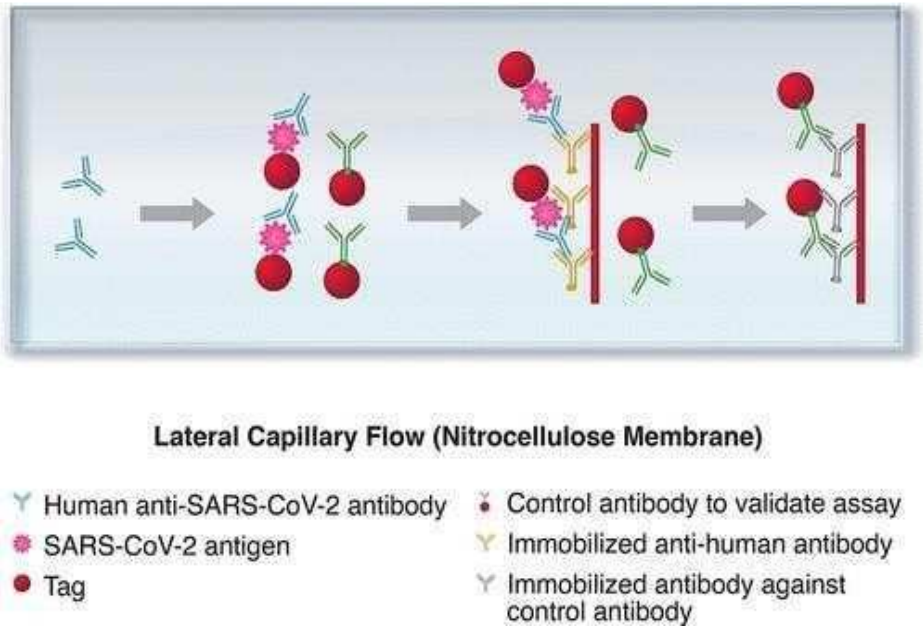

FIGURE 3 : LATERAL FLOW IMMUNOASSAY FOR DETECTION OF ANTI-SARS-COV-2 ANTIBODIES.

The lateral flow immunoassay detect samples move via capillary flow on the nitrocellulose membrane. When anti-sars-cov-2 antibodies are present, they bind to the labeled antigen and continue to move until they are captured by the immobilized antihuman antibodies. The presence of the captured antibody–antigen complex is visualized as a colored test band. The labeled control antibodies comigrate until they are captured at the control band.

Rapid lateral flow assays for antibodies (igm and igg) produced during covid-19 infection have been developed23. Seroconversion occurred after 7 days in 50% of patients (14 days in all), but was not followed by a rapid decline in viral load24. Serological methods will play an important role in the epidemiology of covid-19 and in determining the immune status of asymptomatic patients, but are unlikely to play any role in screening or for the diagnosis of early infections20, 21.

Viral sequencing : In addition to providing confirmation of the presence of the virus, regular sequencing of a percentage of specimens from clinical cases can be useful to monitor for viral genome mutations that might affect the performance of medical countermeasures, including diagnostic tests. Virus whole genome sequencing can also inform molecular epidemiology studies. Many publicaccess databases for deposition of genetic sequence data are available, including gisaid, which is intended to protect the rights of the submitting party.

Viral culture : In rare circumstances, viral cultures can also be used, but they are not normal tools in clinical practice and virus isolation is not recommended as a routine diagnostic procedure.

Reporting of cases and test results : Laboratories should follow national reporting requirements. In general, all test results, positive or negative, should be immediately reported to national authorities. All the testing laboratories should ensure real-time reporting the test results of covid-19 to icmr hq database.

CONCLUSION

The ongoing, unprecedented outbreak of covid-19 infections globally has emphasized the importance of the laboratory diagnosis of human coronavirus infections in order to limit thespread as well as appropriately treat those patients who have a serious infection.because covid-19 exhibits a range of clinical manifestations, from mild flu-like symptoms to life-threatening conditions, it is important to have efficient testing during the early stages of infection to identify covid-19 patients from those with other illnesses.

Diagnostic testing for covid-19 is critical to tracking the virus, understanding epidemiology, informing case management, and to suppressing transmission. It is ultimately important to highlight that different tests serve different purposes in the management of this pandemic: while viral rna testing enables point-of-care, acute detection of those infected with sars-cov-2, as time goes on the potential of immunological tests i.e. Elisa or lateral flow immunoassay for contact tracing and surveillance will be increasingly valued, with efforts to produce them on a large scale already beginning to ramp up.

The urgent need for accurate and rapid diagnosis of sarscov-2 infection remains critical as global healthcare systems continue to operate during the course of the covid-19 pandemic. In particular, serological and immunological testing ofinfected asymptomatic and symptomatic individuals, and their close contacts, is expected to be in high demand.

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