Formulation rationale for the development of SARS-COV-2 immunochromatography rapid test kits in India

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ABSTRACT

Humanity has been continuously threatened by epidemics and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has shown greater epidemic potential. According to the World Health Organization, measures such as rapid diagnosis, immediate isolation, and precautionary contact tracing are key tools of epidemic control. The method of detection or testing is critical in this epidemiological control, where SARS-CoV-2-positive cases are increasingly growing, leading to community infection. Immunochromatographic test kits have been described for the diagnosis of various infectious diseases because of their rapid scalability, convenient use, and prompt validation. The benefits of the immunochromatographic test kits include evaluation of the sample in approximately 20 minutes, lower cost per sample, and simple directions to use. Such test kits are composed of an uncut sheet structure that is protein conjugated, labeled with markers, and reagents coated in a nitrocellulose membrane. The need for thorough vetting is a major concern for the Central Drugs Standard Control Organization, Indian Council of Medical Research and other regulators, as more businesses are rushing to produce serologic test kits for SARS-CoV-2 worldwide. Initial reports of 86%−89% sensitivity and 84.2%–98.6% specificity were reported for these kits. Nonetheless, many national reports show variability in test accuracy significantly among various commercial suppliers. The virus's more recent mutated strains (B.1.1.7, B.1.617, and B.1.351) have also raised concerns about their detectability using these test kits. In India, manufacturers are developing rapid test kits to detect SARS-CoV-2 by importing pre-antigen or antibody-coated uncut sheets from vendors and then cutting them into strips. Such sheets also have problems with specificity and are expensive. The possibility of developing kits with an indigenous protein coating and conjugation to detect antigens and antibodies needs to be explored by Indian researchers. This communication describes techniques to develop precise rapid test kits for detecting SARS-CoV-2.

INTRODUCTION

In December 2019, the World Health Organization declared an outbreak of febrile respiratory illness of an unknown etiology detected in Wuhan, China. Later, Chinese authorities confirmed a new type of coronavirus. Several strains of coronaviruses exist, but seven of them can infect humans. These are severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2, human coronavirus (HKU1, NL63, These are

severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2, human coronavirus (HKU1, NL63, OC43, and 229E) with mild symptoms (Andersen *et al.*, 2020; Corman *et al.*, 2018; ICMRa, 2020). Since the disease outbreak, linked to the seafood market in Wuhan city, China (Cascella *et al.*, 2020; ECDPC, 2020; ICMRa, 2020), the disease has spread to over 180 countries. The infection spreads by coughing and sneezing of an infected patient or through prolonged contact with an infected patient (ICMRa, 2020). Coronavirus has caused two large-scale epidemics in the last two decades, viz., SARS and MERS found mainly in bats (Drosten *et al.*, 2003; Zaki *et al.*, 2012; Zhou *et al.*, 2020). Chan *et al.* (2020) carried out a bioinformatics-based analysis in 2019 on the latest novel coronavirus of 2019 (nCoV-2019) virus genome isolated from a Wuhan, China, patient cluster with atypical pneumonia and compared it with other associated

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coronavirus genomes. The genomes of nCoV-2019 showed 89% nucleotide identity with bat SARS-like-CoVZXC21 and 82% with human SARS-CoV. For this reason, the new virus was called SARS-CoV-2. The origin of the virus is still not clearly known. These genomic analyses suggested that SARS-CoV-2 probably evolved from a strain found in bats (Cascella *et al*., 2020).

Earlier, the most widely used standard test method for diagnosing the disease was real-time reverse–transcription polymerase chain reaction (qRT--PCR). qRT-PCR cannot be used as a point-of-care testing because it requires long-time, highly qualified workers and specialized types of equipment. Coronavirus disease (COVID-19) is currently expanding rapidly in geographical terms with almost half the population is at risk in the world. Among the various available diagnostic solutions, rapid diagnostic tests (RDTs) are the most convenient and prompt. Therefore, there is a need to develop indigenous, cost-effective, and RDT kits that are more convenient and accurate. The immunochromatographic test kits appear to fulfill the requirement as these can be rapidly scaled up during an emergency. Researchers are employing their efforts in developing effective point-of-contact test kits and efficient laboratory techniques for molecular and serological diagnosis. These kits can be used in large populations for SARS-CoV-2 screening and can verify vaccine efficacy. The few approved SARS-CoV-2 lateral flow/rapid test (serological) kits from different countries are listed in Table 1.

Immunochromatographic test kits

Lateral flow immunochromatography assay is based on antigen–antibody reactions intended to detect small quantities of biological markers or target substances in the liquid sample without any need for specialized and expensive equipment. These tests are economic, simple, easy to use, and usually give results within 5–30 minutes. The analytics are identified by antigen–antibody label complexes using different labels, where gold nanoparticles (AuNPs) are used as a standard (Manta *et al*., 2015). The principle of the lateral flow immunochromatographic strip is shown in Figure 1. The immunochromatographic test strips work on the sandwich assay. The sandwich test kit is made up of three pads (sample, conjugate, and absorbent pads) and one nitrocellulose membrane (NCM) (Zhao *et al*., 2018). An antibody coupled with AuNPs was applied to the glass fiber membrane used as a conjugated pad. The test and control sample dots of antibodies were coated on the NCM. Natural untreated glass fiber membranes and cellulose absorbent pads were employed as sample and absorbent pads, respectively (Manta *et al*., 2015). In addition to speed, price, and ease of use, the rapid chromatographic immunoassay is more acceptable because of its simplicity and modality (Li *et al*., 2020). The immunochromatographic test kit consists of several components, viz., glass fiber sample pads, AuNPs-labeled conjugate pads, NCM, test lines, control lines, adsorbent pads, polyvinyl chloride support, and proteins (Zhao *et al*., 2018).

A recent study reported by Grifoni *et al*. (2020) found that unexposed human's blood samples have 40%−60% of SARA-CoV-2-specific CD4-T cells, which leads to cross-reactivity. Therefore, to prevent cross-reactivity with prior human coronavirus (HCoV), MERS-CoV, SARA-CoV, and non-CoV infection, the selection of kit components and their masking is critical. The masking of a conjugate, absorbent pad, and AuNPs can be easily accomplished using various buffers and chemicals, as previously reported by different workers. However, the use for specific protein/reagents is a key for developing immunochromatographic test kits.

### Key components of immunochromatographic test kits

Protein

Coronaviruses are single-stranded positive-polarity ribonucleic acid (RNA) genomes that code for 9,860 amino acids containing 29,891 nucleotides. The various biomarkers are membrane (M) protein, envelope (E) protein, nucleocapsid (N) protein, spike (S) protein, angiotensin-converting enzyme 2 (ACE2), and protein generated during thrombosis. The nucleocapsid (N) and spike (S1 + S2) proteins, around which most kits are developed, are indeed the two most important proteins. Figure 2 shows the various SARS-CoV-2 RNA proteins. In a research undertaken by Okba *et al*. (2020), human serum

Table 1. Few approved immunochromatographic-based rapid diagnostic test kits for SARS-CoV-2 in different countries.

Developer	Description	Country/agency	References
Guangzhou Wondfo Biotech	IgM and IgG antibodies detection kit in 15 minutes directed against SARS-CoV-2	China and CE mark in Europe	Sheridan, 2020
Innovita Biological Technology	Lateral flow immunoassay for the detection of IgM and IgG antibodies directed against SARS-CoV-2	National Medical Products Administration EUA in China	Sheridan, 2020
Pharmact	POC 20-minute test for detecting SARS-CoV-2 and for identification of IgG and IgM antibodies	CE-marked and shipping	Sheridan, 2020
Abbott Core Laboratory	Serum/plasma/whole blood-based chemiluminescent microparticle immunoassay for IgG detection	United States	Carter *et al*., 2020
SD Biosensor	Standard Q COVID-19 IgM/IgG duo lateral flow test kits. Detects results in 10 minutes.	South Korea/India	Carter *et al*., 2020; ICMR, 2020
CTK Biotech Inc.	OnSite COVID-19 IgG/IgM lateral flow rapid immunoassay. Detects results in 10 minutes.	Australia	Carter *et al*., 2020
Everest Links Pte Ltd.	VivaDiag COVID-19 IgM/IgG rapid test. Detects results in 15 minutes.	Singapore	Carter *et a*l., 2020
PharmACT	SARS-CoV-2 rapid test detects IgG/IgM in 20 min. This uses N protein, S1, and S2 subunits used as antigens	Germany	Carter *et al*., 2020
Snibe Diagnostic	MAGLUMI IgG/IgM de 2019-nCoV (CLIA) chemiluminescence immunoassay. Provides results in 30 minutes.	Brazil	Carter *et al*., 2020
LabCare Diagnostics Ltd	COVID-19 Antigen Lateral Test Device	India	ICMRb, 2020
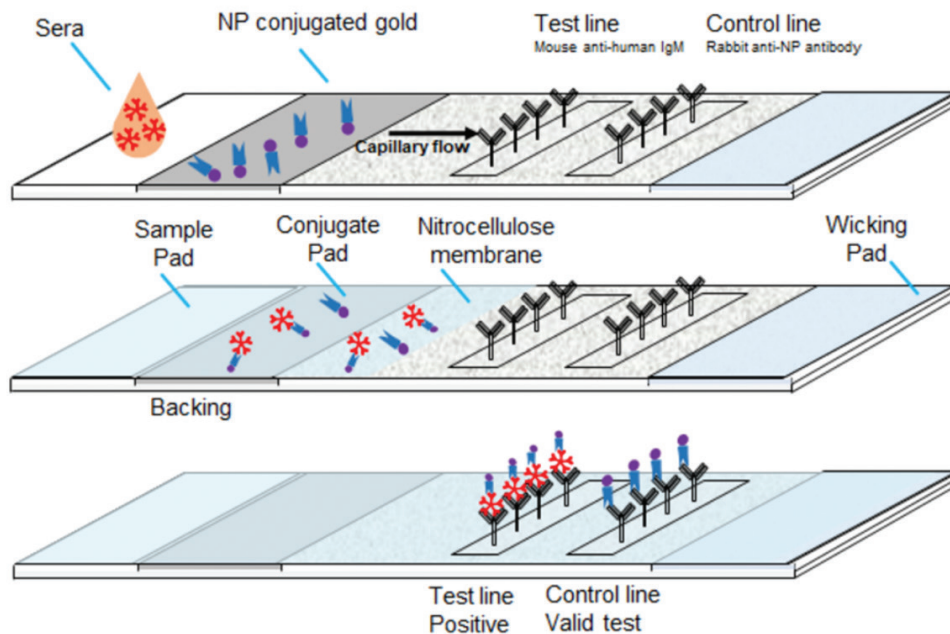
Figure 1. The principle of the lateral flow immunochromatographic test strip sandwich assay (NP = Nanoparticles; IgM = Immunoglobulin M).
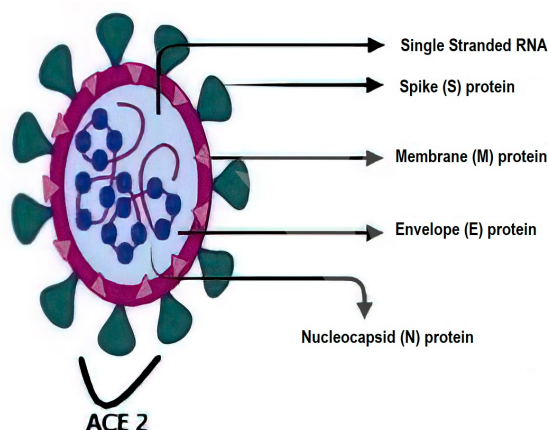
Figure 2. The different proteins of SARS-CoV-2 RNA (RNA = Ribonucleic acid; S = Spike; N = Nucleocapsid; M = Membrane; E = Envelope; ACE2 = Angiotensin-converting enzyme 2).

Figure 3. N protein domains (RBD = RNA-binding domain; IDR = Intrinsically disordered region; SR = Serine–arginine-rich; NLS = Nuclear localization signal; RNA = Ribonucleic acid).

samples infected with HCoV, MERS-CoV, SARS-CoV, non-CoV, and SARS-CoV-2 found that S1 protein has a high specificity compared to N and S2 proteins. The N protein is also highly sensitive to antibody detection. They have also observed that SARS-CoV (coronavirus-2003) cannot be separated from SARS-CoV-2 (coronavirus-2019) in enzyme-linked immunosorbent assay (ELISA)enzyme-linked immunosorbent assayenzyme-linked immunosorbent assay by both of these proteins (Kilic *et al.*, 2020; Okba *et al.*, 2020).

Demers-Mathieu *et al.* (2021) found that human immunoglobulin A (IgA), secretory immunoglobulin A (SIgA), secretory immunoglobulin M (SIgM), immunoglobulin M (IgM), and immunoglobulin G (IgG) antibodies can be induced by COVID-19 infection. The antibodies produced from SARS-CoV-2 infection in human milk react to N and S proteins and SIgM/IgM reactive to the nucleocapsid was found to be 1.2 times greater than the spike (S1 + S2) reactive protein. SIgA/IgA reactive to the nucleocapsid was 1.7 times lower to spike (S1 + S2). The detection of IgG reactive to the nucleocapsid was similar to spike protein. They do not disregard the risk of cross-reactivity either (Demers-Mathieu *et al.*, 2020).

Therefore, particular proteins need to be chosen for the production of immune chromatographic test kits based on the abundance of SARS-CoV-2 reactive antibodies to a specific protein.

N protein (nucleocapsid phosphoprotein)

The nucleoprotein is a significant antigen of SARS-CoV with abundant antigenic sites. The N protein binds to RNA genome and interacts with the viral membrane. It also helps in RNA synthesis. Figure 3 shows a representation of the N protein structure. In comparison to other viral structural proteins, the low variable N protein number, as well as its period of existence, tests virus survival. N protein of SARS-CoV-1 (2003) and SARS-

CoV-2 (COVID-19) are also very similar in their amino acid sequence. Therefore, tests developed using N protein detects both coronavirus (2003 & COVID-19) infections as they share 94% identity and 97% similarity to their amino acid sequence (Renkom Biotech, 2020).
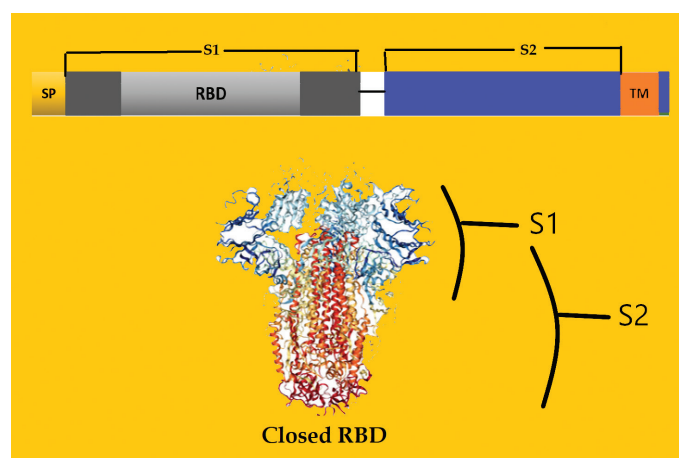
Spike protein S1 & S2

The virus envelope membrane has three or four proteins, viz. membrane (M), envelope (E), and spike (S) (de Haan and Rottier, 2005). The S protein with the 1,160–1,422 amino acid type 1 glycoprotein chain is the largest of all membrane proteins. In some coronaviruses, during maturation, the S protein splits into S1 and S2 subunits and is non-covalently associated. S1 has a globular head and S2 has a stalk-like region (Cavanagh, 1995; de Groot *et al.*, 1987; de Haan *et al.*, 2006).

S1 subunit recognizes the host-receptor binding, while S2 subunit facilitates the fusion between the viral envelope and host cell membrane. S1 has two domains in the coronavirus; one is the N-terminal domain (S1-NTD) and the other is the C-terminal domain (S1-CTD). S1 receptor binding domain (RBD) binds to the ACE2. Figure 4 shows a representation of the S protein structure. One or both of these S1 domains bind to the receptors and function as the RBD. With ectodomain S1 of the spike glycoprotein, it is possible to differentiate COVID-19 from SARS-CoV 2003, as this protein is less similar than nucleocapsid with 70% identical and 81% similarities. For better test specificity, the best option is to select the S1 region of the spike protein. Considering these two domains of S1, S1-NTD has 66% identical and 79% similarities, while S1-CTD has 79% identical and 89% similarities (Renkom Biotech, 2020). The median incubation time is estimated to be 5.1 days. IgM antibodies specific for SARS-COV-2 can be detected 3–5 days after the onset of symptoms (Biopanda Reagents, 2020).

Approaches for the development of SARS-CoV-2 immunochromatographic test kit

For developing the SARS-CoV-2 immunochromatographic test kit, three alternative approaches and combinations can be used.


Figure 4. S protein domains (RBD = Receptor-binding domain; SP = Signal peptide; SR = Serine–arginine-rich; TM = Transmembrane domain; S1= S1 subunit of Spike protein; S2 = S1 subunit of Spike protein).
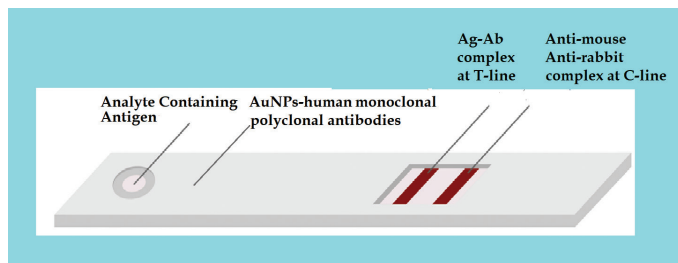
Alternative I (the antibody detection kit)

This alternative is based on antibody detection. The first method detects specimen antibodies by binding to antigen-conjugated markers. The strips will be coated with IgG/IgM mAbs (monoclonal antibodies) and IgG/ IgM pAbs (polyclonal antibodies) of N and S proteins on the "T1 and T2" test line. Figure 5 depicts the kit developed from alternative 1 module. The "C" control line will be coated with an anti-chicken IgY antibody. The test strip contains recombinant SARS-CoV-2 antigen conjugated to colored gold nanoparticles. Free colloidal gold-labeled antibodies, mouse anti-human mIgM/mIgG, and chicken IgY are present in the release pad section. Diluted serum, plasma, or whole blood samples were applied to the release pad section. The mIgM/ mIgG antibody binds to the coronavirus IgM/IgG antibodies and forms an IgM–mIgM / IgG–mIgG complex. The antibodies of the specimen move through the capillary action in the strips. If the sample contains coronavirus IgM/IgG antibodies, the test lines T1 and T2 will bind and develop the color through the IgM–IgM/ IgG–IgG complex. If there are no coronaviruses present in the sample, the free mIgM/mIgG test line will not link to T1 and T2 and no color will develop. The free mIgM/mIgG then binds the chicken IgY antibody at control line C. This control line should be clear after sample testing as it indicates the proper functioning of the kit (Franzen, 2020; Gerbers, 2013; Manta *et al.*, 2015). The Chinese CDC (center for disease control and prevention) used this kind of test kit widely during the COVID-19 outbreak in China (BioMedomics Inc., 2020). Various manufacturers also received CE + certification for the European market.

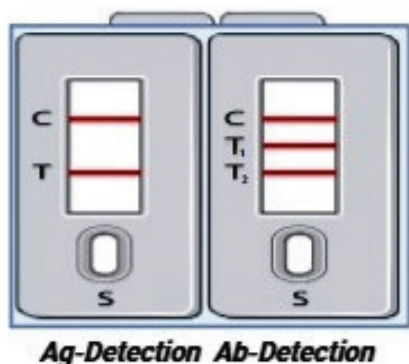*Alternative II (antigen detection kit)*

The second alternative method involves the development of an antigen detection kit. This technique is considered to be more accurate than the antibody detection method. COVID-19 antigens are detectable in the nose and throat of the individual from the onset of symptoms. In this approach, N and S anti-human monoclonal or polyclonal antibody protein is conjugated with gold nanoparticles. After collecting the sample from the nose and throat, it is diluted with the diluent and applied to the sample pad section. The antigen present in the sample pad binds to the monoclonal or polyclonal antibody and forms an antigen–antibody complex at the conjugate pad section (Fig. 6). This mixture subsequently migrates along the membrane to the test area. Then, the complex will bind to the anti-mouse / rabbit SARS-CoV-2 antigen and develop color on the test line. If there is no coronavirus antigen in the sample, the
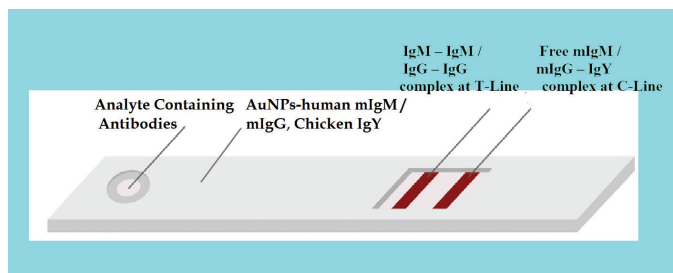
Figure 5. The alternative 1 approach for IgG/IgM antibodies detection kit (IgG = Immunoglobulin G; IgM = Immunoglobulin M; IgY = Immunoglobulin Y; m = Anti-human; AuNPs = Gold nanoparticles; T = Test line; C = Control line).

Figure 6. The alternative 2 approaches for antigen detection kit (Ag = Antigen; Ab = Antibodies; AuNPs = Gold nanoparticles; T = Test line; C = Control line).

Figure 7. The alternative 3 approaches for antigen and antibody detection kit in a single housing (Ag = Antigen; Ab = Antibodies; T = Test line for antigen detection; C = Control line; T1 = Test line 1 for immunoglobulin M detection; T2 = Test line 2 for immunoglobulin G detection; S = Sample port).

free antigen will not link to the test line and no color will develop. In such a case, the free antigen will bind to the anti-chicken IgY antibody of the control line and the color of the control line ensures that the kit is working correctly.

Alternate III (antigen and antibody detection kit in single housing)

For more precision, alternative III can be used. Both antibodies and antigen detection strips can be placed in a single house for comparative and simultaneous detection of antibodies and antigens from specimen samples. In alternative III, alternatives I and II are combined to form the test kits with two separate chambers. One section of test kits detects antibodies (Alternate I) and the other part detects antigens (alternate II) of the COVID-19. Figure 7 depicts the kit developed from alternative III module.

The test license for the manufacture of the COVID-19 kit is to be taken from the Central Drugs Standard Control Organization (CDSCO), Delhi. The test kit should be validated for sensitivity and specificity at the National Institute of Virology in Pune after it has been designed. The CDSCO is then approached for a commercial manufacturing license.

CONCLUSION

The urgent requirement in India is to meet the challenge of SARS-CoV-2. In India, there are currently no commercially available indigenous sources of reagents/protein for the production of rapid test kits. The reagents/proteins are imported for the kits designing. These imported reagents/proteins can cause false results because their selection and validation are not based upon

risk analysis. Besides, pre-protein-coated uncut sheets can lead to false results in a selected population genotype. India needs to develop indigenous reagents/proteins that can have specificity for Indian pollution genotype. The use of pre-coated uncut sheet needs to be avoided. Utilizing imported reagents/proteins for antibodies detection kits, N protein reactive to IgA, secretory IgA (SIgA), secretory IgM (SIgM), and IgM can improve test sensitivity but can have poor specificity. N protein can also not distinguish between SARS-CoV and SARS-CoV-2 infection, but this consideration can be overlooked since SARS-CoV infection occurred 19 years ago and now there is a negligible likelihood of the presence of these antibodies. Using S1 protein is a better choice to increase the specificity of kits. On the contrary, N and S proteins have no role in IgG sensitivity and specificity. In addition, the combined antigen and antibody detection kit in a single housing would be a better choice for comparative screening of SARS-CoV-2.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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