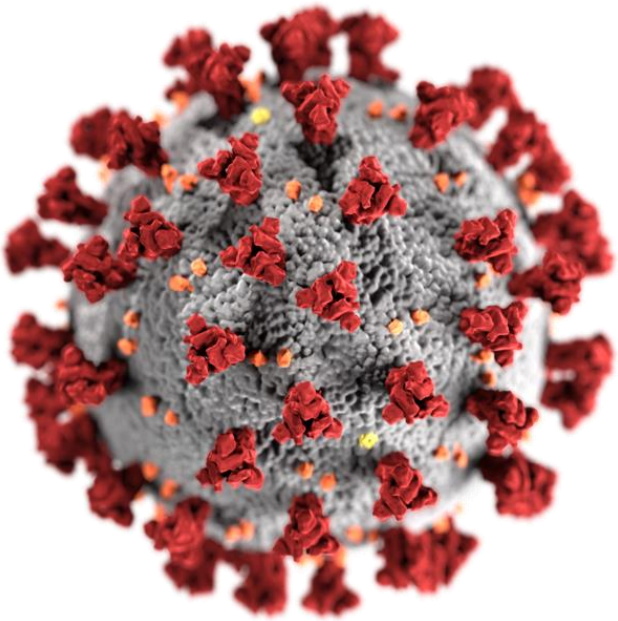

The Diagnostics of Covid-19 Infections

Tech Note

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The design and optimisation of serological tests

On April 17th, 2020, the ICMR prescribed a protocol for using rapid antibody tests for epidemiological studies and surveillance. The advisory is enclosed at the end of this document. Wherein, the rapid antibody tests play a vital role in epidemiological studies, and based on their test results, the patient care is decided along with further course of action. This document attempts to present facts before the reader, along with up-to-date scientific knowledge based on the latest information on hand, through available scientific publications duly referenced. The facts are laid out point-by-point in the document.

Introduction

In the classical infectious serology model, that is well understood through various “older infections” and “viral agents”, it is generally understood that in the first phase of the infection, i.e. Day 0 of infection, free virus (antigen) may be found in the blood stream, and as the immune response develops, it is the IgM class of antibodies that start to increase in concentration. After some delay, it is the IgG response to the viral agent that takes over to give an exponential peak in terms of concentration. So, before seroconversion, it is the antigen that could be detected, during the acute phase the IgM antibodies can be detected, and in the later phase, the IgG antibodies can be detected to provide indirect proof of exposure to and infection with the viral agent. Of course, it is understood that host immuno-competence, the viral dose at the time of exposure also play an important role in the generation of the immune response. Hence, patient-to-patient variation is expected. However, broadly, the understanding is that, presence of IgM antibodies only, signify an acute phase of infection, whereas, detection of IgM and IgG classes both may indicate an ongoing infection moving towards convalescence. Whereas, presence of only IgG class of antibodies indicates the recovery phase. It is also widely expected that, the IgG antibodies to the viral agent are protective in nature, preventing future reinfection from the same viral agent.

Sero-response in the SARS-CoV-2 infections (COVID-19)

❖ Peculiarity of sero-response to SARS-CoV-2

"During the COVID infections, various studies have shown that the sero-response does not follow the traditional sequential antibody response as observed in other viral infections and in fact, IgM and IgG class of antibodies can be detected almost simultaneously in the early phase of infection." [1]

"The positive rate of IgG reached at 100% at around 17-19 days, after the onset of symptoms, while, IgM sero-conversion rate reached its peak of 94.1% at around 20-22 days after the onset of symptoms." [1]

"This finding indicates that SARS-CoV-2 infection can be ruled out if antibody against SARS-CoV-2 is still undetectable after 20 days of onset of symptoms, or after 23 days of exposure (20 days + a median incubation of 3 days). Secondly, there is no rule for the chronological order of IgM and IgG sero-conversion…this supports the detection of IgM and IgG both simultaneously, rather than the single antibody alone." [1]

"When comparing the onset of sero-positivity between IgG and IgM, more patients had earlier sero-conversion for IgG than IgM for anti-NP." [2]

Hence, it is clear, based on the available scientific evidence, that the SARS-CoV-2 antibody response in patients during infection does not follow the classical sequential antibody switch, and, in fact, IgG antibodies in most studies have preceded or appeared simultaneously with the IgM response, hence, separate measurement of IgM or IgG via a diagnostic device is neither helpful in staging of the infection, nor grading of the disease progression since, many PCR-negative and IgG sero-positive patients have suffered reinfections, there is a valid concern whether the sero-response

is protective in nature (from reinfections) and if its measurement from this perspective has any merit.

"Furthermore, the serum IgA level positively correlates with COVID-19 severity." [8]

❖ Important role of IgA class of Antibodies

There seems to be a very strong piece of evidence that has emerged recently that, the measurement of IgA levels in patients would be of great value in the diagnosis of the SARS-CoV-2 infection.

"IgA is considered a major effector molecule in the defence mechanism against viruses and infections with respiratory viruses, can induce efficient IgA responses in secretions as well as in sera. Our data suggests that IgA antibodies are valuable diagnostic markers that show strong signals early after onset of mild COVID-19 associated symptoms, and that, IgA antibodies may be crucial for the efficient clearance of SARS-CoV-2." [2]

"We found that commercial S1 IgG or IgA ELISAs were of lower specificity while sensitivity varied between the two, with IgA showing higher sensitivity." [1]

"It is likely that SARS-CoV-2 behaves as other respiratory viruses yielding the production of protective secretory IgA efficient in asymptomatic or mild infections." [3]

"The concentrations varied widely among different patients. Median 75 concentration of IgA and IgM reached peaks at 16-20 days after illness onset at 8·84 μg/mL and 7·25 76 μg/mL, respectively, while median concentration of IgG peaked during 21-25 days after illness onset at 77 16·47 μg/mL. [8]

Hence, it is extremely clear that in the COVID-19 disease, for early detection, there is a distinct and important role for the detection of IgA class of antibodies. Hence, assay

development and design must take care that the IgA class of antibodies are not missed out by the devices used in detection

❖ Importance of detecting the Total Antibody response (IgM + IgG + IgA)

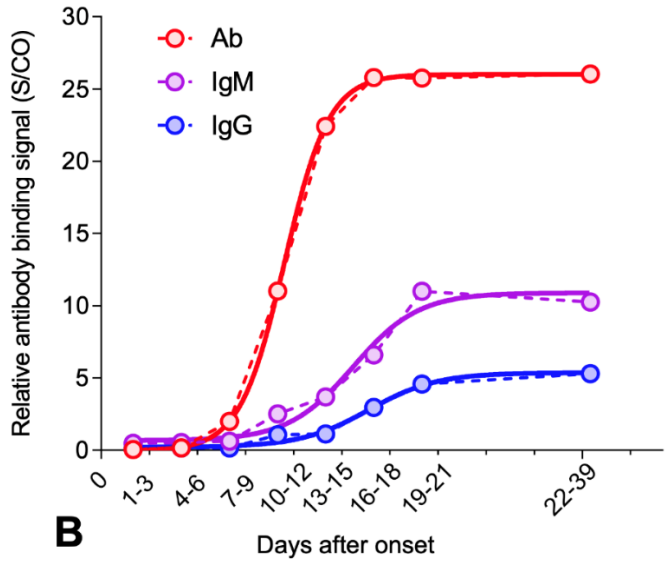
Any sero-response to a viral agent or infectious agent can be classified into IgM, IgG, or IgA, and, when these antibodies are detected together without differentiation, the term ‘total antibody’ or ‘Ab’ has been used in the references.

“The production of IgM, IgA and IgG antibodies against SARS-CoV-2 were positive as early as Day 1 after the symptom onset.” [4]

“To be expected the total antibody was first detected, followed by IgM and IgG.” [5]

“Our data showed that the sensitivity of total antibody testing is higher than IgM or IgG. Therefore, the total antibody detection should be given high priority to be implemented in current clinical and public health practice.” [5]

When the assay system detects the Total Antibody (IgM + IgG + IgA), the detection is much earlier than when individual isotypes are detected.



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Double-Antigen Sandwich Assay

"First, the authors respectively assayed all anti- SARS-CoV-2 antibodies in a double-sandwich method or specifically detected IgM and IgG. Of note, the first assay provided the best results, especially 100% positivity by day 8 in subjects with no viral RNA detectable any longer. The authors briefly suggest that this test also assessed IgA levels. This is corroborated by another recent study where 92.7% of the subjects tested presented with anti- SARS-CoV-2 nuclear capsid IgA, while only 85.4% had IgM and 77.9% IgG. Data from both publications are consistent with what is known of mucosal immune responses, characterized first by the production of secretory IgA, systemic antibodies occurring later." [3]

"Overall, the seroconversion of Ab was significantly quicker than that of IgM ($p = 0.012$) and IgG ($p < 0.001$), that possibly attributed to the double-antigen sandwich form of the assay used which usually show much higher sensitivity than capture assay (IgM) and indirect assay (IgG). Moreover, all isotypes of antibodies against viral antigen, including IgM, IgA and IgG, can be detected by double-sandwich based assay, which may also contribute to the superior performance of Ab test" [5]

"A recent study showed that the sensitivity of the total antibody (IgA, IgM & IgG) test is higher than that of the IgM or IgG test" [7]

"We believe the total antibody test would be of great help in the diagnosis of COVID-19 and the epidemic survey of SARS-CoV-2 infection [6]

"Secondly, there is no general rule for the chronological order of IgM and IgG seroconversion for a specific patient, which resembles the conditions in SARS and MERS. This supports the detection of both the IgG and IgM simultaneously rather than the single antibody alone" [6]

"Rapid detection methods are urgently required for the early diagnosis of patients with fever, and for pre-symptomatic or asymptomatic carriers infected with HCoV-19. However, nucleic acid-based molecular diagnosis tools (e.g., real-time PCR and loop-mediated isothermal amplification), depend on there being a sufficient viral load in the patient's upper respiratory tract and reasonable sample quality" [7]

"Serological assays are supposedly a powerful approach for achieving timely diagnosis of COVID-19, as is nucleic acid testing, especially for patients with undetectable viral RNA" [7]

"The GICA (gold immunochromatography) tests were highly sensitive (86.89%) and specific (99.39%), which should help with diagnosing COVID-19 in RT-PCR-negative patients who are clinically confirmed COVID-19 positive by CT" [7]

"The GICA sandwich used to detect total antibodies is a powerful complement to the current standard RNA-based tests" [7]

"Table 2. Performance of different detections in samples at different time since onset of symptoms" [5]

	Day 1 -7	**Day 8-14**	**Day 15-39**
Viral RNA	66.7%	54.0%	45.5%
Total Antibodies (IgA, IgG, IgM)	38.3%	89.6%	100%
IgM antibodies	28.7%	73.3%	94.3%
IgG Antibodies	19.1%	54.1%	79.8%
RNA+ Total Antibodies	78.7%	97.0%	100%

This table is modified for ease of understanding- Reference [5] of Bibliography

Assay design and optimisation considerations

"The combination of IgA/IgG or IgA/IgM/IgG provides improved diagnostic reliability as compared to conventional IgM/IgG combinations. In addition, we observed that IgA levels in serum correlate positively with COVID-19 severity. [8]

"Although IgM reached its peak at early stages, its detecting sensitivity is lower than that of IgA and IgG. Our data suggest that IgM has the lowest diagnostic power among

the three types of antibodies for diagnosing SARS-CoV-2. Adding IgA into a diagnostic kit that contains IgG and IgM improves the serologic testing power at both early and late stages." [8]

"Therefore, we highly recommend the use of RBD-specific IgA/IgG or IgA/IgM/IgG combinational serological test supplementing nucleic acid detection to provide a more accurate diagnosis of COVID-19. "[8]

Based on the scientific evidence available so far, it is evident that, any immune-assay, whether rapid or otherwise needs to pick up the total antibody (IgM + IgG + IgA) sero-response during the SARS-CoV-2 infection, and must be based on a double-antigen sandwich principle to enable it to do so. In the double-antigen sandwich assay it is possible to increase the antigen coating on the capture phase as well as the conjugate phase. This ensures smaller quantities of sero-responses are picked up during the assay run, simultaneously ensuring that all isotypes are picked up. Thus, a double-antigen sandwich assay provides a testing system that not only has high sensitivity but, also high specificity. Usually, in IgM-IgG differentiation assays where anti-human IgG and IgM response to SARS-CoV-2 is being measured, there is a limit as to how sensitive the detection can be since a balance has to be maintained between the conjugate density and the concentration of the anti-human IgG and IgM on the capture phase (IgM-IgG differentiation assays) being used in the testing system. Beyond a limit, if either component is increased, one might be able to improve the sensitivity but, suffer loss of specificity, which, during epidemiological studies could lead to unnecessary false positives and undesired action from the clinical and patient point of view, when used as per the algorithm specified by ICMR.

It may also necessitate unnecessary confirmatory testing by the use of resource intensive RT-PCR. Another aspect that needs to be highlighted, especially for the rapid

tests is that, the serum matrix of Indian patient population is unique, and probably contains a spectrum of diverse antibodies due to our socio-economic conditions. Hence, it is extremely important that apart from extremely conserved and key components of the assay system, the optimisation of the assay from a sensitivity and specificity point of view is done in India. Imported products which are already finished abroad and have been tested in the population of country of origin, or, manufacturers who import finished sheets where the assay cannot be changed or optimised any further would be unable to modify the assay chemistry suitably to work well for Indian patient samples and in the hands of Indian users. Hence, locally designed, optimised, manufactured, and validated double antigen assays hold the key for effective sero-diagnosis of COVID infections in India in preference over IgM and IgG differentiation assays.

The Utility of Rapid Antibody Assays alongside RT-PCR based tests

“The current method of diagnosis by qPCR or deep sequencing-based technologies rely on the presence of replicating virus in sufficient amount to ensure sufficient quantitates of virus is collected. This method often fails to detect the viral infection if collection procedure is not optimal, or if the patient has low viral load due to early stage of the disease or suppressed by host immunity, or if the samples were obtained at a late stage in the course of infection.” [4]

“Many cases that were strongly epidemiologically linked to SARS-CoV-2 exposure and with typical lung radiological findings remained RNA negative in their upper respiratory tract samples. There are four potential reasons: 1) the viral loads in upper respiratory tract samples are much lower than that in lower respiratory tract samples in COVID-19 patients. 2) the releasing viral loads of patients in different stage of infection varies

with a wide range. 3) the collection of high-quality swab specimen requires skillful health-workers; and 4) PCR reagents from different sources have high variance." [5]

"We propose to conduct antibody testing when qPCR test is negative despite other indications of COVID-19 including symptoms and epidemiology. Our data show that the supplementary IgM test can provide a better sensitivity than qPCR-based method alone. This is especially important at this stage of the pandemic, where proper diagnosis is essential to limit the viral spread." [4]

It may also be added that, anecdotal evidence points to sample collection errors, differences in LOD in different PCR reagents give rise to up to 35% false negative PCR results, thereby, increasing the role of serological tests alongside PCR results for case detection.

"Antibody test aided to confirm 4 patients with COVID-19 from 52 suspects who failed to be confirmed by RT-PCR and 7 patients from 148 close contacts with negative RT-PCR" [6]

"The 16 RT-PCR confirmed cases were positive in IgG or/and IgM. Strikingly, 7 of the 148 cases who were excluded previously by negative nucleic acid results also showed positive results in IgG or/and IgM, indicating that 4.3% (7/162) of close contacts were missed by nucleic acid test. In addition, about 6.1% (10/164) of this cohort were asymptomatic infection" [6]

"Table 3. Serological presence of antibodies against SARS-CoV-2 in patients with undetectable viral RNA at different time since onset of disease.

Days after Onset	No. of patients with undetectable RNA*	Detectable antibody in plasma, n (%)		
		Ab	IgM	IgG
1-3	7	2 (28.6)	2 (28.6)	2 (28.6)
4-7	28	15 (53.6)	12 (42.9)	8 (28.6)
8-14	57	56 (98.2)	45 (78.9)	40 (70.2)
15-39	30	30 (100)	28 (93.3)	22 (73.3)

* RNA was tested using throat/nasal swab sample." [5]

Summary

Since sero-response to SARS-CoV-2 is peculiar and unique, and, the role of IgA detection apart from IgM and IgG isotypes is important, the preferred immunoassay method for rapid diagnostics of total SARS-CoV-2 antibodies (IgM + IgG + IgA) is a double-sandwich based assay system, which, can increase sensitivity without compromising specificity. It is also extremely important due to assay design considerations that the product is designed, optimised, manufactured and validated locally, so as to serve its role effectively for epidemiological studies and surveillance as recommend by the ICMR for India.

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D.O.No. VIR/4/2020/ECD-I (Vol.I)
Dated: 17th April 2020

Addl.Chief Secretaery/Secretary/Principal Secretary Health (All States)

Sub: **Protocol for using 'Rapid antibody test' in Hot area – epidemiological studies and surveillance**

I am writing to you with reference to the rapid antibody test kits for COVID-19 testing. It is understood that many States intend to use these kits in affected areas.

2. The National Task Force at ICMR has carefully reviewed the data evolving from various countries on use of such kits. Based on available evidence, the testing strategy for COVID-19 has been revised further. The revised document is enclosed for your reference.

3. It is critical to understand the following key facts while using the rapid antibody tests:

- Gold standard frontline test for COVID-19 diagnosis is **real time PCR based molecular test**, which is aimed at early virus detection.
- The rapid antibody test cannot replace the frontline test.
- The rapid Antibody test is a **supplementary tool** to assess the prevalence of the diseases within a specific area / perimeter.
- The rapid antibody test will **only be of utility after a minimum of 7 days of onset of symptoms**.
- Data about these rapid tests is emerging and understanding of their utility for diagnosis is still evolving.
- The rapid tests are useful for **epidemiological studies and surveillance purposes**.
- THE TEST HAS TO BE DONE UNDER STRICT MEDICAL SUPERVISION.

4. The enclosed ICMR advisory is for Hot spots. In case your state does not have a Hot spot, these tests may be used for:-

a) Any hotspot which may emerge in future
OR
b) As a surveillance tool for epidemiological purposes in such areas where cases have not emerged so far.

5. Before starting the rapid test, it should be registered on covid19cc.nic.in/ICMR and data related to the test should be reported on the same.

With best regards

Yours sincerely

Balram Bhargava

(Balram Bhargava)

Enclosed: As above

CC: **Chief Secretary/Administrators**

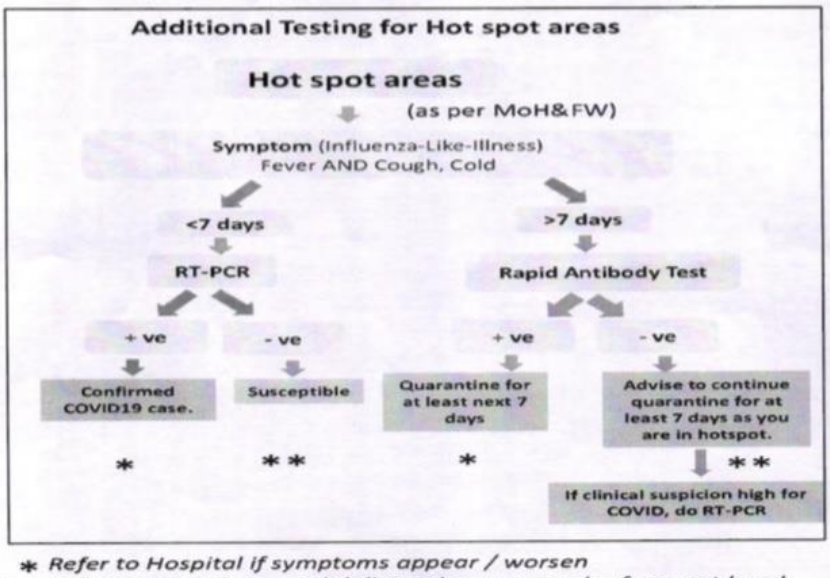
Tele.: 26588204, 26589620, Fax (Off.) : 91-11-26588662, E-mail: secy-dg@icmr.gov.in

A. COVID-19 Testing Strategy for India (Recommended for the entire country)

Real-Time PCR (RT-PCR) test and Point-of-Care molecular diagnostic assays are recommended for diagnosis of COVID-19 among individuals belonging to the following categories:

- All symptomatic individuals who have undertaken international travel in the last 14 days
- All symptomatic contacts of laboratory confirmed cases
- All symptomatic health care workers
- All patients with Severe Acute Respiratory Illness (fever AND cough and/or shortness of breath)
- Asymptomatic direct and high-risk contacts of a confirmed case should be tested once between day 5 and day 14 of coming in his/her contact

B. Additional (in addition to A) Testing recommended in hot spots



* *Refer to Hospital if symptoms appear / worsen*

** *Follow precautions, social distancing, use masks, frequent hand washing, avoid unnecessary travel)*

Balan Bhargava