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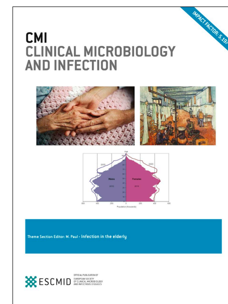

How to interpret and use COVID-19 serology and immunology tests

David S.Y. Ong, Paraskevi C. Fragkou, Valentijn A. Schweitzer, Roy F. Chemaly, Charalampos D. Moschopoulos, Chrysanthi Skevaki, on behalf of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for Respiratory Viruses (ESGREV)

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Narrative Review

How to interpret and use COVID-19 serology and immunology tests

David S. Y. Ong[1,2], Paraskevi C. Fragkou[3], Valentijn A. Schweitzer[4], Roy F Chemaly[5], Charalampos D. Moschopoulos[3], Chrysanthi Skevaki[6], on behalf of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for Respiratory Viruses (ESGREV)

1. Department of Medical Microbiology and Infection Control, Franciscus Gasthuis & Vlietland, Rotterdam, The Netherlands;

2. Department of Epidemiology, Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands;

3. Fourth Department of Internal Medicine, Medical School of Athens, National and Kapodistrian University of Athens, Attikon University Hospital, Athens, Greece;

4. Department of Medical Microbiology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands;

5. Department of Infectious Diseases, Infection Control, & Employee Health, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA;

6. Institute of Laboratory Medicine, Universities of Giessen and Marburg Lung Centre (UGMLC), Philipps University Marburg, German Centre for Lung Research (DZL) Marburg, Germany;

Corresponding author: David S. Y. Ong, MD, PharmD, PhD, Kleiweg 500, 3045 PM Rotterdam. E-mail: davidsyong@gmail.com

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Abstract

Background: Although molecular tests are considered the gold standard for coronavirus disease 2019 (COVID-19) diagnostics, serological and immunological tests may be useful in specific settings.

Objectives: This review summarises the underlying principles and performance of COVID-19 serological and immunological testing.

Sources: Selected peer-reviewed publications on COVID-19 related serology and immunology published between December 2019 and March 2021.

Content: Serological tests are highly specific but heterogeneous in their sensitivity for the diagnosis of COVID-19. For certain indications, including delayed disease presentations, serological tests can have added value. The presence of antibodies against SARS-CoV-2 may indicate a recent or past COVID-19 infection. Lateral flow immunoassay (LFIA) antibody tests have the advantages of being easy and fast to perform, but many have a low sensitivity in acute settings. Enzyme-linked immunosorbent assay (ELISA) and chemiluminescence immunoassays (CLIA) have higher sensitivities. Besides humoral immunity, cellular immunity is also essential for successful host defences against viruses. Enzyme-linked immunospot (ELISpot) assays can be used to measure T-cell responses against SARS-CoV-2. The presence of cross-reactive SARS-CoV-2-specific T-cells in never exposed patients suggests the possibility of cellular immunity induced by other circulating coronaviruses. T-cell responses against SARS-CoV-2 have also been detected in recovered COVID-19 patients with no detectable antibodies.

Implications: Serological and immunological tests are primarily applied for population-based seroprevalence studies to evaluate the effectiveness of COVID-19 control measures and increase our understanding of the immunology behind COVID-19. Combining molecular diagnostics with serological tests may optimise the detection of COVID-19. As not all infected patients will develop antibodies against SARS-CoV-2, assessment of cellular immunity may provide complementary information on whether a patient has been previously infected with COVID-19. More studies are

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needed to understand the correlations of these serological and immunological parameters with protective immunity, taking into account the different circulating virus variants.

Word count abstract (maximum 300): 294

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Introduction

Diagnostics for coronavirus disease 2019 (COVID-19) are mostly performed in case of a suspected acute respiratory infection or for screening of asymptomatic cases as part of outbreak management. Both aim to detect COVID-19 during the early phase of infection. However, in some cases with negative molecular or antigen tests for COVID-19 but remaining high suspicion, it can be relevant to determine whether a patient has previously been infected with COVID-19. In those cases, serological tests may explain a particular clinical presentation, although it does not assess infectiousness. Moreover, serological tests are important to assess seroprevalence and evaluate the effectiveness of applied containment strategies at the community level. However, humoral immunity is just one part of our immune system. Cellular immunity also plays a potential role in the protection against COVID-19. This review summarises the basic principles of serological and immunological tests for COVID-19 and provides recommendations for its application.

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Humoral immunity

The humoral immunity is characterised by the production of antibodies by B cells as a response to antigens [1]. Immunoglobulin (Ig)M quickly appears but has a short half-life (Figure 1). IgA is most abundant in mucosal surfaces but can also be found in serum, and arises within the first week of symptom onset. IgG is the most abundant antibody type and provides longer-lasting immunity. About 7 to 14 days after symptom onset, IgG against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is detectable in most patients [2,3]. IgG titres remain stable for at least four to six months following diagnosis among COVID-19 polymerase chain reaction (PCR)-confirmed individuals, whereas IgA and IgM titres rapidly decay [4-6]. Antibody titres remain negative in about 5% of symptomatic PCR-positive patients [7], whereas for asymptomatic PCR-positive patients 15% to 40% are seronegative [8,9]. Several studies show that severe cases are associated with higher titres of antibodies and may have a later-onset antibody response in comparison to milder or asymptomatic patients [9-11].

The presence of neutralising antibodies against SARS-CoV-2 after natural infection indicates protection against reinfection [12-14]. However, the cut-off levels of neutralising antibodies for protection against reinfection remain to be elucidated. Vaccination also induces neutralising antibody production, causes protection against COVID-19 infection and reduces the severity of infection. However, the relative independent contributions of humoral and cellular immunity to this protection are difficult to determine [15-17].

The presence of neutralising antibodies is considered a functional correlate of immunity and provides at least partial resistance to subsequent infections by virus antigen binding to prevent interaction with host cells [1,18]. Although some serological assays showed a high correlation between IgG and neutralising antibodies [19], other assays have poor correlation [20]. Therefore, comparison to virus neutralising tests is important as part of the validation of new serological assays. Most convalescent plasma samples obtained from individuals who recovered from COVID-19 do not contain high levels of neutralising activity [21]. The antibodies targeting the spike glycoprotein, especially the receptor-binding domain (RBD) within the S1 subunit, show the highest neutralising capacity [1,22]. The degree of cross-reaction to other epidemic and common human coronaviruses largely depends on the target sites to which the antibodies are directed [23]; the S1 subunit is most specific and has the least homology regarding amino acid sequence with other coronaviruses, whereas the S2 subunit is more conservative.

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Previously infected individuals develop higher (neutralising) antibody responses in comparison to infection-naïve individuals after one dose of mRNA vaccine vaccination [24,25], and this single-dose response is comparable or even stronger than in infection-naïve individuals who received two vaccinations [26].

It is of great concern that the presence of neutralising antibodies against one virus variant after natural infection or vaccination does not automatically mean equally effective protection against other variants [27]. When vaccine-induced neutralising antibodies are in-vitro tested against different SARS-CoV-2 variants, reduced or abolished neutralising capacity was observed for the K417N, E484K and N501Y virus mutations. This heterogeneity is in line with findings from vaccination

studies showing that some vaccines were less effective against infections by these variants in comparison to wildtype [28].

Serological tests

In contrast to molecular diagnostic tests that detect the presence of SARS-CoV-2 RNA, serological tests detect anti-SARS-CoV-2 antibodies. The most commonly used serological tests include lateral flow immunoassays (LFIA), enzyme-linked immunosorbent assays (ELISA) and chemiluminescence immunoassays (CLIA) (Table 1). Depending on the assay used, they may detect IgM, IgA, IgG or total antibodies [29]. In addition, assays vary in the specific antibodies they detect; these include antibodies against the RBD, nucleocapsid (N) protein, spike (S) protein, or nucleocapsid and spike (NS) proteins.

LFIA is a rapid immunochromatography based method, which uses colloidal gold conjugated SARS-CoV-2 antigens [30]. Usually, it requires only a few drops of whole blood from a finger prick placed onto the test strip, whereafter the sample migrates towards fixed bands of bound SARS-CoV-2 antigens [31]. If the sample contains SARS-CoV-2-specific antibodies, these will bind with the antigens, resulting in a visible band. Advantages of LFIA include their speed (~15 minutes) and ease of use.

ELISA is a plate-based assay of which the microtiter wells are coated with SARS-CoV-2 antigens [32]. After adding the sample, antigen-specific antibodies will bind to these antigens. After washing, a conjugate that binds to the antigen-antibody complex is added. A substrate is added, which will react with the conjugate, resulting in a colour change. The amount of colour change is a quantitative measure of the number of antibodies present in the sample. ELISA is easily adaptable to automation for high throughput.

CLIA utilises chemiluminescence to quantify the level of antibodies present in the sample [33]. SARS-CoV-2 antigens are conjugated with fluorescein isothiocyanate and bound to magnetic particles. Antibodies in the sample bind to antigens and are then visualised by chemiluminescence

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using a detection antibody. Advantages of CLIA include the wide dynamic range, high signal intensity, absence of interfering emissions, and high stability of reagents.

In contrast to LFIA, which generates only qualitative results, ELISA and CLIA also yield quantitative results. For any serological method, false-positive results due to cross-reactivity are uncommon, with a reported specificity ranging from 96% to 100% [29]. In a meta-analysis, pooled sensitivity of LFIA was 78% (95% confidence interval (CI) 71%-83%), of ELISA 86% (95%CI 82% – 89%), and of CLIA 92% (95%CI 86% – 95%) [29]. Assays detecting antibodies against the RBD may be more sensitive than assays using other antibodies. LFIAs have the possibility of point-of-care application and do not require highly equipped devices or trained laboratory staff to perform the test. Nevertheless, it will also depend on the a-priori probability whether such tests are useful. In high endemic settings and among persons having symptoms longer than one week, the test could be useful to decrease time to result and improve hospital logistics, in which positive results confirm the presence of COVID-19 and could accelerate decision-making in emergency rooms and routing to appropriate hospital wards [2].

Although most currently available serology tests assess antibodies against S and N proteins, other antigenic epitopes could also induce strong immune responses. Among 15 different SARS-CoV-2 antigens, nucleocapsid and open reading frame (ORF)8 and ORF3b induce the strongest specific antibody responses [34]. The combined ORFs had a specificity of 99.5%, suggesting that second-generation diagnostics using novel targets, like non-structural proteins, might improve the performance of serological assays in the future.

Neutralising antibodies can be detected by plaque reduction neutralisation tests [1]. Alternatively, cell-free and protein-based pseudo-neutralising antibody assays or surrogate virus neutralisation tests have been developed, where cells are replaced by receptors, and the virus is replaced by surface proteins [20]. Surrogate virus neutralisation tests have the advantage that no biosafety level 3 containment is needed as these do not require live viruses and cells, while having a very high correlation with plaque reduction neutralisation tests [35].

Cellular immunity

Cellular immunity is of paramount importance in containing SARS-CoV-2 infection [1]. Lymphopenia is a characteristic feature in moderate and severe COVID-19. It correlates with disease severity and mortality [36], thus raising questions about the adequacy and effectiveness of T-cell responses in severe cases. The cause of lymphopenia could be the recruitment and sequestration of activated lymphocytes in the lungs [37], induction of cell death or immune dysregulation [38,39]. The latter, manifesting either as immunosuppression or excessive immune activation and cytokine release syndrome, is characterised by increased IL-6 production and has been a major concern as it correlates with increased severity and mortality in COVID-19 [38,40,41]. The chronic pro-inflammatory state that accompanies old age and obesity may contribute to the immune imbalance seen in COVID-19, putting these populations at higher risk for severe infection [42].

Robust SARS-CoV-2 T-cell responses were observed in acute COVID-19 as well as in the majority of convalescent individuals [43-46]. Both CD4+ and CD8+ responses were characterised by the secretion of IFNγ, IL-2 and TNFα, indicative of T helper (Th)-1 polarisation, and weak Th2 and Th17 responses [43,44,46,47]. SARS-CoV-2 S-specific CD4+ T-cell responses were detected in the majority of COVID-19 cases, with a substantial fraction representing T follicular helper (T_{FH}) cells required for effective humoral immunity and affinity-matured B cell memory [41,43,45,48]. Moreover, there is a positive correlation between S-specific T-cell responses and anti-S antibody titres [47]. CD8+ specific responses were also identified both in acute COVID-19 and during convalescence, characterised by the secretion of IFNγ, granzyme B and perforin, and the expression of degranulation marker CD107a [43,46]. After stimulation with SARS-CoV-2 peptides in COVID-19 patients 26 days post symptom onset, specific T-cell responses were elicited against the membrane (M) and N protein, and to a lesser extent, non-structural proteins (e.g., nsp3, nsp4, ORF8) [1,46].

Memory T-cell responses are detectable during early convalescence (1-2 months post symptom onset) in the majority of infected individuals, and they are accentuated in those with more severe disease [40-42,44]. In addition, dominant central memory differentiation among CD4+ T-cells and effector memory differentiation among CD8+ T-cells can be found [49]. Another study identified

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an early differentiated memory phenotype with stem-like properties (CCR7^{+} CD127^{+} CD45RA$^{-/+}$ TCF1^{+}) among CD8+ T cells [43]. Memory T cells show a preferential specificity for S protein epitopes, but reactivity against M, N and non-structural proteins has also been observed [49]. Robust T-cell immunity is substantial even 6 to 8 months after SARS-CoV-2 infection, with CD4+ responses being the most frequent [50].

Importantly, cross-reactive SARS-CoV-2-specific T cells are present in 20-50% of unexposed healthy donors, possibly induced by previous exposure to other circulating endemic coronaviruses [43,44,46,49,51]. Whether these T-cell responses could influence clinical outcomes in COVID-19 remain unclear. Responses against S protein primarily aim at the S2 domain, which shows great homology to the S2 domain of endemic coronaviruses [44]. Cross-reactivity has also been observed against M, N and other non-S proteins and was more common among CD4+ T cells [43,46]. T-cell responses against SARS-CoV-2 have also been detected in recovered COVID-19 patients with no detectable antibodies, indicating that in some cases, cellular immunity could be maintained independently of antibody responses [42,43]. This finding is consistent with previous reports regarding SARS-CoV and MERS [52,53]. Interestingly, using a bioinformatics approach, *in silico* data showed the presence of T-cell cross-reactivity between SARS-CoV-2 peptides and several allergens that could be beneficial against COVID-19 in atopic individuals [54].

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To achieve protective immunity, vaccines against SARS-CoV-2 should elicit effective and lasting T-cell responses in addition to the induction of neutralising antibodies [39]. In clinical trials, mRNA-1273 (Moderna™) vaccine induced CD4+ Th1-biased and CD8+ T-cell responses, with a lack of Th2 cytokine responses, demonstrating a favourable immunological signature [16]. Similar results have been reported for another mRNA vaccine (i.e., BNT162b2, Pfizer/BioNTech™) and a chimpanzee adenovirus-vectored vaccine (i.e., ChAdOx1, University of Oxford/AstraZeneca™) [15,17]. In the latter, S-specific T-cell responses peaked at day 14 of vaccination and were still detectable on day 56 [17]. Previously infected individuals develop much stronger T-cell responses against spike protein peptides in comparison to infection-naïve individuals after one dose of mRNA vaccine vaccination [24].

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ELISpot

Enzyme-linked immunospot (ELISpot) is an antigen-specific T-cell functional assay that can measure the proportion of T-cells producing a specific cytokine [55] (Table 1). It is a highly sensitive approach and has been used to assess SARS-CoV-2-specific T-cell responses [36,43,51,56,57]. IFNγ-secreting T cells were reactive against M, N and S peptides in 70% to 100% of convalescent COVID-19 patients depending on the specific antigens and techniques used in the test [47,51,57-59]. Illness severity is correlated with anti-M and anti-S T-cell responses [56,57]. IFNγ-ELISpot assays have also been used for the assessment of cellular responses after SARS-CoV-2 vaccination, with vaccine candidates reporting robust S-specific T-cell responses after vaccination [60]. As ELISpot assay cannot provide further information about the exact cytokine-producing cell types, intracellular cytokine staining with flow cytometry can be used to identify specific cell subpopulations and the presence of polyfunctional cells [61].

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Recommendations for clinical practice

Although several aspects of the hosts' immune responses against SARS-CoV-2 remain to be further unravelled, some serological and immunological tests can be used to gain valuable information. Humoral immunity responses are variable and highly dependent on various assay-based and host factors. Hence, the optimisation of a standardised approach regarding the correct timing and the appropriate type of serology test that should be performed in different disease phases, severity classes, patient ages and settings remains challenging.

Although the diagnostic utility of serological testing in the acute phase of illness is limited, it can be used for SARS-CoV-2 diagnostics in case of unavailability of molecular diagnostic tests in, e.g., resource-deprived settings. However, in such settings also rapid antigen tests can be considered. Antibody tests may also be utilised when molecular test results are inconclusive or in cases of late-onset post-infectious complications, such as the multisystem inflammatory syndrome in children

(MIS-C) [62]. In patients who are highly suspected of COVID-19 but in whom the molecular test was negative, serological testing may be helpful to yet establish the diagnosis.

As point-of-care serological tests enable a timely and convenient way of antibody testing, they may be preferred when rapid results are needed or when access to central laboratories requires precarious logistics, like community-based screening, non-referral hospitals, outpatient practice or in home-based settings. Self-sampling approaches might be useful in population-based screening studies, but further validation is needed.

Most importantly, serological tests for SARS-CoV-2 are necessary for population-based epidemiological surveillance. Serological tests are useful for public health policymaking to address the extent of SARS-CoV-2 spread in the community and assess the effectiveness of infection control strategies.

The introduction of community-wide vaccination programs may complicate the interpretation of serological test results. The majority of currently available vaccines induce anti-S protein or anti-RBD neutralising antibody response [1]. Consequently, assays targeting only anti-S protein or anti-RBD antibodies will not be able to discriminate between natural and post-vaccination induced immunity. Nevertheless, the measurable effect of post-vaccination immune response is of great significance for the affirmation of SARS-CoV-2 immune communities. As of writing this review, community-wide monitoring of antibody levels after vaccination to determine whether sufficient protection levels have been obtained, is not recommended according to most guidelines. Nevertheless, it is conceivable to consider measuring vaccine response in patients' groups that are at high risk for reduced immune responses, e.g., immunocompromised patients, and to identify those with insufficient protection despite completing the standard vaccination schedule. However, this needs to be further evaluated in clinical studies, and more supporting evidence to reach consensus on cut-off values for presumed protection is mandatory.

The choice of antibody testing strategy and interpretation of the results should be based on the assay performance characteristics and serological tests should only be used in settings where the prevalence is not too low. Otherwise, even a very high specific test can lead to a substantial absolute

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number of false-positive results when the prevalence is very low. Moreover, when tests have low specificities, combining different tests may be applied to increase overall specificity and maintain high sensitivity rates [63].

Finally, many information gaps need to be clarified regarding long-term antibody kinetics and T-cell responses after natural infections by various SARS-CoV-2 variants and vaccination strategies. Longitudinal studies should be employed in order to delineate the clinical sensitivity and specificity rates of serology and immunology tests in various settings with different prevalence.

Conclusion

Serological tests provide information about previous COVID-19 infections or vaccinations. They are not suitable as stand-alone diagnostics for acute-phase infections. However, in cases of more prolonged existing symptoms and when molecular diagnostic results are unavailable or inconclusive, serological diagnostics could identify additional COVID-19 cases among suspected patients. Awareness of the limitations of serological and immunological tests in a particular setting is required, because of the large heterogeneity in test performance of different assays. Currently, it remains uncertain how serological and immunological parameters are precisely correlated with the extent of protective immunity.

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Conflict of interest

RFC: Research Grants from Gilead, Pulmotec, Janssen, Karius, Chimerix, Merck, Viracor, Takeda/Shire, and Ansun Pharmaceuticals. Advisory Board/Consultant for ADMA Biologics, Pulmotec, Ablynx, Janssen, Merck, ReViral, Kyorin, Chimerix, Partner Therapeutics, and Ansun Pharmaceuticals. All of RFC disclosures are not related to the topic discussed.

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Figure Legends.

Figure 1. Antibody and T-cell responses over time after SARS-CoV-2 infection

Immune responses can be highly heterogenous depending on various factors including patient characteristics and severity of illness. The presented figure is a simplified representation to increase general understanding, but can be variable for different individuals in different settings.

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Table 1. Overview principles of serological and immunological tests

	Detection targets	Advantages	Limitations	When to apply
Lateral flow immunoassay (LFIA)	IgM, IgA, IgG or total antibodies	- Suitable as point-of-care test - Rapid and easy testing	- Heterogeneous performance with overall limited sensitivity during acute phase of disease - Only qualitative results	- Population-based epidemiological surveillance - For individual patient care in case of unavailability of molecular diagnostic tests, inconclusive molecular test results, late presentations during disease course or late-onset post-infectious complications - Implications for interpretation after vaccination and correlation with protective immunity remain to be determined
Enzyme-linked immunosorbent assay (ELISA)		- Overall higher sensitivity in comparison to LFIA - Suitable for high throughput and automation - Some assays generate quantitative results	- Not suitable for rapid testing - Need for trained laboratory staff - Batchwise workup in laboratory process	
Chemiluminescence immunoassay (CLIA)				
Plaque reduction neutralisation tests (i.e., conventional virus neutralisation test)	Total antibodies (that can inhibit viral replication)	- Presumably high correlation with protective immunity - Gold standard for quantification of neutralising antibodies	- Only in biosafety level 3 laboratories possible - Time consuming test	- To increase scientific understanding regarding immunity - Implications for interpretation after vaccination and correlation with protective immunity remain to be determined
Pseudo-neutralising antibody assays / surrogate virus neutralisation test (sVNT)		- High correlation with plaque reduction neutralisation tests - Rapid and safe (no need for live biological material)	- Not considered as gold standard for quantification of neutralising antibodies	
ELISpot	Antigen specific T-cells (producing a specific cytokine, e.g., IFNγ)	- Quantitative measurements - Commonly used for evaluation of immunity in vaccination trials	- No information regarding exact cytokine-producing cell types	- To increase scientific understanding regarding immunity - Implications for interpretation after vaccination and correlation with protective immunity remain to be determined
Flow cytometry	Different cell types, including T-cells	- Identification of specific cell subpopulations and presence of polyfunctional cells	- Test is (relatively) complex	

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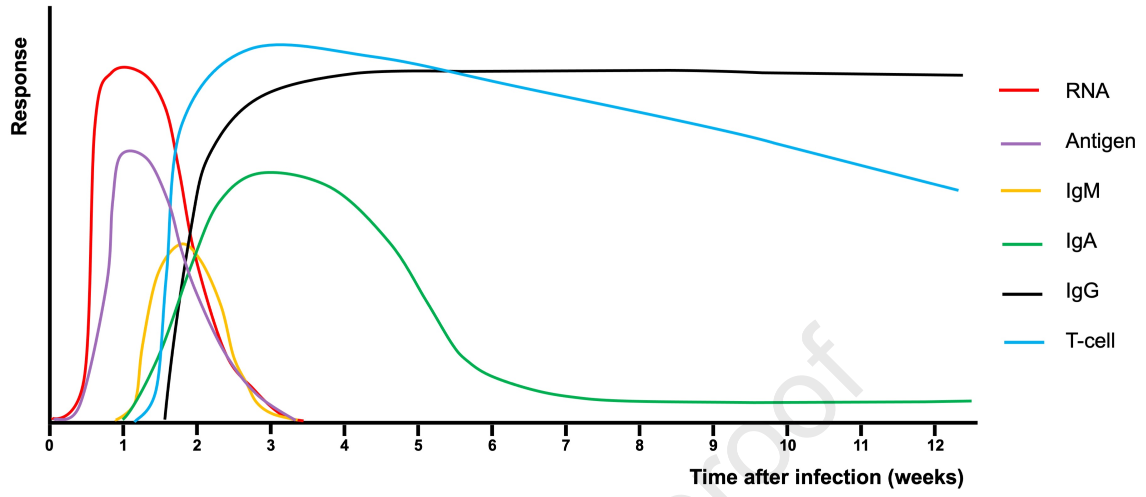

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