Original Article

Development of an ELISA for SARS-CoV-2 Detection Focusing on Antibodies against Nucleocapsid Protein

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How to cite this article: **Golchinfar F, Madani R, Hezarosi M, Emami T, Ghanizadeh A. Development of an ELISA for SARS-CoV-2 Detection Focusing on Antibodies against Nucleocapsid rotein. Archives of Razi Institute. 2025;80(1):85-92.** DOI: 10.32592/ARI.2025.80.1.85

ABSTRACT

In late 2019, a novel viral disease, designated as SARS-CoV-2, emerged in China and rapidly propagated, ultimately resulting in a global pandemic. This virus has had a profound impact on human health and has caused significant financial losses for various societal sectors. Consequently, researchers are endeavoring to expedite the identification and control of this pathogen. The ELISA method has emerged as a valuable tool in the screening of large patient populations during infectious epidemics. In this study, the nucleocapsid protein (NP) of the SARS-CoV-2 virus was utilized to measure serum antibodies, which were obtained from the Avicenna Research Institute. The antigen was coated on each well of the plate, followed by the addition of serum samples from medical diagnostic laboratories (positive and negative sera measured by ELISA and PCR). To optimize the ELISA assay, a checkerboard titration was performed for all serum samples and antigens. The ELISA test was an indirect assay that could detect antibodies against NP.Finally, the cut-off, sensitivity, and specificity of the ELISA test were measured. The findings of the study indicated a 95% sensitivity and 92% specificity rate. Additionally, the intra-assay and inter-assay coefficient of variation (CV) values were recorded at 0.263% and 0.41%, respectively. These outcomes substantiate the remarkable precision and reliability of the ELISA test.In summary, the efficacy and precision of our kit in detecting antibodies targeting NP hold considerable promise. This innovative approach enhances diagnostic accuracy and holds significant potential for advancing antibody detection methodologies in the fields of virology and immunology.

Keywords: SARS-CoV-2, Nucleocapsid Protein, ELISA, COVID-19, Diagnosis.

Article Info:
Received: 14 June 2024
Accepted: 23 September 2024
Published: 28 February 2025

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1. Introduction

In December of 2019, an outbreak of pneumonia of unknown etiology was reported in Wuhan, China. Subsequent genome analysis of the virus revealed its classification as a novel coronavirus, related to SARS-CoV and designated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a betacoronavirus belonging to the Sarbecovirus subgenus. The WHO declared a pandemic on March 12, 2020, due to the thousands of deaths caused by the disease and the subsequent global spread of SARS-CoV-2 (1, 2). The diagnosis of SARS-CoV-2 is imperative for the management of the virus's propagation. Conventional diagnostic methodologies encompass RT-PCR tests, which detect the virus's genetic material, rapid antigen tests, which detect viral proteins, and antibody tests, which identify the immune system's response to the virus. However, each method possesses inherent limitations and challenges. A notable challenge in the diagnosis of SARS-CoV-2 is the potential for false negative or false positive results, which can impact patient care and the implementation of public health measures.Polymerase chain reaction (PCR) tests, which detect the RNA of the virus through a polymerase chain reaction (PCR), are regarded as the gold standard for diagnosing SARS-CoV-2 due to their high sensitivity and specificity (3, 4). However, the real-world sensitivity of RNA tests has been found to fall short of expectations. The presence of false-negative cases has been linked to issues related to sample transportation, collection, and the RT-PCR methodology itself. The necessity of specialized equipment and trained personnel, in addition to the extended processing time required by this test, underscores its many limitations (5). Conversely, the Rapid Antigen Test offers rapid results but may exhibit reduced sensitivity compared to RT-PCR, leading to an increased probability of false negatives, particularly in individuals with low viral loads (6). A prompt and accurate diagnosis is imperative for effective contact tracing, isolation, and treatment. In this regard, the Enzyme-Linked Immunosorbent Assay (ELISA) test plays a pivotal role.The ELISA test is a sensitive laboratory technique that can detect and quantify proteins (antibodies or antigens) in a sample.During the SARS-CoV outbreak, many serological tests, including Western blot (WB) analysis, ELISA, and immunofluorescence assays (IFA), were developed. Viral-based IFA and ELISA were reported to be highly sensitive but lacking in specificity, with results that were falsely positive being caused by cross-reaction with autoantibodies in autoimmune diseases and well-preserved antigens across various CoV species (7-9).Therefore, the foundation of numerous serological assays used in laboratory diagnosis was recombinant antigens derived from both spike (S) and nucleocapsid protein (NP). The utilization of recombinant antigens, in addition to their enhanced suitability for assay standardization, offers the advantage of being exempt from stringent biosafety regulations.The N protein, being devoid of glycosylation sites and characterized by its compact size, facilitates straightforward cloning into prokaryotic or eukaryotic expression plasmids.Studies have demonstrated that ELISA and Western blot assays based on recombinant proteins exhibit low to moderate specificity and are highly sensitive (10-15). In the context of the ongoing study on the novel coronavirus, SARS-CoV-2, ELISA tests can be strategically designed to specifically identify the antibodies against the N protein of the virus. Among the structural proteins, the nucleoprotein (NP) is an immunodominant antigen that is highly conserved in the CoVs genus and is one of the most abundant structural proteins in virus-infected cells (16). The NP has a role in packaging the viral genome RNA into a long helical ribonucleocapsid (RNP) complex and participating in the assembly of the virion through its interactions with the viral genome and membrane protein M (17). Consequently, the ELISA test, which is a valuable diagnostic tool, can provide crucial information about the infection, especially in the early stages when viral RNA levels might be low. This is due to the fact that the NP can assist in the early detection of patients and monitoring the levels of antibodies against this protein in patients to track the progression of the infection and assess treatment efficacy. This can improve the accuracy of diagnosis and help healthcare professionals make informed decisions regarding patient care and disease management. Consequently, the ELISA test for detecting antibodies against the NP antigen can contribute to more precise and reliable SARS-CoV-2 diagnosis, ultimately aiding in the control and prevention of the spread of the virus. Therefore, in this research, we developed an ELISA kit based on NP to facilitate the diagnosis of COVD-19.

2. Materials and Methods

2.1. Preparation of Recombinant Proteins

The recombinant proteins of N were purchased from the Avicenna Research Institute, Tehran, Iran.

2.2. Lowry Method for Measuring Proteins

The protein concentration of the virus solution purchased was measured using Lowry's protein assay method (18). Subsequently, the spectrophotometer was utilized to measure the optical density (OD) of 0 (Blank), 10, 25, 50, and 100 µl of the standard protein (bovine serum albumin (BSA) 1 mg/ml), as well as 1:5 and 1:10 diluted protein solutions, against a reagent blank (19, 20).

2.3. ELISA test

The ELISA test was performed by setting up the appropriate chequerboard titration for all serum samples and antigens.The NP of SARS-CoV-2 was diluted with coating buffer (carbonate and bicarbonate), and 100 microliters of this solution were coated in each well of the strip. The plates were then incubated for one night at 4 °C. Subsequently, the plates were washed with PBST (phosphate-buffered saline with Tween 20).In the blocking stage, 300 microliters of 5% skim milk were utilized to block the spaces between the antigens in the bottom of the wells.The plates were then incubated for 90 minutes at 37°C in the incubator.Following the incubation, the plates

were washed with PBST three times. Checkerboard titrations were prepared for serum samples, with four dilutions of serum prepared for this purpose: 1:25, 1:50, 1:100, and 1:200.At this stage, the samples were incubated for 75 minutes at 37°C, and the washing steps were repeated four times. Subsequently, 100 µL of Goat Anti Human whole IgG HRP conjugate was added to each well, and the incubation was continued for an additional 75 minutes at 37°C. Following this step, the plates were washed with PBST. Then, 100 µL of BM Blue, a specific substrate, was added to each well and placed in the dark for 20–30 minutes at room temperature.Subsequently, 50 µL of 1M sulfuric acid was added to each well as a stop solution. Immediately thereafter, the absorbances were measured using an ELISA reader at 450 nm.

2.4. Calculating Cut-Off

To calculate this level, the mean of the optical density (OD) of fifteen true positive samples and fifteen true negative samples was determined. The threshold limit was then calculated using the following formula: Threshold Limit = Mean ± xSD.The positive and negative samples were identified by the index value, which is obtained by dividing the light absorption of the sample by the threshold limit: Index Value (IV) = OD of sample/Threshold Limit.2.5. Reproducibility Test (Intra-assay and Inter-assay):This test is used to measure the validity of the system designed in ELISA, so that several samples are randomly selected so that their reproducibility can be tested once simultaneously in one day.This method is called Inter-assay(four positive samples and four negative samples were evaluated for this test), and the same number of samples are selected so that their reproducibility can be tested on different days. The former approach is referred to as "intra-assay" testing, and the latter as "inter-assay" testing.In both cases, the average, standard deviation, and coefficient of variation parameters are calculated. According to the definition of the standard, the coefficient of variation is considered acceptable for the reproducibility of the tests if its value is lower than 10.

2.6. The Diagnostic Sensitivity, Specificity

A total of 200 sera were received from medical diagnosis laboratories, and the optical density (OD) of these sera was measured in the medical diagnosis laboratory with a commercial enzyme-linked immunosorbent assay (ELISA) system. The results were used as true positives and negatives. Then, the results were compared with the results of a designed ELISA. According to the definition of the sensitivity and specificity equation, the following calculation was made:

Number of true positives + number of false negatives/number of true positives = sensitivity

Number of false positives + number of true negatives/number of true negatives = specificity

3. Results

3.1. Protein Measurement

Subsequent to measuring the optical absorbance of each solution at 750 nm, the concentration of the available protein was determined based on dilutions of 1:5 and 1:10. This concentration was found to be 350 microgram per milliliter (µg/ml) (Table 1 and Figure 1).

3.2.ELISA results

3.2.1 checkerboard

A serum sample from a patient group exhibiting elevated antibody titers in ELISA was identified as a "true positive" sample, as it demonstrated a higher titer compared to other serum samples. This serum sample was then confirmed as a "true positive" through PCR testing. An analogous serum sample from a healthy group was selected as the "true negative" sample. This sample was found to be negative in both ELISA and PCR tests. The checkerboard test was then performed, guided by the parameters outlined in Table 2. In this method, the concentrations of antigen were set at 4 µg/ml, 2 µg/ml, and 1 µg/ml, while the dilutions of sera ranged from 1:25 to 1:200. The optimal concentration of recombinant NP of the SARS-CoV-2 as an antigen was determined to be 2 µg/ml, and the optimal serum dilution was identified as 1:100.

3.2.2.Determining the Cut off Threshold

To this end, the average optical density (OD) of 15 positive sera and 15 negative sera was measured using the ELISA system, and their standard deviation (SD) was subsequently calculated (Table 3).The average OD of positive and negative standards was determined to be 1.19586 and 0.145, respectively. The corresponding SDs were 0.336358 and 0.035106, respectively. Consequently, employing the Cut-off Index (COI), samples with OD values greater than 1/1 are classified as positive, while those with OD values less than 0.9 are designated as negative.Samples exhibiting an OD value between these thresholds are considered suspicious and require re-evaluation with fresh serum at a later time.

3.2.3. Intra-assay and Inter-Assay

A total of eight samples were evaluated in this test: four positive and four negative. The results of the average, standard deviation, and coefficient of variation for the upper and lower limits are presented in Tables 4, 5, 6, and 7. Finally, the average of the upper and lower limits (intra-assay) for the coefficient of variation of the controls was obtained as 0.263. In a similar manner, the results of the parameters such as mean, standard deviation, and coefficient of variation for the upper and lower limits were measured in inter-assay for control samples with upper and lower limits (Tables 8, 9, 10, and 11). Ultimately, the average of the upper and lower limits for the coefficient of variation of controls was obtained to be 0.41. As demonstrated in Figure 1, the intra-assay value of the coefficient of variation (CV) was 0.263%, and the inter-assay value was 0.41%. These results indicate that the ELISA test exhibited both high accuracy and durability.

Table 1. Optical absorption of standard protein and diluted samples.

	Concentration (µg)	OD
Standard	Blank	0
	10	0.061
	25	0.185
	50	0.322
	100	0.639
Sample	1:5	0.446
	1:10	0.220

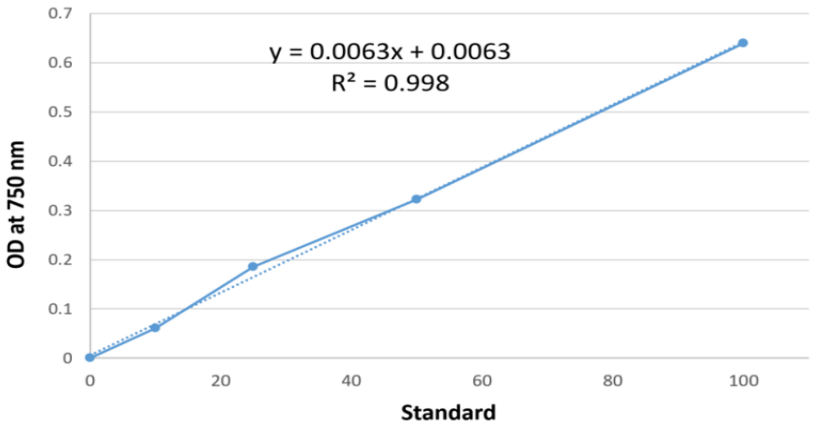

Figure 1. Optical absorbance of standard proteins.

Table 2. Checkerboard results

Ag Concentration (µg/well) / Serum dilution	1 µg/well -	1 µg/well +	2 µg/well -	2 µg/well +	4 µg/well -	4 µg/well +
1:25	0.622	1.174	0.583	1.500	0.530	1.885
1:50	0.635	1.068	0.635	1.323	0.560	1.643
1:100	0.611	1.004	0.537	1.212	0.505	1.656
1:200	0.649	0.917	0.618	1.132	0.576	1.536

Table 3. Optical absorbance of positive and negative standard samples.

Positive standard OD	Negative standard OD
1.036	0.123
1.003	0.129
1.044	0.132
1.167	0.133
1.223	0.114
1.012	0.121
1.400	0.153
1.247	0.115
1.632	0.112
1.959	0.242
1.684	0.171
0.816	0.162
0.912	0.185
0.859	0.159
0.934	0.124

Table 4. Intra-assay results for high-limit control samples.

Control	Result 1	Result 2	Plate	Plate mean
high	2.379	1.180	1	1.779
high	1.821	0.964	2	1.392
high	1.709	1.436	3	1.572
high	1.668	2.517	4	2.0925

Table 5. Mean, standard deviation and coefficient of variation for high-limit.

Mean of means	1.709
Std Dev	0.3004
Cv of mean	0.175

Table 6. Intra-assay results for low-limit control samples.

Control	Result 1	Result 2	Plate	Plate mean
Low	0.325	0.296	1	0.310
Low	0.157	0.218	2	0.187
Low	0.372	0.391	3	0.381
Low	0.426	0.509	4	0.467

Table 7. Mean, standard deviation, and coefficient of variation for lower limit

Mean of means	0.3367
Std Dev	0.1184
CV of mean	0.351

Intra assay = average of high and low control
CV = 0.175 + 0.35 / 2 = 0.263

Table 8. Inter-assay results for high-limit control samples.

Control	Result 1	Result 2	Plate	Plate mean
high	1.180	0.993	1	1.086
high	0.964	0.918	2	0.941
high	1.436	1.330	3	1.383
high	2.517	2.352	4	2.432

Table 9. Mean, standard deviation and coefficient of variation for high-limit in inter-assay.

Mean of means	1.461
Std Dev	0.674
Cv of mean	0.46

Table 10. Inter-assay results for low-limit control samples in inter-assay.

Control	Result 1	Result 2	Plate	Plate mean
Low	0.296	0.265	1	0.280
Low	0.218	0.194	2	0.206
Low	0.391	0.351	3	0.371
Low	0.509	0.486	4	0.497

Table 11. Mean, standard deviation and coefficient of variation for low-limit in inter-assay.

Mean of means	0.338
Std Dev	0.125
CV of mean	0.37

Inter assay = average of high and low control CV = 0.46 + 0.37 / 2 = 0.41

3.2.4.Sensitivity and Specificity of ELISA Test

In general, from the test of 200 serum samples that were evaluated, the results were as follows:
Actual positive number = 100, Actual negative number = 100
Number of false positives = 8, number of false negatives = 5
Therefore, the sensitivity was 95% and the specificity was 92%.

4. Discussion

In the wake of the global dissemination of SARS-CoV-2, the urgency of immediate diagnosis has become a paramount concern. Various diagnostic assays, such as viral gene detection by RT-PCR and antibody detection methods (ELISA), have emerged as standard methods for patient detection. ELISA, in particular, has been instrumental in detecting antibodies produced by the immune system in response to the virus, offering insights into past infections and immune responses. However, this test is subject to a significant limitation: the immune system requires a certain duration to produce antibodies against antigens. Conversely, PCR is a highly sensitive, specific, and rapid method of amplifying and detecting the genetic material (RNA) of the virus. However, it is more complex and generally more expensive than ELISA.In the context of epidemiological studies, the identification of contact with viruses is essential. ELISA can assist in tracing contacts with antibodies against the virus, thereby highlighting the immune system's memory (21-23). The immune system

can produce multiple antibodies against individual virus particles; however, for the present study, we selected NP for its capacity to coat the plates.While the spike protein is widely regarded as a critical antigen for neutralization and is incorporated into vaccines, diagnostic assays are predominantly based on other proteins to enhance sensitivity and specificity. This is due to the potential for antibodies against other SARS viruses to cross-react with the spike protein of SARS-CoV-2 (24). A study by Tan, Goh (25) on SARS-CoV-1 demonstrated that antibodies against the N-protein exhibited a longer lifespan in serum compared to antibodies against the S-protein. Consequently, the detection of antibodies against this protein can facilitate the identification of patients and the monitoring of their condition over extended periods. In the present study, an ELISA assay targeting nucleoprotein was developed and validated for the detection of SARS-CoV-2 antibodies in blood samples. The specificity of this assay was found to be 92%, which is consistent with the specificity values reported in analogous assays in other studies (26–29). The assay yielded eight false positive results when testing 200 serum samples from individuals, likely attributable to immunity from influenza vaccines or severe respiratory conditions not related to SARS-CoV-2. The possibility of cross-reactivity with serum samples from individuals previously infected with common cold coronaviruses was also considered. While some cross-reactivity may exist between antibodies to seasonal coronaviruses and SARS-CoV-2, its impact on the context of the pandemic is considered minimal (30, 31). In contrast to our study, which did not allow for the evaluation of cross-reactivity, the study by Tozetto-Mendoza and Kanung (32) examined cross-reactivity with sera from individuals with previous confirmed infections of the dengue, zika virus, other respiratory viruses, or bacterial and fungal infections that cause pneumonia. Notably, their study did not report any false negative results, thereby demonstrating the high specificity of their ELISA kit. Notwithstanding the superior specificity of the ELISA kit utilized in our study (97.9%), the cross-reactivity evaluation conducted by Tozetto-Mendoza et al. (32) yielded more favorable outcomes. This is attributable to the fact that the aforementioned study exclusively focused on individuals with documented past exposure to dengue, zika, other respiratory viruses, or bacterial and fungal pathogens that lead to pneumonia. The absence of false negative results in this particular study further substantiates the high specificity of the ELISA kit employed. Our kit demonstrated higher sensitivity compared to the aforementioned kit. According to the findings of our study, the sensitivity of our kit was 95%, while the sensitivity of the kit designed by Tozetto-Mendoza, Kanunfre (32) was approximately 90%. The research conducted by Chang, Sue (33) revealed that S1-subunit and N-protein-based ELISA assays exhibited moderate sensitivity, with values of 91.8% and 84.8%, respectively. While the sensitivity of the N-protein-based ELISA in the study by Chang et al. (33) was lower than that of the ELISA kit designed in this study, the combination of S- and N-protein-based ELISA increased the sensitivity of the latter to 93.2%.According to the results of Chang et al. (33), S- and N-protein-based ELISA are complementary to each other. Consequently, further studies may facilitate the enhancement of the sensitivity of our designed kit by integrating the recombinant NP with the recombinant S protein. In summary, the ELISA assay developed in this study, which was based on NP, demonstrated a high degree of sensitivity in detecting antibodies specific to SARS-CoV-2. This ELISA kit has the potential to enhance diagnostic efficiency, reduce dependence on costly or sophisticated equipment, and optimize resource allocation, making it a cost-effective solution for large-scale testing initiatives.

Acknowledgment
Expressions of gratitude are extended to the Razi Vaccine and Serum Research Institute for their sustained endeavors in the realm of public health promotion.

Authors' Contribution
Conceptualization: R.M.
Data curation: F.G & M.H.
Formal analysis: F.G & M.H.
Methodology: F.G &M.H & A.Gh & T.E.
Software: M.H.
Validation: R.M.
Investigation: F.G &M.H & A.Gh & T.E.
Writing - original draft: M.H.
Writing - review & editing: R.M & F.G.

Ethics
The present study was approved by the Ethics Committee of Razi Vaccine and Serum Research Institute.

Conflict of Interest
No potential conflicts of interest relevant to this article were reported.

Funding Sources
The project was granted funding by the Razi Vaccine and Serum Research Institute.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

References

1.Yuki K, Fujiogi M, Koutsogiannaki S. COVID-19 pathophysiology: A review. Clinical Immunology. 2020;215:108427.
2.Ciotti M, Ciccozzi M, Terrinoni A, Jiang W-C, Wang C-B, Bernardini S. The COVID-19 pandemic. Critical reviews in clinical laboratory sciences. 2020;57(6):365-88.

3.Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. New England journal of medicine. 2020;382(8):727-33.
4.The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. Nature microbiology. 2020;5(4):536-44.
5.Sidiq Z, Hanif M, Dwivedi KK, Chopra KK. Benefits and limitations of serological assays in COVID-19 infection. Indian Journal of Tuberculosis. 2020;67(4, Supplement):S163-S6.
6.Høeg TB, Prasad V. Rapid antigen testing for COVID-19: Decreasing diagnostic reliability, potential detrimental effects and a lack of evidence to support continued public funding of community-based testing. Public Health Pract (Oxf). 2023;6:100451.
7.Che X-y, Qiu L-w, Liao Z-y, Wang Y-d, Wen K, Pan Y-x, et al. Antigenic cross-reactivity between severe acute respiratory syndrome—associated coronavirus and human coronaviruses 229E and OC43. Journal of Infectious Diseases. 2005;191(12):2033-7.
8.Dijkman R, Jebbink MF, Gaunt E, Rossen JW, Templeton KE, Kuijpers TW, et al. The dominance of human coronavirus OC43 and NL63 infections in infants. Journal of Clinical Virology. 2012;53(2):135-9.
9.Wang Y, Sun S, Shen H, Jiang L, Zhang M, Xiao D, et al. Cross-reaction of SARS-CoV antigen with autoantibodies in autoimmune diseases. Cell Mol Immunol. 2004;1(4):304-7.
10. Carattoli A, Bonito PD, Grasso F, Giorgi C, Blasi F, Niedrig M, et al. Recombinant protein-based ELISA and immuno-cytochemical assay for the diagnosis of SARS. Journal of medical virology. 2005;76(2):137-42.
11. Guan M, Chen HY, Foo SY, Tan Y-J, Goh P-Y, Wee SH. Recombinant protein-based enzyme-linked immunosorbent assay and immunochromatographic tests for detection of immunoglobulin G antibodies to severe acute respiratory syndrome (SARS) coronavirus in SARS patients. Clinical and Vaccine Immunology. 2004;11(2):287-91.
12. Leung DTM, Chi Hang TF, Chun Hung M, Sheung Chan PK, Cheung JLK, Niu H, et al. Antibody response of patients with severe acute respiratory syndrome (SARS) targets the viral nucleocapsid. The Journal of infectious diseases. 2004;190(2):379-86.
13. Liu X, Shi Y, Li P, Li L, Yi Y, Ma Q, et al. Profile of antibodies to the nucleocapsid protein of the severe acute respiratory syndrome (SARS)-associated coronavirus in probable SARS patients. Clinical and vaccine immunology. 2004;11(1):227-8.
14. Shi Y, Yi Y, Li P, Kuang T, Li L, Dong M, et al. Diagnosis of severe acute respiratory syndrome (SARS) by detection of SARS coronavirus nucleocapsid antibodies in an antigen-capturing enzyme-linked immunosorbent assay. Journal of clinical microbiology. 2003;41(12):5781-2.
15. Woo PC, Lau SK, Tsoi H-w, Chan K-h, Wong BH, Che X-y, et al. Relative rates of non-pneumonic SARS coronavirus infection and SARS coronavirus pneumonia. The Lancet. 2004;363(9412):841-5.
16. He Y, Zhou Y, Wu H, Kou Z, Liu S, Jiang S. Mapping of antigenic sites on the nucleocapsid protein of the severe acute respiratory syndrome coronavirus. J Clin Microbiol. 2004;42(11):5309-14.
17. Masters PS, Sturman LS. Background paper. Functions of the coronavirus nucleocapsid protein. Adv Exp Med Biol. 1990;276:235-8.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265-75.
19. Golchinfar F, Madani R, Hezarosi M. Study the Profile of Foot-and-mouth Disease Virus Protein by Electrophoresis and Identification of the Immunodominant Proteins. Archives of Razi Institute. 2023;78(5):1563-71.
20. Madani R, Hezarosi M, Golchinfar F. Unveiling indirect ELISA test against nucleoprotein of H9N2 comparing with hemagglutination inhibition test. Archives of Razi Institute. 2024:-.
21. Yüce M, Filiztekin E, Özkaya KG. COVID-19 diagnosis —A review of current methods. Biosensors and Bioelectronics. 2021;172:112752.
22. Rai P, Kumar BK, Deekshit VK, Karunasagar I, Karunasagar I. Detection technologies and recent developments in the diagnosis of COVID-19 infection. Applied Microbiology and Biotechnology. 2021;105(2):441-55.
23. Zhai P, Ding Y, Wu X, Long J, Zhong Y, Li Y. The epidemiology, diagnosis and treatment of COVID-19. International Journal of Antimicrobial Agents. 2020;55(5):105955.
24. Zhao L, Han N, Zheng Y, Rao H, Li J, Chen Y, et al. Detection of Antibodies Against the SARS-CoV-2 Spike Protein and Analysis of the Peripheral Blood Mononuclear Cell Transcriptomic Profile, 15 Years After Recovery From SARS. 2021;11.
25. Tan YJ, Goh PY, Fielding BC, Shen S, Chou CF, Fu JL, et al. Profiles of antibody responses against severe acute respiratory syndrome coronavirus recombinant proteins and their potential use as diagnostic markers. Clinical and diagnostic laboratory immunology. 2004;11(2):362-71.
26. Bastos ML, Tavaziva G, Abidi SK, Campbell JR, Haraoui L-P, Johnston JC, et al. Diagnostic accuracy of serological tests for covid-19: systematic review and meta-analysis. bmj. 2020;370.
27. Böger B, Fachi MM, Vilhena RO, Cobre AF, Tonin FS, Pontarolo R. Systematic review with meta-analysis of the accuracy of diagnostic tests for COVID-19. American journal of infection control. 2021;49(1):21-9.
28. Caini S, Bellerba F, Corso F, Díaz-Basabe A, Natoli G, Paget J, et al. Meta-analysis of diagnostic performance of serological tests for SARS-CoV-2 antibodies up to 25 April 2020 and public health implications. Eurosurveillance. 2020;25(23):2000980.
29. Deeks J, Dinnes J, Takwoingi Y, Davenport C, Spijker R, Taylor-Phillips S, et al. Ruffano LFd, Harris IM, Price MJ, Dittrich S, Emperador D, Hooft L, Leeflang MM, Bruel AVd, Cochrane COVID-19 Diagnostic Test Accuracy Group.

Antibody tests for identification of current and past infection with SARS-CoV-2. Cochrane Database Syst Rev. 2020.
30. Ng KW, Faulkner N, Cornish GH, Rosa A, Harvey R, Hussain S, et al. Preexisting and de novo humoral immunity to SARS-CoV-2 in humans. Science. 2020;370(6522):1339-43.
31. Tso FY, Lidenge SJ, Peña PB, Clegg AA, Ngowi JR, Mwaiselage J, et al. High prevalence of pre-existing serological cross-reactivity against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in sub-Saharan Africa. International Journal of Infectious Diseases. 2021;102:577-83.
32. Tozetto-Mendoza TR, Kanunfre KA, Vilas-Boas LS, Sanchez Espinoza EP, Paião HGO, Rocha MC, et al. Nucleoprotein-based ELISA for detection of SARS-COV-2 IgG antibodies: Could an old assay be suitable for serodiagnosis of the new coronavirus? Journal of Virological Methods. 2021;290:114064.
33. Chang C-k, Sue S-C, Yu T-h, Hsieh C-M, Tsai C-K, Chiang Y-C, et al. Modular organization of SARS coronavirus nucleocapsid protein. 2006;13:59-72.