IJID Regions 10 (2024) 1–8

Contents lists available at ScienceDirect

IJID Regions

journal homepage: www.elsevier.com/locate/ijregi

Usefulness of receptor binding domain protein-based serodiagnosis of COVID-19

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ARTICLE INFO

Keywords:
SARS-CoV-2
Serodiagnosis
Recombinant RBD protein
Antibody detection
Diagnostic performance

ABSTRACT

Objectives: This study evaluated the performance of recombinant receptor binding domain (RBD) protein-based enzyme-linked immunosorbent assays (RBD-ELISAs) for detecting anti-SARS-CoV-2 immunoglobulin (Ig) G and IgM antibodies.

Methods: In this study, 705 sera from SARS-CoV-2-infected individuals and 315 sera from healthy individuals were analyzed.

Results: The RBD-ELISA IgG exhibited high specificity (99.1%) and moderate sensitivity (48.0%), with an overall diagnostic accuracy of 73.5%. RBD-ELISA IgM demonstrated specificity at 94.6% and sensitivity at 51.1%, with an accuracy of 72.8%. Both assays displayed improved performance when analyzing samples collected 15-21 days post-symptom onset, achieving sensitivity and accuracy exceeding 88% and 90%, respectively. Combining RBD-ELISA IgG and IgM in parallel analysis enhanced sensitivity to 98.6% and accuracy to 96.2%. Comparing these RBD-ELISAs with commercially available tests, the study found overlapping sensitivity and similar specificity values. Notably, the combined RBD-ELISA IgG and IgM showed superior performance. Cross-reactivity analysis revealed low false-positive rates (4.4% for IgG, 3.7% for IgM), primarily with viral infections.

Conclusion: This research underscores the potential of RBD-based ELISAs for COVID-19 diagnosis, especially when assessing samples collected 15-21 days post-symptom onset and utilizing a parallel testing approach. The RBD protein's immunogenicity and specificity make it a valuable tool for serodiagnosis, offering an alternative to polymerase chain reaction-based methods, particularly in resource-limited settings.

Introduction

SARS-CoV-2, a beta coronavirus belonging to the Coronaviridae family, was first identified in Wuhan, China, in December 2019 following reports of viral pneumonia cases with an unknown origin [1]. COVID-19, resulting from SARS-CoV-2 infection, can present with various symptoms or remain asymptomatic. The clinical presentation includes nonspecific signs such as fever, cough, fatigue, body aches, and respiratory distress, with the potential to progress to viral pneumonia [2].

The current standard diagnostic method for acute-phase COVID-19 involves detecting viral sequences using real-time reverse transcription polymerase chain reaction (rRT-PCR) in nasopharyngeal fluids [3]. However, this collection method poses challenges, causing patient discomfort and carrying the risk of errors and contamination. Implementing rRT-PCR as a point-of-care test faces obstacles due to its high cost, labor-intensive nature, and the requirement for specialized personnel and laboratory infrastructure [4]. Additionally, molecular tests may exhibit reduced sensitivity as the disease progresses [5,6] or due to the emergence of SARS-CoV-2 variants [7,8]. In this context, serological as-

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https://doi.org/10.1016/j.ijregi.2023.11.001
Received 11 October 2023; Received in revised form 31 October 2023; Accepted 5 November 2023

says play a crucial role by providing additional information regarding infection stages and past infections, serving as an epidemiological surveillance tool. Moreover, serological assays can be employed as point-of-care tests to address the aforementioned challenges [4,9]. Therefore, the detection of antibodies against specific viral components using indirect immunoassays is of utmost importance [10].

The spike (S) protein of SARS-CoV-2 is highly immunogenic and has been extensively studied. It consists of two subunits, with the receptor binding domain (RBD) located in subunit one (S1). The RBD plays a crucial role in initiating the infection by binding to the host cell [11]. This protein holds significant potential as an antigen for serological tests and serves as a target for neutralizing antibodies [12–14]. Despite the development of several commercial serological tests for COVID-19, diagnostic performance still varies, particularly with low sensitivity during the first week after symptom onset. This variability is further complicated by the lack of a standardized antibody production and duration profile among patients [15]. Hence, our objective was to assess the performance of recombinant RBD protein in COVID-19 serodiagnosis using indirect enzyme-linked immunosorbent assay (ELISA) for the detection of anti-SARS-CoV-2 immunoglobulin (Ig)G and IgM antibodies.

Material and methods

RBD protein production

The RBD protein utilized in this study was provided by the Institute for Protein Design in Seattle, WA, USA. To create the recombinant protein, a synthetic gene encoding the WA-1 strain of SARS-CoV-2 RBD, along with a 28 amino acid N-terminal signal sequence and an 8-histidine (His) C-terminal tag, was inserted into a mammalian expression vector. This gene encoded the following amino acids: "MGILPSPGMPALLSLVSLLSVLLMGCVAETGTRFPNITNLCPFGEVFNAT RFASVYAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPTKLNDLCFTNV YADSFVIRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSK VGGNYNYLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVEGFNCYFPLQS YGFQPTNGVGYQPYRVVVLSFELLHAPATVCGPKKSTHHHHHHHH". For optimal expression, we produced the protein as a soluble form in transiently transfecting HEK293 mammalian cells. Subsequently, the protein was purified using immobilized metal chromatography binding the 6-His-C-terminal tag. Further purification was achieved through size-exclusion chromatography. The purified protein was then stored at −20°C at a concentration of 0.5 mg/ml.

Sample size and sampling

The required sample size for this study was determined using the open-source software OpenEpi [16]. Assuming an infinite population, a 95% confidence interval (CI), a 1.1% absolute error, and an expected sensitivity and specificity of 99%, the minimum sample size for this study was estimated to be 315 sera from SARS-CoV-2-infected individuals and 315 sera from healthy individuals. A total of 840 samples were used, divided into two panels. Panel A comprised 705 samples specifically selected for this investigation, including 354 samples collected from 128 hospitalized patients at Aliança and Aeroporto hospitals, as well as José Maria de Magalhães Neto Maternity in the metropolitan region of Salvador between March and October 2020. Samples from SARS-CoV-2 patients were classified by symptom onset as follows: 0-7 days post-symptom onset (PSO), 8-14 days PSO, and 15-21 days PSO [5]. Additionally, 351 samples were obtained from the Bahia State Blood Bank Foundation (HEMOBA). Positive samples in panel A were from patients who tested positive for SARS-CoV-2 using RT-PCR and exhibited clinical presentation consistent with COVID-19, while negative samples were obtained from healthy individuals prior to the pandemic. Commercial tests, namely GOLD ELISA COVID-19 IgG + IgM (REM Diagnóstica SA, São Paulo, Brazil), Euroimmun Anti-SARS-CoV-2 NCP IgG ELISA (EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany), and Anti-SARS-CoV-2 NCP ELISA (IgM) (EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany), were employed to detect the presence of anti-SARS-CoV-2 IgM and IgG antibodies. Panel B consisted of samples collected prior to the COVID-19 pandemic, which tested positive for various infectious and parasitic diseases, including dengue (n = 20), syphilis (n = 20), HIV-1/2 (n = 20), human T-cell lymphotropic virus-1/2 (n = 20), hepatitis C virus (n = 20), hepatitis B virus (n = 20), Chagas disease (n = 5), filariasis (n = 5), and leishmaniasis (n = 5).

Receptor binding domain-enzyme-linked immunosorbent assay

The optimal dilutions for antigen coating, antibody-enzyme conjugate (HRP), and serum were determined through checkerboard titration. Final conditions were selected based on the signal-to-noise ratio (SNR) and the maximum difference in average optical density (OD) values between positive and negative samples plus three standard deviations (SD). Acceptable results were defined as positive samples with an average OD above 0.8 and negative samples below 0.15. Following optimization, the RBD protein was added to transparent flat-bottomed polystyrene microplates (Corning® 96-well, Costar, Glendale, Arizona, USA) at a concentration of 100 ng per well in a coating buffer of PBS (Phosphate Buffered Saline) with a pH of 7.4. For RBD-ELISA IgG, the microplates were incubated overnight (16 ± 2 hours at 8°C), while for RBD-ELISA IgM, incubation was performed at room temperature for 15 minutes. The microplates were then blocked using Well ChampionTM synthetic blocking buffer (Ken-En-Tec Diagnostics A/S, Taastrup, Denmark) according to the manufacturer's instructions. Serum samples (100 μl) were added at dilutions of 1:50 (RBD-ELISA IgG) and 1:200 (RBD-ELISA IgM) in PBS buffer containing 0.05% Tween-20 (PBS-T, pH 7.4). After a 30-minute incubation at 37°C, the microplates were washed with PBS-T to remove unbound antibodies. Subsequently, 100 μl of HRP-conjugated goat anti-human antibody (Bio-Manguinhos, Fiocruz/RJ, Brazil) diluted at 1:10,000 (RBD-ELISA IgG) or 1:20,000 (RBD-ELISA IgM) in PBS-T were added to each well. The microplates were incubated at 37°C for 30 minutes, followed by another wash with PBS-T. To detect the immunocomplexes, 100 μl of TMB Plus solution (tetramethylbenzidine; Ken-En-Tec Diagnostics A/S, Taastrup, Denmark) was added to each well. After a 15-minute incubation at room temperature in the dark, the reactions were stopped with 50 μl 0.3 M H_2SO_4, and absorbance was measured at 450 nm using a microplate spectrophotometer (SPECTRAmax 340PC®, San José California, USA).

Laboratory assays

All samples were included in the study to compare the performance and agreement between the ELISA-RBD and commercially available SARS-CoV-2 ELISA tests. The selection of commercial tests was based on their availability and licensing for use in Brazil. Accordingly, three specific COVID-19 enzyme immunoassays were chosen: the Anti-SARS-CoV-2 NCP IgM ELISA (Euroimmun Medizinische Labordiagnostika AG, Lübeck, Germany), which detects IgM antibodies against SARS-CoV-2 using the virus nucleocapsid (N) protein; the Anti-SARS-CoV-2 NCP IgG ELISA (Euroimmun Medizinische Labordiagnostika AG, Lübeck, Germany), which detects IgG antibodies against SARS-CoV-2 using the virus nucleocapsid (N) protein; and the GOLD ELISA COVID-19 IgG + IgM (REM Diagnóstica, São Paulo-SP, Brazil), which detects both IgG and IgM antibodies using the spike protein S1 and S2 domains and the N protein. The tests were performed following the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.5.1 software (San Diego, California, USA). Descriptive measures, in-

Table 1
Comparison of sensitivity, specificity, and accuracy in series vs parallel analysis of RBD-ELISA test results.

Days PSO	RBD ELISA	Type	Sen (%) (95% CI)	Spe (%) (95% CI)	Acc (%) (95% CI)
0-7	IgM	Individual	34.7 (27.5-42.7)	94.6 (91.7-96.5)	76.9 (73.0-80.4)
	IgG	Individual	22.4 (16.5-29.8)	99.1 (97.5-97.7)	76.5 (72.6-80.0)
	IgM + IgG	Series	7.8 (4.5-12.7)	99.5 (99,8-100)	53.7 (52.0-56.2)
		Parallel	49.3 (39.5-59.8)	93.7 (89.4-96.2)	71.4 (64.3-77.9)
8-14	IgM	Individual	60.6 (52.1-68.5)	99.1 (97.5-97.7)	85.3 (81.9-88.2)
	IgG	Individual	54.1 (45.7-62.4)	99.1 (97.5-97.7)	86.8 (83.5-89.5)
	IgM + IgG	Series	33.6 (23.6-42.6)	99.5 (99.8-100)	66.1 (61.5-71.2)
		Parallel	81.8 (73.8-88.1)	93.7 (89.4-96.2)	87.7 (81.6-92.1)
15-21	IgM	Individual	88.2 (73.4-95.3)	99.1 (97.5-97.7)	94.0 (91.2-96.0)
	IgG	Individual	88.2 (73.4-95.3)	99.1 (97.5-97.7)	98.2 (96.3-99.1)
	IgM + IgG	Series	77.8 (53.9-90.8)	99.5 (99.8-100)	88.2 (76.7-95.4)
		Parallel	98.6 (92.9-99.8)	93.7 (89.4-96.2)	96.2 (91.1-98.0)
> 21	IgM	Individual	50.0 (35.2-64.8)	99.1 (97.5-97.7)	90.0 (86.7-92.6)
	IgG	Individual	87.5 (73.9-94.5)	99.1 (97.5-97.7)	98.0 (96.0-99.0)
	IgM + IgG	Series	43.7 (26.0-61.2)	99.5 (99.8-100)	71.7 (62.7-80.5)
		Parallel	93.7 (83.1-98.1)	93,7 (89.4-96.2)	93.7 (86.2-97.1)

Acc, accuracy; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; PSO, post-symptoms onset; RBD, receptor binding domain; Sen, sensitivity; Spe, specificity.

cluding arithmetic and geometric means, along with SD, were used to analyze the variables. Geometric means were calculated with a 95% CI. The normality of the data was assessed using the Shapiro-Wilk test. If the null hypothesis was rejected, either the Wilcoxon-Mann-Whitney or Kruskal-Wallis test was applied. In cases where data normality was confirmed, the Student's *t*-test was used. A significance level of $P < 0.05$ was employed for all conclusions. Statistical significance was determined by the absence of overlapping 95% CI values. Cut-off (CO) values were determined by adding two SDs to the mean of negative samples (RBD-ELISA IgG) or three SDs (RBD-ELISA IgM). Results were normalized by calculating the reactivity index (RI), which represents the ratio between the OD of the samples and the CO. Samples with an RI ≥ 1.0 were considered positive, while samples falling within $\pm$ 10% of 1.0 were classified as inconclusive (gray zone).

The overall accuracy of RBD-ELISA was evaluated using the area under the receiver operating characteristics curve (AUC) and categorized as low (0.51-0.61), moderate (0.62-0.81), elevated (0.82-0.99), or outstanding (1.0) [17]. Performance parameters of RBD-ELISA, including sensitivity (Sen), specificity (Spe), accuracy (acc), likelihood ratios (LR), and diagnostic odds ratio (DOR), were determined and compared. To comprehensively assess the diagnostic performance of the RBD-ELISA IgG and RBD-ELISA IgM, multiple tests, including series and parallel approaches, were employed [18]. In parallel tests, multiple tests are conducted simultaneously, and a positive result in any of the tests indicates the presence of the disease. In sequential tests (series), new tests are requested based on the results of previous tests, and all results need to be positive to establish a disease diagnosis. Cohen's kappa (κ) analysis was used to determine the agreement strength between the standard tests and RBD-ELISA. The interpretation of κ values was as follows: $1.0 \leq \kappa \geq 0.81$ (almost perfect agreement), $0.80 \leq \kappa \geq 0.61$ (substantial agreement), $0.60 \leq \kappa \geq 0.41$ (moderate agreement), $0.40 \leq \kappa \geq 0.21$ (fair agreement), $0.20 \leq \kappa \geq 0$ (slight agreement), and $\kappa = 0$ (poor agreement) [19]. A flowchart (Figure 1) and a checklist (Supplementary Table 1) are provided in accordance with the Standards for the Reporting of Diagnostic Accuracy Studies (STARD) guidelines [20].

Results

The study comprised 705 previously collected and anonymized unvaccinated human serum samples. Among the 128 individuals who tested positive for SARS-CoV-2, the median age was 45 years (interquartile range: 32.5-66.0 years), and the female-to-male ratio was 0.85 to 1. Among intensive care unit-admitted patients, 85.2% (104/122) successfully recovered and were discharged, while 14.8% (18/122) did not survive. For SARS-CoV-2-negative individuals (n = 315), the female-to-male ratio was 1:1.6. All blood donors were residents of Bahia, with no available age-related information for these individuals.

Optimal dilutions for antigen coating, antibody-enzyme conjugate (HRP), and serum were determined through checkerboard titration. The best condition was selected based on achieving a higher signal-to-noise ratio (SNR) and a greater difference in median values between SARS-CoV-2-positive and negative samples. The predefined criteria (OD < 0.15 for negative samples and OD > 0.80 for SARS-CoV-2-positive samples) were successfully met by using antibody-enzyme conjugate at dilutions of 1:10,000 for RBD-ELISA IgG and 1:20,000 for RBD-ELISA IgM. Regarding antigen quantity in each well, all tests showed a more significant difference in median values when 100 ng per well was utilized. In contrast, the serum dilution for ELISA-RBD IgG was determined to be 1:50, while for ELISA-RBD IgM, it was established at 1:200.

Following optimization, we assessed the RBD-ELISA performance and RI distributions using 705 sera from SARS-CoV-2-positive and -negative individuals, as illustrated in the overall analysis of Figure 2 (individual RI data points are shown in Supplementary Table 2). RBD-ELISA IgG exhibited high specificity at 99.1%, with only two false-positive samples, whereas RBD-ELISA IgM yielded the lowest specificity at 94.6%, with 19 false positives. Both assays showed relatively low sensitivity at 48.0% (IgG) and 51.1% (IgM), identifying 170 and 181 of the 354 positive samples, respectively. However, the AUC values were 86.7% (IgG) and 76.5% (IgM), indicating elevated and moderate diagnostic capacity, respectively. This suggests that both assays can effectively differentiate between positive and negative samples despite their lower sensitivity. Both tests demonstrated similar accuracy, as evidenced by overlapping CIs. Cohen's Kappa values indicated that the RBD-ELISA IgG and IgM presented moderate agreement with the RT-PCR results of the samples included in the study. Notably, the detection of IgG antibodies for RBD exhibited a higher DOR of 107.2 compared to the detection of the IgM antibody (18.3), signifying superior diagnostic capacity due to the LRs found.

The performance of RDB-ELISA IgM and RDB-ELISA IgG was assessed at various infection stages, categorizing samples based on symptom onset as follows: 147 samples within 0-7 days, 133 within 8-14 days, 34 within 15-21 days, and 40 after 21 days (see the analysis stratified according to symptoms onset of Figure 2). Notably, both assays exhibited improved performance, particularly during the 15-21 days post-symptom onset (PSO) and beyond 22-day PSO. During the 15-21 days PSO, we observed remarkably high sensitivity and accuracy for both RBD-ELISA IgM and RBD-ELISA IgG, achieving a sensitivity of 88.2% and accuracy exceeding 90%. The DOR for RBD-ELISA IgG notably increased to 870 compared to IgM, which reached 131.05.

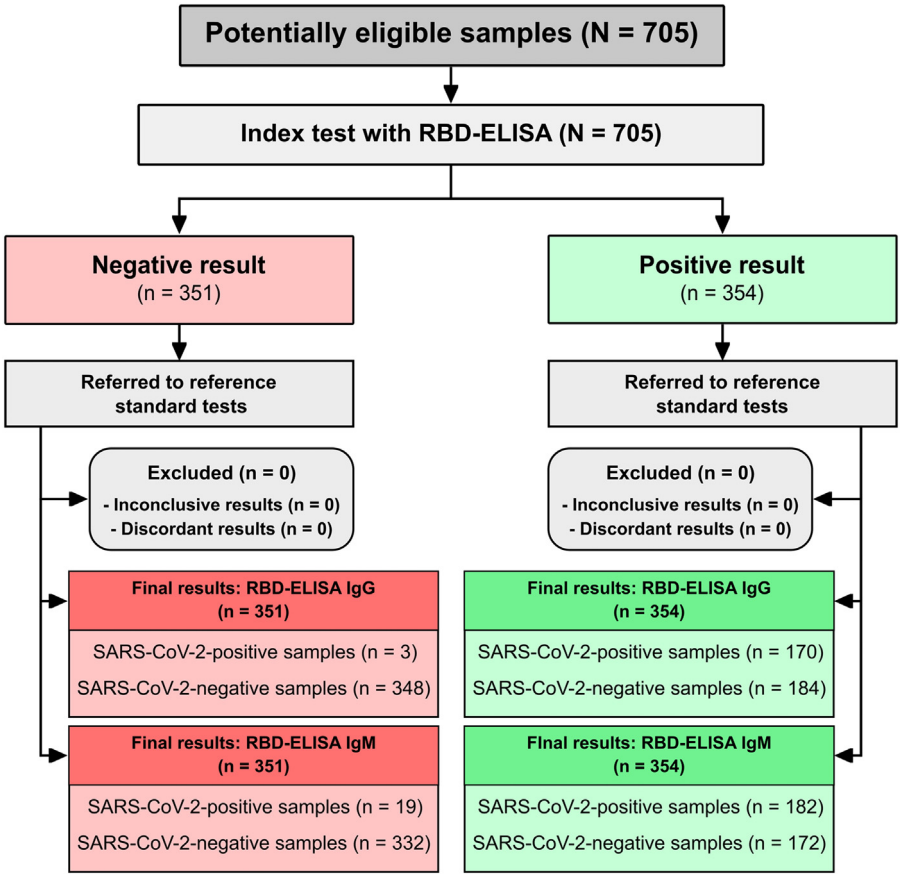


Figure 1. Flowchart illustrating study design in conformity with the Standards for Reporting of Diagnostic Accuracy Studies (STARD) guidelines. ELISA, enzyme-linked immunosorbent assay; RBD, receptor binding domain.

Table 2
Comparison of performance metrics for RBD-ELISAs and commercial COVID-19 tests.

Assays	Sen (%) (95% CI)	Spe (%) (95% CI)	Acc (%) (95% CI)	DOR (95% CI)
RBD-ELISA IgG	48.0 (42.9-53.2)	99.1 (97.5-99.7)	73.5 (70.1-76.6)	107.2 (33.8-340.3)
RBD-ELISA IgM	51.1 (45.9-56.3)	94.6 (91.7-96.5)	72.8 (69.4-76.0)	18.3 (11.0-30.4)
GOLD ELISA	57.6 (52.4-62.7)	99.4 (96.6-99.9)	70.7 (66.7-74.5)	**219.0** (30.3-1,581.7)
NCP ELISA IgG	41.4 (36.3-46.6)	99.4 (96.5-99.9)	59.4 (55.1-63.5)	111.4 (15.4-805.2)
NCP ELISA IgM	46.5 (41.3-51.7)	96.2 (92.0-98.3)	61.9 (57.6-66.0)	22.1 (9.5-51.4)

Acc, accuracy; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; RBD, receptor binding domain; Sen, sensitivity; Spe, specificity.

In an attempt to reduce diagnostic uncertainty, we conducted analyses using both series and parallel approaches based on the individual RBD-ELISA results (see Table 1). These approaches involve combining two diagnostic test outcomes. We consistently observed an increase in sensitivity when analyzing ELISA test results in parallel, as opposed to assessing them individually or through a series approach. Particularly noteworthy was the substantial enhancement in performance values, especially after the 15th day, with sensitivity and accuracy exceeding 90% in parallel analyses.

When comparing the performance of the RBD-ELISAs with the commercial tests used in this study, all five assays exhibited overlapping sensitivity CIs and similar specificity values (see Table 2). Notably, the Anti-SARS-CoV-2 NCP ELISA (IgG) and Anti-SARS-CoV-2 NCP ELISA (IgM) displayed lower accuracy values at 41.4% and 46.5%, respectively, compared to the other assays. While the RBD-ELISAs and GOLD ELISA COVID-19 IgG + IgM shared overlapping CIs, considering all performance parameters, the GOLD ELISA COVID-19 IgG + IgM and the RBD-ELISA IgG demonstrated superior performance (see Table 2).

In the cross-reactivity analysis, we utilized 135 samples (Panel B) from various infectious and parasitic diseases, including dengue (n = 20), syphilis (n = 20), hepatitis B (n = 20), hepatitis C (n = 20), HIV-1/2 (n = 20), human T-cell lymphotropic virus-1/2 (n = 20), as well as cutaneous and visceral leishmaniasis (n = 5), Chagas disease (n = 5), and filariasis (n = 5) (Figure 3). In RBD-ELISA IgM, 5 of 135 samples (3.7%) were false positives, and only one was within the gray zone. For RBD-ELISA IgG, 6 of 135 samples (4.4%) produced false-positive results, with four falling within the gray zone. Among these false positives, positive samples were identified for dengue (1 of 20), HIV (1 of 20), and leishmaniasis (4 of 5).

Discussion

Despite their low sensitivity, both ELISAs effectively distinguished positive from negative samples based on AUC values. Notably, when we stratified the samples by the collection date relative to symptoms onset, both ELISAs performed better, especially in the 15–21-day inter-

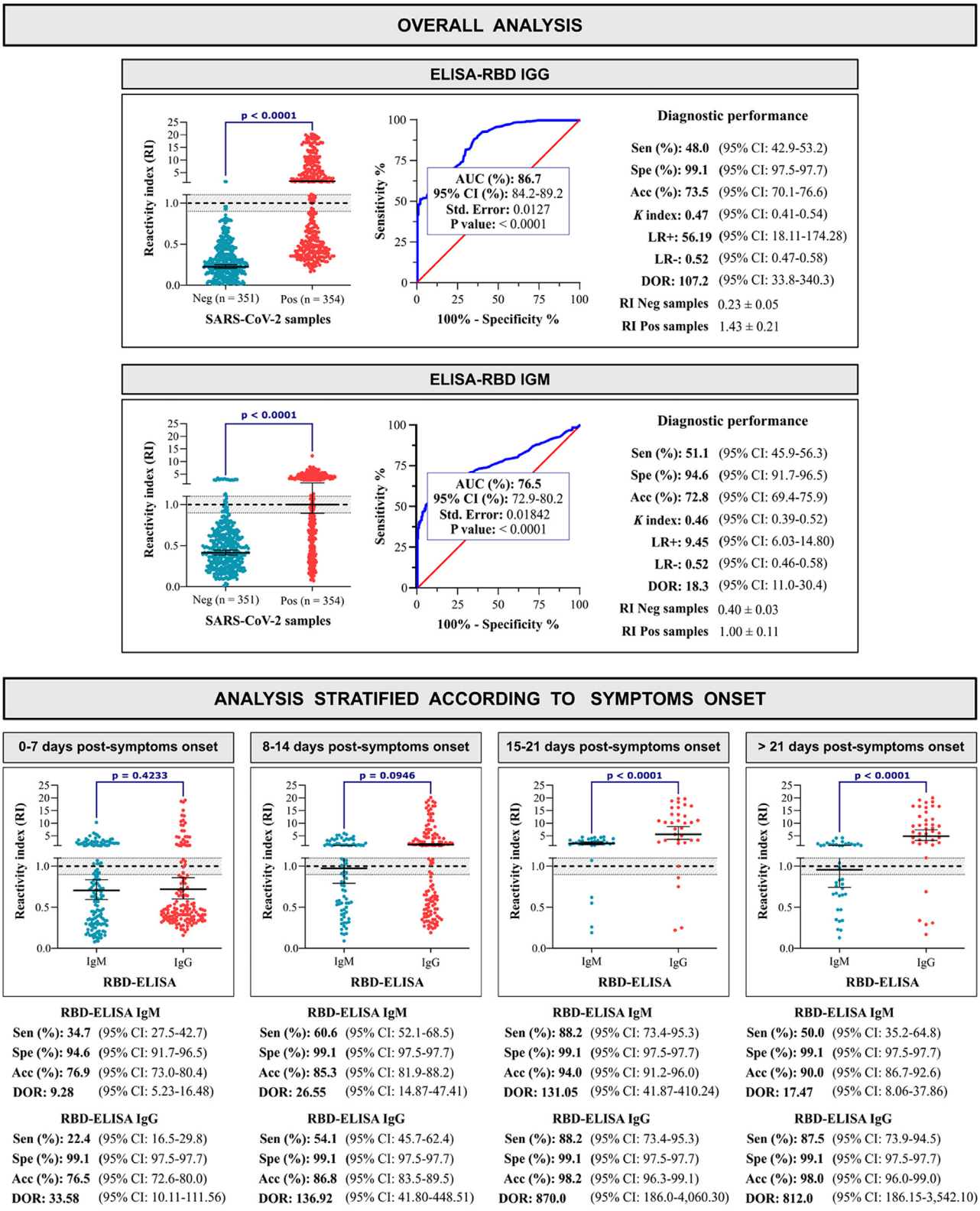
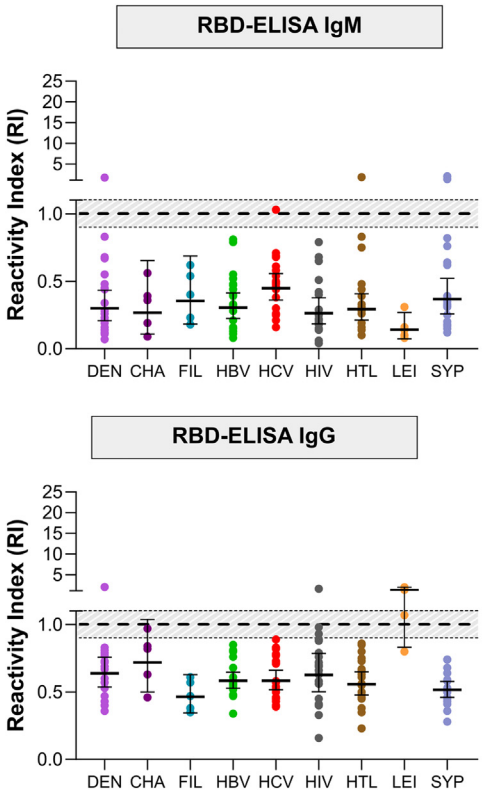


Figure 2. RI and diagnostic performance metrics for SARS-CoV-2-positive (Pos) and SARS-CoV-2-negative (Neg) serum samples (Panel a) and SARS-CoV-2-positive samples categorized by symptom onset (Panel b). The established RI cut-off value was 1.0 (dashed line), with shaded areas denoting gray zones (RI = 1.0 ± 0.10). Geometric mean RI values and corresponding 95% CI values are represented by solid lines.

Acc, accuracy; AUC, area under curve; CI, confidence interval; DOR, diagnostic odds ratio; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; K, Cohen's Kappa index; LR, likelihood ratio; RBD, receptor binding domain; RI, reactivity index; Sen, sensitivity; Spe, specificity.



Disease	RI (95%CI)	Gray zone	Cross reaction
DEN	0.30 ± 0.11	0	n = 1 (5%)
CHA	0.27 ± 0.27	0	0
FIL	0.35 ± 0.25	0	0
HBV	0.31 ± 0.10	0	0
HCV	0.45 ± 0.10	n = 1 (5%)	0
HIV	0.26 ± 0.10	0	0
HTL	0.29 ± 0.10	0	n = 1 (5%)
LEI	0.14 ± 0.10	0	0
SYP	0.37 ± 0.13	0	n = 1 (5%)

Disease	RI (95%CI)	Gray zone	Cross reaction
DEN	0.64 ± 0.11	0	n = 1 (5%)
CHA	0.72 ± 0.27	n = 1 (20%)	0
FIL	0.47 ± 0.14	0	0
HBV	0.58 ± 0.06	0	0
HCV	0.59 ± 0.07	0	0
HIV	0.63 ± 0.14	n = 2 (10%)	n = 1 (5%)
HTL	0.56 ± 0.09	0	0
LEI	1.29 ± 0.59	n = 1 (20%)	n = 3 (60%)
SYP	0.52 ± 0.06	0	0

Figure 3. Cross-reactivity analysis of the RBD protein with sera from various infectious and parasitic diseases. The cutoff value is set at an RI of 1.0, with the shaded region representing the gray zone (RI = 1.0 ± 0.10).
CHA, chronic Chagas disease; DEN, dengue; ELISA, enzyme-linked immunosorbent assay; FIL, filariasis; HBV, hepatitis B virus; HCV, hepatitis C virus; HTL, human T-cell lymphotropic virus 1/2; Ig, immunoglobulin; LEI, leishmaniasis; RBD, receptor binding domain; RI, reactivity index; SYP, syphilis.

val. Combining RBD-ELISA IgG and RBD-ELISA IgM in parallel showed promise for diagnosing COVID-19, achieving a sensitivity of 98.6% and an accuracy of 96.2%. We also conducted interval-based analyses, revealing performance variations depending on the sample collection time. However, evaluating a test's effectiveness solely based on sensitivity, specificity, and accuracy is insufficient. The DOR provides a more comprehensive assessment by measuring the likelihood of positive results in sick individuals compared to non-ill individuals [21]. Both RBD-ELISA IgG and RBD-ELISA IgM yielded high DOR values, with probabilities of 107 and 18 times, respectively. Calculating DOR is crucial for antigen evaluation and validation studies as it is independent of disease prevalence in the population. Both ELISAs demonstrated high specificity. The RBD-ELISA IgM showed 19 false-positive samples, whereas the RBD-ELISA IgG exhibited only two false positives. Unfortunately, the limited information available for the negative samples prevented us from assessing the potential for cross-reactivity with the rheumatoid factor.

The RBD portion of the protein S plays a pivotal role in binding to host cells, enabling membrane fusion machinery activation. Given its importance, researchers have extensively studied it, observing a high rate of neutralizing antibodies against the RBD [12,22]. Literature search results indicate varying performance in studies using RBD as an antigen for COVID-19 diagnosis, influenced by the chosen protocols, secondary antibody detection methods, and vector expression of recombinant protein [23]. For IgG detection, sensitivity ranged from 76.31-94.7%, while IgM detection displayed more variability, ranging from 47.0-81.6%. However, all studies consistently achieved high specificities above 95% [24–29]. In one study, immunoassay accuracy using RBD for IgG detection reached 94.0%, surpassing our study's results [29]. Commercial RBD-based tests, including VIDAS SARS-COV-2 RBD IgG (BioMérieux, Marcy-l'Etoile, France), Siemens SARS-CoV-2 RBD Total (COV2T) (Siemens, NY, USA), and the Access SARS-CoV-2 RBD IgG assay (Beckman-Coulter, CA, USA), reported specificities of 99.9%, 99.8%, and 99.8%, respectively. However, they estimated sensitivity based on the days after PCR positivity. A study demonstrated this variation, where values of sensitivity found were 89.3% (VIDAS), 81.5% (Acess), and 85.9% (Siemens) [26].

Variations in performance of serological assays utilizing RBD as an antigen can be attributed to the kinetics of antibodies during SARS-CoV-2 infection. Many authors have reported increased reactivity and sensitivity in samples collected more than 10 days after symptom onset [5,30,31]. SARS-CoV-2 infection leads to seroconversion to various antibody isotypes, deviating from the typical pattern seen in other diseases, sometimes taking up to 20 days [15,32]. These data corroborate our results of performance parameters when performing the stratification of positive samples for COVID-19 according to the date of the collection after the onset of symptoms. Our results align with this pattern, as we observed differing sensitivities and accuracies when stratifying positive COVID-19 samples based on the collection date. When considering both ELISAs in parallel analysis, with a positive result determined if at least one test was positive, we achieved higher sensitivity (74.60%) and accuracy (84.10%). Interval-based analyses yielded a superior sensitivity (98.6%) and accuracy (96.2%) in the 15–21-day interval. These values exceeded those obtained by screening tests, notably the GOLD ELISA COVID-19 IgG + IgM, which had a sensitivity of 57.6% and an accuracy of 70.7%, even when using three proteins as antigenic matrixes to detect IgG and IgM antibodies. With the ongoing pandemic, continuous transmission of SARS-CoV-2, and the emergence of new variants capable of evading antibodies, cases of reinfection have arisen [33].

Both RBD-ELISA IgG and RBD-ELISA IgM exhibited low cross-reactivity, with rates of 4.4% (IgG) and 3.7% (IgM). False-positive samples were primarily of viral origin, with exceptions such as syphilis and

leishmaniasis. Cross-reactivity may be due to antibodies against other circulating coronaviruses, which cause mild flu-like symptoms [34]. Existing data in the literature indicate a low cross-reactivity rate in individuals infected with arboviruses, particularly in those actively infected with dengue [35].

The primary limitation of this study is the lack of samples from individuals infected with SARS-CoV-2 variants, asymptomatic individuals, and those who have received multiple vaccines. The assays described here were mainly suitable for individuals infected during the first COVID-19 wave before the vaccine was accessible. Additionally, we did not assess cross-reactivity with other arboviruses like Zika, yellow fever, and chikungunya. Further research is required to investigate and address these specific scenarios and questions.

In summary, using the RBD portion of the Spike protein as an antigen in indirect serological assays holds promise for COVID-19 diagnosis, especially when employing the RBD-ELISA IgG and RBD-ELISA IgM in parallel combinatorial analysis.

Declarations of competing interest

The authors have no competing interests to declare.

Funding

This research was supported by the Coordination for the Improvement of Higher Education Personnel in Brazil (CAPES; Finance Code 001) and the National Council for Scientific and Technological Development-Brazil (CNPq; 88887.637869/2021-00 and 88887.805768/2023-00). ICS and FLNS are CNPq research grantees (processes no. 316456/2021-7 and 309263/2020-4, respectively). This work was also supported in part through National Institutes of Health USA grant U01AI151698 for the United World Antiviral Research Network (UWARN). The funders had no influence on the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Ethical approval

This study was approved by the Institutional Review Board for Human Research at the Gonçalo Moniz Institute (IRB/IGM/Fiocruz-BA), Salvador-Bahia, Brazil, under protocol number 33552720.0.0000.0040. Written informed consent was obtained from all participants, and patient data were fully anonymized prior to access by the investigator.

Acknowledgments

The authors express their gratitude to the teams at Hospital do Aeroporto and Hospital Aliança for their warm reception and invaluable assistance in patient and sample selection. Special thanks go to all the individuals who generously donated samples for this study. We extend our heartfelt appreciation to the patients who participated in the study and tragically lost their lives during the pandemic, as well as to their families and friends.

Author contributions

All authors made significant contributions to the work described in this article. ICS and FLNS were responsible for designing the experimental procedure. LCMV and FLNS played a key role in organizing the database. AAC, ACM, ALFO Jr, and ACB were responsible for patient selection at the hospitals. LCMV, LML, YSFM, and AOD contributed to the serum panel. LCMV and LML conducted the ELISA tests. LCMV and FLNS were the primary authors of the paper. ICS and FLNS provided laboratory facilities and secured funding for the study. FLNS oversaw the entire project. All authors have reviewed and approved the final version of the manuscript for publication.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijregi.2023.11.001.

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