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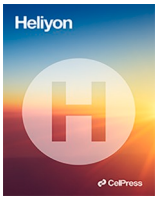

Research article

Development and evaluation of a Lateral flow immunoassay (LFIA) prototype for the detection of IgG anti-SARS-CoV-2 antibodies

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ABSTRACT

Lateral flow immunoassays (LFIA) for antibody detection represent cost-effective and user-friendly tools for serology assessment. This study evaluated a new LFIA prototype developed with a recombinant chimeric antigen from the spike/S and nucleocapsid/N proteins to detect anti-SARS-CoV-2 IgG antibodies. The evaluation of LFIA sensitivity and specificity used 811 serum samples from 349 hospitalized, SARS-CoV-2 RT-qPCR positive COVID-19 patients, collected at different time points and 193 serum samples from healthy controls. The agreement between ELISA results with the S/N chimeric antigen and LFIA results was calculated. The LFIA prototype for SARS-CoV-2 using the chimeric S/N protein demonstrated 85 % sensitivity on the first week post symptoms onset, reaching 94 % in samples collected at the fourth week of disease. The agreement between LFIA and ELISA with the same antigen was 92.7 %, 0.827 *kappa* Cohen value (95 % CI [0.765–0.889]). Further improvements are needed to standardize the prototype for whole blood use. The inclusion of the novel chimeric S + N antigen in the COVID-19 IgG antibody LFIA demonstrated optimal agreement with results from a comparable ELISA, highlighting the prototype's potential for accurate large-scale serologic assessments in the field in a rapid and user-friendly format.

1. Introduction

Lateral flow immunoassays (LFIA), especially for the detection of SARS-CoV-2 antigens, have been essential for COVID-19 diagnosis both in rich and resource-constrained settings with unprecedented large scale use during the pandemic [1]. Simultaneously, the

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development and deployment of vaccines have been crucial for mitigating the pandemic's impact, leading to reductions in severe cases, hospitalizations, and mortality rates worldwide [2–6]. In the current scenario of controlled COVID-19 pandemic, the availability of antibody LFIA for accurate population-wide serologic assessments represents an important epidemiologic tool for surveillance and decision-making policies.

LFIA is considered reliable, user and field-friendly, cost-effective, suitable for point-of-care testing (POCT), not requiring laboratory infra-structure or any specialized equipment [7,8]. LFIAs can be develop to detect antigen or antibody and the design of conventional LFIA is based on membrane capillarity, formation of antigen-antibody complexes, which can be visualized by the use of a colorimetric label [9]. Further integration of automated readers for objective result interpretation and the use of smart reader applications can amplify test capabilities allowing real time transfer of data to a cloud and download at specific centers, identifying hot spots of transmission [10,11]. LFIA has been recognized as an established technology for worldwide use for diagnosis and monitoring of infectious diseases [1]. However, the scarcity of biotech development and production hubs worldwide remains challenging, particularly during pandemic scenarios, restricting accessibility for low- and middle-income countries.

In this context, our study describes a new LFIA prototype for the detection of anti-SARS-CoV-2 IgG antibodies, using a chimeric antigen that merged the highly immunogenic regions of the Spike (S) and nucleocapsid (N) proteins. The sensitivity and specificity of this LFIA prototype was evaluated using a robust serum bank of hospitalized COVID-19 patients and of healthy controls. Agreement of results with an equivalent ELISA are reported.

2. Material and methods

2.1. Samples

A serum bank of 1004 samples was used in this study (Table S1). The sensitivity assessment of the prototype LFIA used a serum bank of 538 samples from 349 COVID-19 patients, RT-qPCR-confirmed that were hospitalized at Instituto de Gestão Estratégica de Saúde do Distrito Federal (IGESDF, central west Brazil). Serum samples were collected from March to November 2021. The mean age of patients was 56.2 years [SD 16.5], ranging from 18 to 93 years. Serum samples were collected at different time points since the onset of symptoms, with a mean of 13 days [SD 6.1 days] following the first symptoms. SARS-CoV-2 RT-qPCR was used to confirm diagnosis at hospital entry and during subsequent monitoring and sample collections no additional molecular test was performed. Samples were distributed in five groups according to collection period (days post symptoms onset, PSO) as 4–7 days PSO: 54 samples; 8–14 days PSO: 216 samples; 15–21 days PSO: 155 samples; 22–28 days PSO:70 samples; >28 days PSO (29–65 days) 43 samples. To evaluate the LFIA test specificity, 193 serum samples collected before 2019 from healthy controls living in the same geographical area, were used as control. Agreement and *kappa* analyses between LFIA and ELISA results were performed using the 193 samples from healthy individuals and 811 samples from the 349 COVID-19 patients regardless of sample collection period.

2.2. SARS-CoV-2 antigens

The QCoV9 chimeric antigen used in the LFIA was based on the SARS-CoV-2 monomer spike (S) with receptor binding domain RBD (449aa – 711aa) linked to the nucleocapsid (N) protein (160–406aa). The chimeric antigen was produced using *Escherichia coli* expression system at Universidade Federal de Pelotas, Brazil (UFPel). The antigen was suspended on urea 8 M at 600 μg/mL. . Quality control measures were implemented for both proteins, including assessment of protein concentration through Bicinchoninic acid assay (BCA) and evaluation of the molecular weight and the protein integrity by Western Blotting (data not shown).

2.3. SARS-CoV-2 RNA extraction and RT-qPCR

RNA extraction and RT-qPCR for SARS-CoV-2 detection were conducted as described [12]. Briefly, the QIAamp® Viral RNA Mini Kit (Qiagen, Germany) was utilized for RNA extraction, and the Promega GoTaq® Probe 1-Step RT-qPCR System was employed for amplification, following manufacturer instructions. Primers and probes targeting two regions of the SARS-CoV-2 N gene (N1 and N2), and the human RNAse P (RP) gene, were provided by Integrated DNA Technologies (Coralville, IA, USA). Samples with a cycle threshold (Ct) value lower than 40 (for N1, N2, and RP targets) were considered positive for the presence of SARS-CoV-2 RNA.

2.4. Enzyme-linked immunosorbent assay (ELISA) IgG anti-SARS-CoV-2

Indirect ELISA was performed using high-binding microtiter 96 well plates (Nunc MaxiSorp™, Thermo Fisher, Denmark, Cat. No. 449824). To eliminate the impact of any unspecific binding, half of the wells were coated with SARS-CoV-2 chimeric antigen QCoV9 (1 μg/mL in 0.05 M carbonate/bicarbonate buffer pH 9.6; 100 μL/well), and the other half with buffer only, then plates were incubated (overnight, 4 °C). ELISA plates were washed (PBS 0.05 % Tween 20-PBS-T; 200 μL/well), blocked (1 % bovine serum albumin-BSA; Probumin, Millipore, USA, Cat. No 82-045-1) in PBS-T (200 μL/well, 37 °C, 60 min). The blocking solution was poured out and, human serum was added in duplicates (1:200 in PBS-T with 1 % BSA, 100 μL/well; 37 °C, 60 min). Plates were washed and horseradish peroxidase labeled goat anti-human IgG was added (100 μL/well, 1:50.000-v/v; Sigma-Aldrich, USA, Cat. No. A0170) (60 min, 37 °C). Plates were washed and incubated with 3,3′,5,5′-tetramethylbenzidine fresh substrate solution (TMB) Sigma-Aldrich; 15 min, room temperature). The reaction was stopped (2 N sulfuric acid, Sigma-Aldrich, USA). The 450 nm optical density (OD) (iMark™ BIO-RAD, USA). The cut-off was previously calculated based on the Receiver Operator Characteristics (ROC) curve: 0.525 for QCoV9 (data not

shown). The interpretation of results was based on the mean OD values of duplicate samples on antigen wells minus the mean OD values of duplicate samples on buffer wells only. To assure the reliability and repeatability of the results, positive, negative, and standard samples were used on each plate.

2.5. SARS-CoV-2 lateral flow immunoassay prototype

2.5.1. Colloidal gold nanoparticle production and conjugation to anti-human IgG

Colloidal gold nanoparticles of 40 nm diameter were produced *in-house* using the citrate reduction method as described previously [13]. Nanoparticle size was measured based on dynamic light scattering (DLS) and analyzed by polydispersity index (PDI) on Zetasizer Lab equipment and ZS Xplorer software (Malvern Panalytical, UK). Anti-human IgG Fcγ specific antibodies (Jackson ImmunoResearch, West Grove, USA, Cat. No.109-005-008) were conjugated to 40 nm colloidal gold nanoparticles by physical adsorption as described [14]. In parallel, the conjugate pad (Millipore, USA, Cat.No. GFSP223000) was treated with trizma buffer 1 % BSA and incubated (overnight 37 °C). UV–Vis Cary 50 (Varian, Palo Alto, California, EUA) was used to confirm conjugation by wavelength shifts closer to 530 nm. Further, the conjugate solution was diluted for adjusting the final optical density to 30 and deposited on the conjugate pad with 6 μL/cm using Biodot Airjet XYZ3050 equipment (Irvine, CA, USA).

2.5.2. Nitrocellulose membrane coating and LFIA assembly

The chimeric SARS-CoV-2 QCoV9 antigen (70 μg/mL in PBS) was coated on test line and the Protein A (Invitrogen, USA Cat.No. 10–1006; 500 μg/mL) was coated on control lines of HI-Flow™ Plus HF180 nitrocellulose membrane cards (Millipore) using Biodot Dispenser XYZ3050 equipment. The membrane was dried (4 h, 37 °C). Then the sample pad (Ahlmstrom 1662), the conjugate pad coated with detection reagent, and the absorbent pad (Millipore, Cat No. GFSP223000) were assembled on the nitrocellulose membrane adhesive card, with 2 mm overlap of each component, for capillarity maintenance. The cards were cut into 5 mm strips using Biodot CM4000 and placed on a housing cassette using Assembly Roller YK725 (Kinbio Tech, Shanghai, China).

2.5.3. LFIA analytical procedure and interpretation

The LFIA was performed in duplicates for each sample: 20 μL of serum and 120 μL of running buffer (20 mM Tris 1 % BSA 0.1 % Tween, pH 8.8) were added to the sample pad for 20 min. During this development phase, the housing cassettes were opened, and dried, and the nitrocellulose membranes were placed on a sheet and the results were analyzed by naked eye by two independent evaluators. A result was considered positive when there was a clear purple staining of the test line, and of the control line. Negative results were observed when there was no staining in the test line and the control line was stained in purple. Invalid test was defined by the absence of staining in the control line and the sample should be re-tested. The interpretation of LFIA results involved the evaluation of the visual intensity of the test line by assigning a score, ranging from 0 for negative results to +4 for strongly positive results, as demonstrated in Fig. 1.

2.6. Statistical analyses

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the prototype LFIA were calculated (MedCalc Software Ltd. Diagnostic test evaluation calculator). Based on information retrieved from the Brazilian Institute of Geography and Statistics (https://www.ibge.gov.br/) and the Brazilian Coronavirus Panel (https://covid.saude.gov.br/) in December 2022, Brazil's estimated population was around 207.8 million people, with reported COVID-19 cases reaching 37.6 million. This suggests a prevalence rate of approximately 17.5 %. The calculated prevalence value was subsequently utilized for the determination of the PPV, NPV and the overall accuracy in the study. Statistical analysis also used R Studio 3 or GraphPad Prism 9. *Kappa Cohen* coefficient (κ) performed with R Studio 3 was employed to analyze agreement between ELISA and LFIA IgG serology tests'

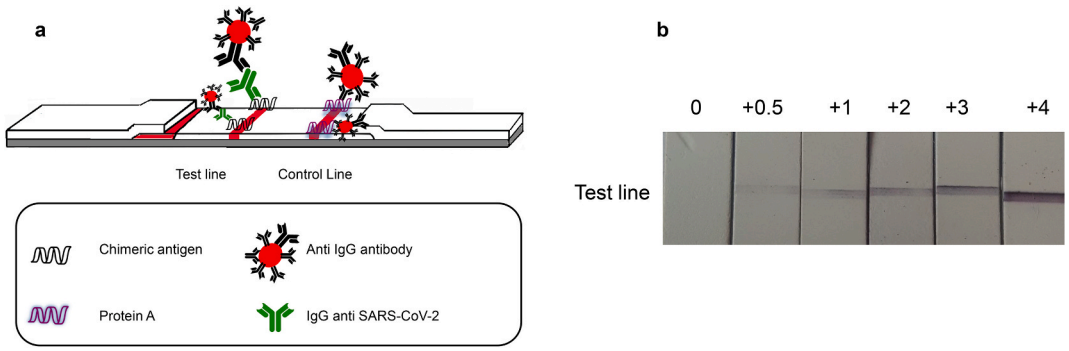

Fig. 1. SARS-CoV-2 LFIA prototype. **1a** Scheme of the LFIA prototype. **1b** Test line intensity score: 0 for negative results; stained positive lines/results ranged from +0.5 to +4 according to subjective naked eye evaluation of color intensity. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

results. The *Kappa* test is an agreement coefficient to correct errors due to chance. The *Kappa* coefficient can range from 0 (complete disagreement) to 1 (complete agreement). In this study, the Landis scale was used, according to which the agreement is classified as: poor (below 0), slight (0–0.2), weak (0.21–0.4), moderate (from 0.41 to 0.6), substantial (0.61–0.8), almost perfect (0.81–1) [15].

3. Results

3.1. LFIA prototype performance for anti- SARS-CoV-2 IgG detection

The main features of the SARS-CoV-2 LFIA prototype performance are depicted in Table 1 and Table S2. The observed total sensitivity using 80.9 %, 95%CI [77.5 %–85.8 %], and the specificity was 93.8 %, 95%CI [89.3 %–96.7 %]. Among the 349 patient's qPCR-positive for COVID-19, 80.9 % (n = 283) were positive by LFIA while 19.1 % (n = 66) were false negatives. Among the 193 healthy controls, 6.2 % (n = 12) tested false positive or cross-reacted with the SARS-CoV-2 LFIA.

Further, we evaluated the LFIA sensitivity observed in samples collected at different days PSO.

The following sensitivity was observed according to days POS: 85 % (95 % CI [72.8 %–93.3 %]), for samples collected during the first week of disease manifestations (4–7 days), for the second week (8–14 days PSO): 81.9 % (95 % CI [76.1 %–86.8 %]), for the third week (15–21 days PSO): 83.8 % (95 % CI [76.1 %–86.8 %]), for the fourth week (22–28 days PSO): 94.3 % (95 % CI [86.0 %–98.4 %]), and after 28 days: 93.0 % (95 % CI [80.9 %–98.5 %]). The overall test accuracy was 92.3 %, the estimated PPV was 73.5 % while the NPV was 95.9 %. For samples collected during the fourth week (22–28 days) PSO, the PPV and the NPV increased to 76.0 % and 98.0 % respectively (Table 1). However, this difference during the weeks was not statistically significant ($p > 0.05$).

Upon testing three different LFIA prototype produced batches using the same samples and assessed by the same evaluators, similar LFIA results were observed, indicating that the new LFIA was reproducible and repeatable (Fig. 2, Fig. S1).

Analysis of total sensitivity was based on the first sample collected from each 349 patient and the time point calculations (days post symptoms onset/PSO) of sensitivity was based on 538 samples from these 349 patients. PSO – Post symptoms onset; Pos – Positive results; Neg – Negative results; PPV – Positive Predictive Value; NPV – Negative Predictive Value; n = number of samples used for analysis.

3.2. Agreement and kappa coefficient of SARS-CoV-2 ELISA and LFIA results

Considering ELISA as a reference assay, comparison of ELISA and LFIA results using 1004 serum samples from COVI-19 patients and the same chimeric antigen showed 92.7 % agreement with a *kappa* coefficient of 0.826, 95 % CI [0.764–0.888] (Table 2). No statistical difference was observed between ELISA and LFIA positivity rates: 70.4 % for ELISA and 69.9 %. for LFIA. ELISA and LFIA results of 1004 samples from COVID-19 patients showed 7.2 % (n = 73) discordant results: 34 (11.4 %) ELISA negative samples were LFIA positives; 39 (5.5 %) ELISA positive samples were LFIA negatives. (Table 2). Comparisons of ELISA and LFIA results with samples of the healthy control group showed that 3.6 % (n = 7) ELISA negative control samples were LFIA positives, and 2.6 % (n = 5) ELISA positive samples were also positive in the LFIA prototype (Table S3).

ELISA and LFIA agreement of 92.7 %, *Kappa Cohen* 0.826, 95 % CI [0.764–0.888]. Positivity percentage for each assay and group are shown in parentheses.

4. Discussion

In this study, we evaluated a new LFIA prototype designed for the detection of IgG antibodies against SARS-CoV-2 using a chimeric antigen derived from the S monomer (449–711aa), with the RBD linked to the N protein (37–402aa). In principle, the chimeric antigen should be able to detect a broader spectrum of antibodies, capturing responses to both highly immunogenic S and N proteins, though we have not compared test results obtained using the chimeric S/N antigen and using individual components S and N. However, the strength of this new rapid test prototype is illustrated by the evidence that the inclusion of the novel chimeric antigen in the COVID-19 antibody LFIA demonstrated optimal agreement with results from a comparable ELISA, highlighting the prototype's potential for accurate large-scale serologic assessments. The new LFIA demonstrated sensitivity and specificity comparable to those already reported in studies that utilized antigens based on SARS-CoV-2 RBD, S, or N proteins [16–21]. We also highlight that in the current study,

Table 1
Performance of SARS-CoV-2 Lateral Flow Immunoassay prototype and reactivity based on 349 COVID 19 patients collected at different days after onset of symptoms and 193 control samples.

Group (Number of samples)	Pos (n)	Neg (n)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Patients (n = 349)	283	66	80.9	93.8	73.5	95.9
Controls (n = 193)	12	181	–	–	–	–
Patients PSO (n = 538)						
1–7 (n = 54)	46	8	85.0	93.8	74.5	96.8
8–14 (n = 216)	177	39	81.9	93.8	73.6	96.0
15–21 (n = 155)	130	25	83.8	93.8	74.1	96.5
22–28 (n = 70)	66	4	94.3	93.8	76.2	98.7
>28 (n = 43)	40	3	93.0	93.8	76.0	98.5

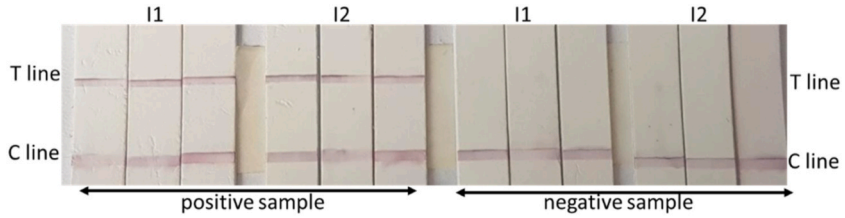

Fig. 2. Reproducibility of results using three different batches of produced SARS-CoV-2 LFIA prototype. Assays were performed on different days with the same samples, in triplicates by two independent investigators (I1 and I2) One positive sample and one negative sample were used. T line = test line; C line = control line.

Table 2
ELISA and LFIA agreement of results and positivity rate for the detection of IgG antibodies anti chimeric SARS-CoV-2 antigen using 1004 COVID 19 serum samples.

	ELISA positive (70.4 %)	ELISA negative (29.6 %)	TOTAL
LFIA positive (69.9 %)	668	34	702
LFIA negative (30.1 %)	39	263	302
TOTAL	**707**	**297**	**1004**

the estimation of sensitivity and specificity was based on the use of a robust serum bank of 811 samples from 349 COVID-19 patients, that represents a significantly larger dataset than used in previous reports. We assume that the large serum bank and the chimeric S/N antigen used certainly contributed to a broader assessment of SARS-CoV-2 IgG antibodies by our LFIA prototype.

Since LFIAs are often used for screening diseases, the specificity is a crucial, parameter requiring further laboratory assays for diagnosis confirmation [1]. Nevertheless, the prototype described in this study exhibited a similar level of specificity compared to reports of other commercial SARS CoV-2 serologic kits marketed in Brazil to detect IgM/IgG antibodies (Eco Diagnostics, Brazil) (Sensitivity: IgM and IgG—87.8 %; specificity: IgM-92.4 %, IgG—92.1 %, based on 70 samples (20 patients and 50 controls). In fact, accurate comparisons of performance among diagnostic tests can be misleading as reliable comparisons should use the same serum bank to avoid potential sampling errors and bias and variations related to disease prevalence [8,22]. Coronaviruses such as MERS-CoV and SARS-CoV-1 are not endemic in Brazil, however, other human coronaviruses (HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoVHKU1) may be circulating in the country and leading to cross-reaction in serology tests [23]. The cross-reactivity of SARS-CoV-2 serology with other circulating human coronaviruses can influence the diagnostic accuracy of serology, thus further evaluation of diagnostic test specificity is necessary to understand the real impact of cross-reactivity in different epidemiological settings in which several coronaviruses may be co-circulating.

The reproducibility and repeatability of the prototype test indicate that the new LFIA was well standardized as different test batches produced yielded comparable results. The repeatability of the negative sample showed that one sample/strip presented a faint line, which may be possibly attributed to nitrocellulose membrane pore obstruction.

The LFIA which is considered a screening test, presented higher false positive rates than the ELISA as expected when patients and control samples were tested. It is important to acknowledge that from the 12 negative control samples that were LFIA positive only two were scored as +1 while all other were scored as 0.5+, exhibiting faint test lines. Several methodologic factors may influence false positivity, such as the long time for immune complexes formation in ELISA which is followed by several washing steps to prevent non-specific bindings, whereas in LFIA the antigen-antibody reaction process happens quickly in less than 30 min [24]. Standardization procedures in LFIA can influence false positivity and sample characteristics such as, antibody affinity, avidity, the presence of interfering/competing antibodies, and borderline serology results may also contribute to disagreement of results among assays [1, 25–28]. LFIA optimization is important to achieve high accuracy, Notably, ELISA results showed a low rate (3.6 %) of discordant results, negative samples that yielded false positives by LFIA, indicating minimal need for refinement in the LFIA protocol to reduce these discrepancies This optimization is crucial to enhance the precision of the LFIA, thereby minimizing both false positive and false negative outcomes, which is essential for the reliable application of LFIA in diagnostic settings. Therefore, optimizations to decrease unspecific bindings of negative samples and increase test line intensity of patient's samples can include adjustments on running and coating buffer parameters [29]. The use of automated readers with well-defined positivity cut off could contribute to more accurate and objective readings, compared to visual evaluation. The potential use of this new LFIA as a POCT requires the standardization for whole blood which can be easily obtained through a 'finger-prick' and applied in the field in diverse settings instead of venous blood collection and serum separation in a laboratory facility.

Despite the pandemic's resolution, the SARS-CoV-2 antibody LFIA prototype may be relevant beyond immediate clinical diagnostics, as sensitive and effective POCT may contribute to massive sero epidemiological studies. POCTs for antibody or antigen detection may be considered cost-effective public health tools for evaluating the spread of a specific pathogen in populations from endemic and non-endemic areas. Thus, the new COVID-19 antibody LFIA prototype can potentially contribute as a user and friendly tool for the surveillance and understanding of humoral responses to SARS-CoV-2.

5. Conclusion

Our study describes the development and the application of a new LFIA prototype to detect anti-SARS-CoV-2 IgG antibodies using a chimeric S/N SARS-CoV-2 recombinant antigen. The parameters of this test evaluated by over 1000 samples from COVID-19 patients and healthy controls showed overall sensitivity of 80.9 % which ranged from 85 % on the first week, 81.9 % on the second week, 83.8 % on the third week, 94.3 % on the fourth week, and 93.0 % after the fourth week post symptoms onset. The LFIA test specificity observed was high, 93.8 %, similar to other commercially available COVID-19 serologic tests. The high agreement observed among the LFIA and ELISA results indicates the reliability of this new prototype in rapidly assessing IgG responses to SARS-CoV-2 infection. Our results suggest that this rapid test seems a promising point-of-care test that can be applied as an accurate field and user-friend diagnosis tool, and also for serology surveillance of COVID-19, especially in resource-constrained settings.

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Data availability

The authors declare that the data will be available upon request.

Declarations

Ethics approval

The study was approved by the Research Ethics Committee of Brazil [No 30794920.7.0000.5317] and, in accordance with the Helsinki Declaration. The collection of data and blood samples was conducted after the individuals selected for the sample had agreed to participate.

Consent for publication

All authors consent for publication.

CRediT authorship contribution statement

Matheus Bernardes Torres Fogaça: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Djairo Pastor Saavedra:** Writing – review & editing, Methodology, Conceptualization. **Leonardo Lopes-Luz:** Writing – review & editing, Conceptualization. **Bergmann Morais Ribeiro:** Writing – review & editing, Resources, Methodology, Conceptualization. **Luciano da Silva Pinto:** Writing – review & editing, Methodology, Conceptualization. **Tatsuya Nagata:** Writing – review & editing, Methodology, Conceptualization. **Fabricio Rochedo Conceição:** Writing – review & editing, Resources, Methodology, Conceptualization. **Mariane Martins de Araújo Stefani:** Writing – review & editing, Methodology, Conceptualization. **Samira Buhrer-Sékula:** Writing – review & editing, Supervision, Project administration, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29938.

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