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Development of a sensitive and affordable alternative ELISA method for detection of human IgG antibodies

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Here we present a platform technology that serve as robust alternative to the traditional enzyme-linked immunosorbent assay (ELISA) approach utilizing protein A and protein G in conjunction with streptavidin-biotin chemistry for the detection of IgG antibodies, obviating the need for anti-human polyclonal antibodies (pAbs) and expensive monoclonal antibodies (mAbs). The target antigen, specific to IgG, is directly immobilized on ELISA plates. Leveraging the high affinity of protein A and protein G for the Fc region of IgG, we enable robust detection of IgG antibodies in diverse biological samples. Through direct immobilization of the IgG antigen and subsequent detection using streptavidin-biotin chemistry, we achieve sensitive and specific quantification of IgG antibody binding. Here, using SARS-CoV 2 Spike protein for validation, our study demonstrates comparable performance to traditional methods and can be adopted for any animal species. This approach offers a practical solution for IgG antibody detection, promising advancements in research and diagnostics.

Keywords: Antibody diagnostics, Biotin-streptavidin chemistry, Protein A/G

The detection of antibodies plays a pivotal role in disease diagnostics, facilitating the identification of past and present infections. Reliable and accurate serological assays are crucial for effective disease surveillance, epidemiological studies, and vaccine efficacy assessments. This need becomes particularly pronounced during pandemics such as COVID-19, where large-scale screening of individuals for immune response status is necessary to control disease spread, evaluate herd immunity, and optimize public health strategies[1]. Among the major immunoglobulin (Ig) subclasses assessed in serological assays, IgM and IgG hold significant importance. IgM is the first antibody produced during an immune response and serves as a marker of early-phase infection, whereas IgG, produced later, has a longer half-life and provides evidence of prior infection or vaccination[2,3]. Due to its higher specificity, affinity maturation, and persistence in circulation, IgG serves as a robust biomarker for long-term immune response evaluation[4,5].

Traditional enzyme-linked immunosorbent assays (ELISAs) remain the gold standard for antibody detection in diagnostic applications due to their sensitivity and adaptability. Standard ELISA protocols employ enzyme-conjugated secondary antibodies raised against human IgG, typically sourced from hyperimmunized animals such as goats, rabbits, or sheep. These polyclonal antibodies recognize the Fc or Fab region of human IgG, facilitating antigen-antibody detection[6-8]. However, the use of polyclonal antibodies introduces several challenges, including batch-to-batch variability, cross-reactivity with non-target immunoglobulins, and potential interference from heterophile antibodies. These factors can contribute to false-positive or false-negative results, thereby reducing assay specificity and reliability[9,10].

To mitigate these limitations, monoclonal antibodies are often employed in ELISA systems due to their high specificity, reproducibility, and ability to target defined epitopes. Despite these advantages, the production and purification of monoclonal antibodies are costly and resource-intensive, making them less feasible in large-scale applications, particularly in low-resource settings or during high-demand situations such as global pandemics[11]. The COVID-19 pandemic underscored the need for cost-effective, scalable, and highly specific serological assays

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capable of providing accurate immune response assessments across diverse populations.

In response to these challenges, we developed an alternative ELISA approach that leverages the high-affinity binding properties of protein A and protein G in conjunction with the streptavidin-biotin amplification system. Protein A, a cell wall protein derived from *Staphylococcus aureus*, and protein G, originating from *Streptococcus* species, are well-characterized immunoglobulin-binding proteins with strong affinity for the Fc region of IgG. Protein A exhibits binding specificity for IgG_1, IgG_2, IgG_4 subclasses and to IgA and IgM to some extent from human as well as several animal species[12].On the other hand, protein G has a broader binding profile, recognizing all IgG subclasses with high affinity[13]. This selective Fc region binding eliminates the need for species-specific secondary antibodies, thereby reducing batch variability and non-specific interactions that could otherwise compromise assay performance.Thus, use of protein A/G enables detection of multiple immunoglobulins and in multiple animal species in a single set-up compared to conventional ELISA and thus is less cumbersome. In fact, in a cross-species comparative study between conventional ELISA and protein A/G based ELISA, protein A/G based ELISA was found superior for detection for anti-*Pythium insidiosum* antibodies[14].

To further enhance detection sensitivity, we incorporated the streptavidin-biotin chemistry system, which is widely recognized for its extraordinarily high binding affinity (K_d= 2.3 × 10^{13} M^{-1}). Streptavidin, a tetrameric bacterial protein derived from *Streptomyces avidinii*, exhibits an almost irreversible binding interaction with biotin, a vitamin cofactor commonly used for molecular labeling[15]. This interaction significantly amplifies assay signal strength while maintaining low background noise, thereby improving detection accuracy.

A critical component of our assay is the use of SARS-CoV-2 spike protein-specific IgG as the detector molecule. The spike (S) protein of SARS-CoV-2 is a trimeric glycoprotein that plays a crucial role in viral entry into host cells. It consists of two functional subunits: S1, which contains the receptor-binding domain (RBD) responsible for binding to the human angiotensin-converting enzyme 2 (ACE2) receptor, and S2, which facilitates membrane fusion and viral entry. The spike protein is highly immunogenic and serves as the primary target for neutralizing antibodies following infection or vaccination[16]. Its pivotal role in viral infectivity and immune recognition makes it an ideal antigen for serological assays aimed at detecting SARS-CoV-2-specific IgG antibodies. By incorporating spike protein IgG as the detector molecule, our assay ensures precise recognition of SARS-CoV-2-specific antibodies, improving both sensitivity and specificity.

The efficacy of protein A- and protein G-based capture systems has been validated in various serological applications, including autoimmune disease diagnostics, bacterial and viral infection detection, and therapeutic antibody quantification[14,17,18]. Building upon these foundations, our adaptation of this approach for SARS-CoV-2 IgG detection provides a cost-effective, specific, and scalable serodiagnostic tool, particularly suited for resource-limited environments. This method offers a practical alternative to conventional ELISA systems, circumventing issues associated with polyclonal and monoclonal secondary antibodies while maintaining sensitivity and specificity.

Materials and Methods

Materials

We procured flat-bottom, clear, polystyrene, high-binding surface 96-well EIA/RIA Assay Microplates from Corning, USA. Chemicals including Non-fat Dry Milk (NFDM), Casein, Bovine Serum Albumin (BSA) Fraction V, 10x Tris Buffer Saline (TBS), and 10x Tris Buffer Saline Tween-20 (TBS-T) were obtained from SRL, India. SARS-CoV-2 Spike (trimeric) (16-1208) Recombinant Protein (8xHis-Tag) was acquired from Cell Signaling Technology, while HRP-Protein G and Biotin-Protein A were sourced from GenScript, USA. Additional reagents such as 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate were procured from Sigma-Aldrich/Merck. Human Immunoglobulin-G (H-IgG) was obtained from GeNei, India, and SARS-CoV-2 Spike Protein (RBD) Recombinant Human Monoclonal Antibody was sourced from Thermo Fisher Scientific, USA. Rabbit anti-human IgG – HRP was procured from GeNei, India.

Serum samples

Serum samples were collected from 10 healthy volunteers vaccinated against SARS-CoV-2. Participants were selected based on the absence of any known autoimmune or infectious diseases, with no recent history of illness or medication. Written informed consent was obtained from all participants before sample collection. All volunteers had received

two doses of the same vaccine (Covishield, manufactured by Serum Institute of India), completed at least 4 weeks prior to blood collection. Since antibody titers may vary depending on the type of vaccine and the number of doses, this uniformity in vaccination ensured consistency across samples. Blood samples (1 mL per participant) were drawn via venipuncture and allowed to clot at room temperature for 30 min. The clotted blood was centrifuged at 5000 × g for 5 min at 4°C to separate the serum. The supernatant (serum) was aliquoted and stored at -80°C until use. This study received ethical approval from the Institutional Ethics Committee at Medical college & S.S.G. Hospital, Baroda, under approval number IECBHR132-2024 ensuring compliance with all relevant guidelines and regulations.

Optimization of Assay Conditions: Polystyrene 96-well plates were coated with 50µL of SARS-CoV-2 Spike protein diluted to 1 ng/ µL in Tris-buffered saline, pH 7.4 (TBS), and incubated overnight at 4°C. After three washes with 200µL per well of TBST (TBS with 0.1% Tween-20), plates were blocked using different blocking buffers prepared in TBS at room temperature (23 ± 2°C) for 1 h. The blocking buffers tested included5% Non-fat Dry Milk (NFDM), 3% BSA, 1% Casein. Subsequent steps involved testing various assay buffers and dilutions of human sera, followed by incubation with Protein G-HRP/Biotinylated Protein A and Streptavidin-HRP. The reaction was developed using the HRP substrate TMB and read at 450 nm using a plate reader[7,8,11].

Comparison with Commercial ELISA Kit

Our optimized ELISA method was compared with the SARS-CoV-2 Spike Protein Serological IgG ELISA Kit from Cell Signaling Technology (Cat No. 20154C), following the manufacturer's instructions.

Statistics analysis

Statistical analyses were conducted using Prism 8 (GraphPad Software). Differences between groups were evaluated using one-way ANOVA. Data were expressed as mean ± standard deviation (SD), and p-values less than 0.05 were considered statistically significant.

Results

The main aim of this study was to develop and optimize an indirect enzyme-linked immunoassay (ELISA) for the reliable detection of specific IgG antibodies in human sera. Specifically, our objective was to develop, optimize, and validate alternative ELISA assays for detecting anti-spike IgG antibodies targeting the spike protein of SARS-CoV-2, which served as a reference antigen. This involved employing an indirect antibody immunoassay format where the spike protein is immobilized on a solid surface. The binding of human antibodies in serum to the immobilized spike protein was detected through two methods:

a) Utilizing HRP covalently coupled to protein G (Fc specific), and
b) Utilizing biotinylated protein A (Fc specific), which further binds to HRP-conjugated streptavidin.

Optimization of assay conditions for Anti-spike SARS CoV 2 IgG ELISA

Optimization experiments were conducted to determine the best blocking agent, sample diluent, serum sample dilution, and incubation temperature for antigen-coated plates. These parameters were optimized based on achieving the lowest OD_{450} values for negative controls and blanks, while maximizing the signal-to-noise ratio for positive samples. Initially, purified human IgG and spike monoclonal antibodies were tested, followed by pre-pandemic negative serum and serum from vaccinated volunteers. The assays were evaluated semi-quantitatively using a purified antibody curve.

Blocking buffers containing non-fat dry milk (NFDM), casein, and bovine serum albumin (BSA) were assessed for both HRP-Protein G and Biotin-Protein A ELISAs. Incubation of samples and conjugates was performed at room temperature (RT) for 60 and 30 min, respectively. Casein was ineffective as a blocking agent (data not shown). For HRP-Protein G, BSA-based blocking yielded a higher signal-to-noise ratio compared to NFDM (Fig. 1A). For Biotin-Protein A, NFDM provided low background and robust performance in both serum and antibody detection curves (Fig. 1D & E).

Protein G and Protein A were optimized independently due to differences in binding properties. Protein G demonstrated higher affinity for human IgG subclasses, making it more suitable for initial evaluation. Protein A, with its broader IgG isotype binding, required distinct conditions to reduce background. BSA and NFDM were used as sample diluents, corresponding to their respective blocking agents.

Various spike protein coating concentrations were tested to determine the optimal amount. A dose-dependent increase in OD_{450} was observed, with 50 ng

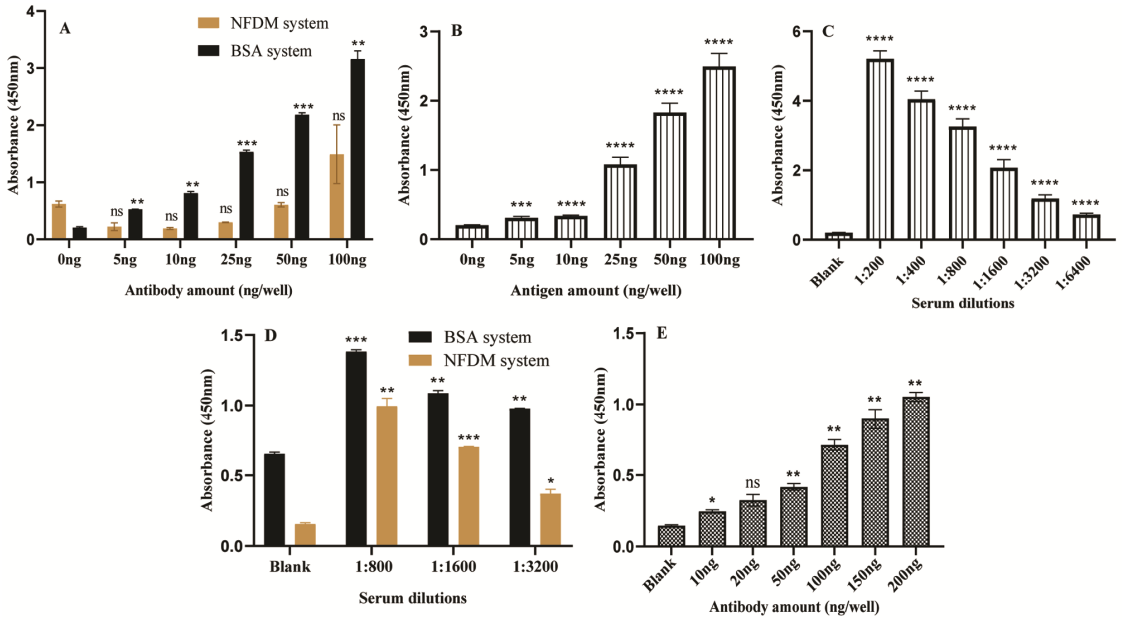

Fig. 1 — Identification of optimal assay conditions for Anti-spike SARS CoV 2 IgG indirect ELISA (A) Human IgG antibody coated onto plate in different amounts and different BSA and NFDM based blockers and sample diluents were tested for HRP-Protein G based ELISA; (B) Spike antigen with varying coating amount per well and using BSA based blockers and sample diluents for HRP-Protein G based ELISA; (C) Serial serum dilution samples with fixed 50ng spike coating amount per well for HRP-Protein G based ELISA; (D) Serum serial dilution sample with different BSA and NFDM based blockers and sample diluents for Biotin-Protein A based ELISA; and (E) hIgG curve with different amounts coated onto the plate, detected by Biotin-Protein A based ELISA. Each assay was carried out in triplicates and error bars depict the standard error of the mean. Unpaired T-test with 95% confidence level was used to calculate statistical significance. ns = not significant ($P > 0.05$); *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$

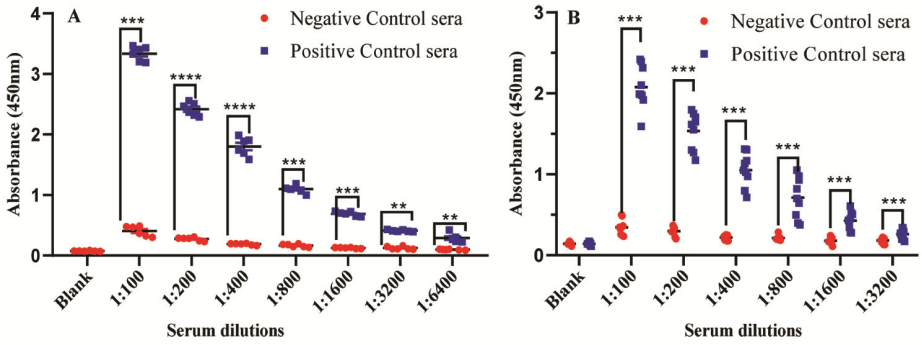

Fig. 2 — Serial dilution serum curve of NC and PC serum samples (A) HRP-Protein G 1:1,00,000 dilution based serial dilution serum curve of Positive and Negative control serum using the previously optimized condition and buffer system; and (B) Biotin-Protein A 1:5,00,000 dilution and Streptavidin-HRP 1:25,000 dilution based serial dilution serum curve of Positive and Negative control serum using the previously optimized condition and buffer system. Each concentration was done in triplicates thrice n=3 and Error bars depict the standard error of the mean. Paired T-test with 95% confidence level was used to calculate statistical significance. ns = not significant ($P > 0.05$); *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$

per well providing sufficient signal while conserving antigen usage (Fig. 1B).

The optimized conditions identified were as follows: 50 ng per well of spike protein incubated at 4°C overnight, 1% BSA in TBS for blocking and 0.5% BSA in 1× TBST as sample diluent for HRP-Protein G ELISAs, and 3% NFDM in 1× TBS for blocking and 1% NFDM in 1× TBST as sample diluent for Biotin-Protein A ELISAs.

After optimization, serum serial dilution assays were repeated with pre-COVID negative control serum to determine the optimal serum dilution. Negative control serum did not significantly affect OD_{450}, while positive sera showed a significant increase at all dilutions (Fig. 2A & B). A serum dilution of 1:800 was selected for both HRP-Protein G and Biotin-Protein A-based ELISAs due to the best signal-to-noise ratio.

Validation of the developed COVID-19 S IgG ELISA

Once the conditions were optimized, validation of the developed ELISAs was carried out using the serum from 10 healthy vaccinated volunteers followed by signal enhancement by varying the dilutions of HRP-Protein G and Biotin-Protein A/Streptavidin HRP. By keeping the optimized conditions and 1:800 sera dilution constant, we confirmed the presence of anti-Spike antibodies in the 10 serum samples from vaccinated individuals. The signal was further enhanced by altering the dilutions of HRP-Protein G and Biotin-Protein A/Streptavidin-HRP. In case of HRP-Protein G based assay, dilutions of 1:50,000 (20 pg/µL) and 1:25,000 (40 pg/µL); and in Biotin-Protein A based assay, 1:50,000 (40 pg/µL) dilution of Biotin-Protein A and 1:10,000 of Streptavidin-HRP showed significant signal enhancement (Fig. 3 A and B).

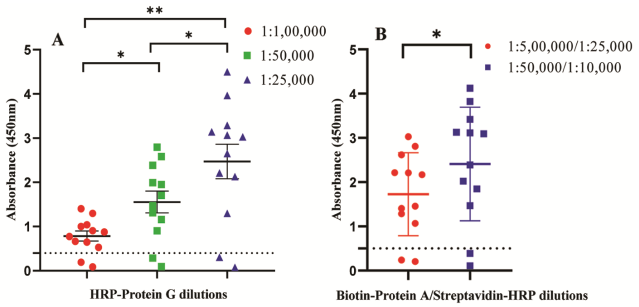

Fig. 3 — Validation of developed indirect ELISA methods with serum sample from vaccinated volunteers (A) HRP-Protein G based ELISA validation using serum samples (1:800 dilution). Signal enhancement was carried out by using indicated dilutions of HRP-protein G *i.e.*, 1:50,000 and 1:25,000; and (B) Biotin Protein-A based ELISA validation was carried out as above by signal enhancement using indicated dilutions of Biotin-Protein A and Streptavidin-HRP i.e., 1:50,000 and 1:10,000 respectively. Each dot represents the antibody status of vaccinated volunteer. Dots below the dotted line represents Blank and Negative Control serum sample of pre-covid volunteer. Paired T-test with 95% confidence level was used to calculate statistical significance. ns = not significant ($P > 0.05$); *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$

Evaluation of performance, stability and comparison of developed ELISA Assay to a commercial kit

After achieving the signal enhancement, we compared our assays with a commercial anti-spike IgG antibody detection kit from Cell Signaling Technology. Although, the commercial kit exhibited higher sensitivity, likely due to proprietary optimizations in the detection system, our lab-developed assays showed good sensitivity and a similar pattern (Fig. 4A).

In order to streamline the testing process and reduce sample processing time, we investigated the use of whole blood as a substitute for serum. Our findings demonstrated that by adding 2 µL of whole blood into 800µL of sample diluent, the resulting signal readout was comparable to that obtained with sera (Fig. 4B).We selected 2 µL of whole blood based on findings from[19], which demonstrated comparable signals to sera while minimizing interference from blood components. We also evaluated the stability of the assays using antigen coated plates (blocked or not blocked) stored at 4°C for one month and compared it with a freshly coated microplate. As shown in Figure

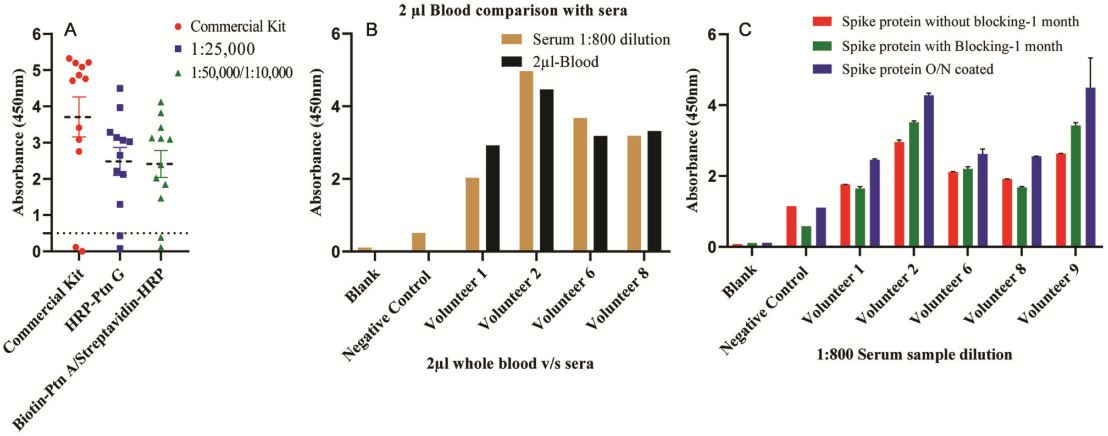

Fig. 4 — Comparison of developed indirect ELISA assays with commercial Kit and testing adaptability to whole blood and stability of assay systems (A) Comparative ELISA with CST commercial indirect ELISA kit with the best optimal condition with 1:25,000 dilution and 1:50,000/1:10,000 dilution of HRP-Protein G and Biotin-Protein A/Streptavidin-HRP respectively. Each dot represents the antibody status of vaccinated volunteer. Dots below the dotted line represents Blank and Negative Control serum sample of pre-covid volunteer; and (B) Comparison of only sera sample and 2µL whole blood sample with the HRP-Protein G 1:25,000 dilution-based ELISA; and (C) Stability evaluation of the antigen pre-coated ELISA plate with and without blocking agent and comparing it with the freshly overnight antigen coated plate using serum samples and 1:25,000 HRP-Protein G based ELISA

4C, the signal was slightly reduced in the stored plates compared to freshly coated plate, but the pattern remains the same. The assay was done using HRP-Protein G system only due to instability of NFDM system used in Biotin-Protein A system.

Discussion

In this study, we describe two distinct and sensitive ELISA based methods using Protein A and Protein G for detecting serum IgG. These assays are based on the reactivity towards the recombinant full-length spike protein of SARS-CoV-2, chosen for its high immunogenicity and significant role in eliciting neutralizing antibodies, particularly targeting its RBD and S1 subunits[20,21].

Certain bacterial species possess Fc receptors for immunoglobulins, such as Staphylococcal Protein A and Streptococcal Protein G, which exhibit high-affinity binding to human, pig, and rabbit IgG with affinity constants (Ka) in the range of 10^{10} to 10^{11} M^{-1} for human IgG[22]. Protein A binds all IgG subtypes except IgG3 and also binds some IgA and IgM. Conversely, Protein G binds all human IgG subclasses but not IgA and IgM[23]. Leveraging these properties, we developed and optimized two separate ELISA based methods utilizing Protein A andProtein G.

Protein G-based ELISA serves as a valuable detection system for human IgG due to its strong binding to IgG subclasses. This system employed HRP-conjugated Protein G as the tracer molecule, which, upon binding to human IgG captured by the spike protein, enabled specific and sensitive colorimetric detection using TMB as HRP substrate[17].

For the Protein A-based ELISA, we utilized biotinylated Protein A to capture IgG, followed by detection with streptavidin-HRP. This method takes advantage of the strong affinity between biotin and streptavidin (low K_d in the femtomolar range), leading to enhanced binding and improved detection limits[15]. This strategic combination allowed us to leverage the strong biotin-streptavidin interaction and the IgG-binding capability of Protein A, resulting in a sensitive, efficient, and cost-effective detection system[24].

Numerous studies have explored whether utilizing Protein A and Protein G conjugates in ELISA could improve antibody detection compared to conventional anti-IgA or anti-IgG conjugates. In a study based on celiac disease (CD) detection, Protein A or Protein G conjugates in ELISA improved sensitivity compared to conventional anti-IgG conjugates for detecting anti-tTG (anti-tissue transglutaminase) IgG in untreated CD patients[23]. Similarly, an indirect form of Protein A-ELISA successfully detected anti-Smith antibodies in the sera of 31 systemic lupus erythematosus (SLE) patients, comparable to a commercial immunodiffusion kit[25]. Similar results were reported in other diseases as well[26,27]. However, to the best of our knowledge, no immunoassay based on Protein G and/or Protein A for detecting SARS-CoV-2 anti-S IgG has been reported to date.

Both ELISA systems were evaluated and optimized independently to ensure maximum sensitivity and specificity. Increasing the detection molecule concentrations enhanced the signal, resulting in sensitivity and specificity comparable to a commercial ELISA kit, validating the accuracy and reliability of our alternative ELISA methods.

We further evaluated the stability of our assays, finding only a minor reduction in sensitivity after one month of storage at 4°C, demonstrating the robustness of our systems. The system was further evaluated using whole blood samples (2µL) instead of serum and found to be as sensitive, highlighting the suitability of the assay even in resource-constrained settings. This approach reduces sample processing time without compromising sensitivity, making it a versatile tool for antibody detection.

While cross-reactivity with other coronaviruses could not be assessed due to a lack of serum samples from individuals infected with other coronaviruses, previous reports suggest that antibodies directed towards the SARS-CoV-2 spike protein do not cross-react significantly with other coronaviruses, supporting the specificity of our assays[28,29].

Further, the developed protocol can also detect anti-spike antibodies in some animal species vulnerable to SARS-CoV-2 infection due to the high conservation of ACE2 (i.e., cats, dogs, ferrets, tigers, and other wild species)[30].

Although the SARS-Cov2 pandemic is largely over, new viral infections are being reported at an alarming rate. The latest addition to the list includes Human Metapneumovirus (HMPV) that was detected in 2024 with cases reported in India as well. The protocol developed in this study can be rapidly adopted to detect human antibodies against any viral infection just by replacing the capture antigen with the specific viral protein. In essence, we have developed a platform technology that can be easily

customized to any condition that require detection of immunoglobulins. This protocol can also be adapted for pool testing, reducing both time and cost for sample processing, with proper optimization and validation under large-scale pooling conditions for assay reliability. These assays represent a robust and versatile tool for detecting IgG and to an extent IgA and IgM antibodies, applicable to various immunological studies beyond SARS-CoV-2 including autoimmune disorders.

Conclusion

Essentially, with this study, we have provided a strong foundation for developing similar ELISA based methods for sero-testing of immunoglobulins, specifically IgG, in various diseases. The Protein G and Protein A conjugates, in conjunction with biotin-streptavidin technology, offer a robust platform that can be adapted to various disease-specific antigens and antibodies. This opens up the possibility of rapidly and accurately detecting antibodies, for a wide range of infectious and non-infectious diseases. Also, the cost-effectiveness makes our method particularly appealing for large-scale sero-testing and surveillance efforts, where cost considerations play a crucial role in resource allocation. Our findings present an exciting opportunity for advancing serological diagnostic methods and contribute to the field of disease surveillance and control.

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Conflict of interest

All authors declare no conflict of interest.

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