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Development and evaluation of a low cost IgG ELISA test based in RBD protein for COVID-19

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ARTICLE INFO

Keywords:
SARS-CoV-2
COVID-19
Serological test
RBD
ELISA

ABSTRACT

Serology tests for SARS-CoV-2 have proven to be important tools to fight against the COVID-19 pandemic. These serological tests can be used in low-income and remote areas for patient contact tracing, epidemiologic studies and vaccine efficacy evaluations. In this study, we used a semi-stable mammalian episomal expression system to produce high quantities of the receptor-binding domain-RBD of SARS-CoV-2 in a simple and very economical way. The recombinant antigen was tested in an in-house IgG ELISA for COVID-19 with a panel of human sera. A performance comparison of this serology test with a commercial test based on the full-length spike protein showed 100% of concordance between tests. Thus, this serological test can be an attractive and inexpensive option in scenarios of limited resources to face the COVID-19 pandemic.

1. Introduction

In December 2019, the coronavirus SARS-CoV-2 produced an outbreak in the Wuhan region of the People’s Republic of China. Only 3 months later, the World Health Organization (WHO) declared COVID-19 as a pandemic disease (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/interactive-timeline). By May 2021, SARS-CoV-2 had infected more than 153 million people and had caused more than 3.2 million deaths worldwide (https://coronavirus.jhu.edu/map.html).

Rapidly, the State-funded consortium of universities together with research institutes and pharmaceutical companies responded to the COVID-19 pandemic, by working around the clock on the development of vaccines, treatments and diagnostic tools. However, middle and low-income countries have had limited access to these biotechnological products. The distribution of vaccines and diagnostic reagents to face this pandemic worldwide has been unequal, with priority on economic, rather than humanitarian, criteria. Thus, it is essential that countries or regions aim for the autonomous development of crucial biotechnological tools.

As many other infectious diseases, diagnosis of COVID-19 can be done by detection of components of the pathogen (such as nucleic acids or proteins) or of the host’s immune response against the pathogen (such as specific antibodies against pathogen’s proteins). RT-qPCR assays are the gold standard tests for COVID-19 for the detection of the SARS-CoV-2 genome, even during the incubation period. Other less sensitive tests can also detect SARS-CoV-2 proteins at the very early onset of the disease (Berger et al., 2021). Since low level of antibodies is produced at early stage of SARS-CoV-2 infection, antibody testing is not the best diagnostic strategy (Ojeda et al., 2021). However, antibody tests can be used for the detection of past SARS-CoV-2 infection or vaccination.

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https://doi.org/10.1016/j.jim.2021.113182
Received 8 June 2021; Received in revised form 8 September 2021; Accepted 3 November 2021
Available online 8 November 2021
Researchers have developed and validated several serological tests for COVID-19 (https://www.cdc.gov/coronavirus/2019-ncov/lab/virus-nCoV) Real-Time Reverse Transcriptase (RT)-PCR Diagnostic Panel (available at https://www.cdc.gov/coronavirus/2019-ncov/lab/virus-s-requests.html). Negative serum samples obtained during a community seroprevalence study of SARS-CoV-2 from asymptomatic adults were subsequently analysed for IgG antibodies against S protein (SARS-CoV-2 IgG II Quant assay, Abbott) and N protein (SARS-CoV-2 IgG assay, Abbott). Regarding the negative serum samples, 59 were regarded as “negative” in all three assays aforementioned. A few samples (17) were only tested with COVIDAR (negative = 10, positive = 7).

All these serum samples were obtained from adult patients from the metropolitan area of Buenos Aires city and Buenos Aires Province during 2020. Most SARS-CoV-2 sequences obtained in this area in 2020 belonged to B lineage, which was responsible for most infections in Europe and North America during the first phase of the pandemic. Up to December 2020, the only variant of concern (VOC) of SARS-CoV-2 circulating in Argentina was B.1.1.28, whose origin was in Rio de Janeiro, Brasil. UK variant (B.1.1.7) was detected for the first time at the end of December from an imported case (http://pais.qb.fcen.uba.ar/reports.php). Additionally, 33 serum samples from people vaccinated against COVID-19 obtained in the metropolitan area of Buenos Aires city during 2021 were also included in this study. These sera were taken 2–3 weeks after the administration of the first or second dose of Sputnik V
(Gamaleya) (27), Sinopharm (3) or (Oxford–AstraZeneca) (3).

2.6. ELISA

The in-house ELISA protocol was adapted from one previously reported (Stadlbauer et al., 2020). Briefly, 96-well microtiter ELISA plates (nuncMaxisorp) were coated with 50 μl of RBD (2 μg/ml) in PBS overnight (ON) at 4 °C. The plates were blocked for 1 h with 200 μl of PBS-T plus 3% milk powder at room temperature (RT) before incubating 100–200 μl of diluted serum samples (1:50) in PBS-T plus 1% milk powder at RT for 2 h. The wells were washed 12 times with 200 μl of PBS-T before adding 100 μl of 1/3000 Goat anti-Human IgG HRP (Promega) in PBS-T + 1% milk power. The plates were further incubated for 1 h at RT and then washed 12 times with 200 μl of PBS-T. Once the wells were completely dried, 100 μl of 1% 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)-ABTS in phosphate citrate buffer plus 0.1 μl of 30% hydrogen peroxide were added to each well and the colour developing was then stopped with 100 μl of stop solution (50% v/v dimethylformamide (Sigma) 20%w/v SDS (Promega)). The optical density at 405 nm was measured with Multiskan plate reader. This in-house ELISA protocol was used to assay 162 serum samples.

The cut off value of the in-house RBD ELISA was calculated as mean + 3xSD of sera from people that were negative to both RT-qPCR of COVID-19 and commercial IgG ELISA (COVIDAR).

All sera were analysed with COVIDAR ELISA following the manufacturer’s recommendations.

3. Results

3.1. Production of RBD from mammalian cells

Transient expression of recombinant proteins in eukaryotic cells demands the production of large quantities of ultrapurified recombinant plasmids as well as expensive reagents and media to optimize the transfection efficiency. Moreover, each round of protein production requires starting all over again beginning at the cellular transfection step. In contrast, semi-stable expression systems use episomal vectors that replicate extra-chromosomally in cells over a long period (Magistrelli et al., 2010, 2012); which allows the expansion of transfected cells at a logarithmic scale.

In this study, the sequence encoding the polypeptide RBD of S protein was cloned in pEAK 8 CMV I Promoter. This vector is an episomal replicative plasmid that confers puromycin resistance to the transfected cells. In the presence of puromycin only transfected cells replicate. The sequence encoding a six histidine tag was added at the end of the gene for purification purposes. The recombinant plasmid was used to transfect 2×10⁶ of PEAKrapid ATCC® CRL-2828™ cells. The transfected culture was expanded by successive passages in standard complete medium or serum-free medium containing puromycin until reaching 8.5x10⁷ cells. Recombinant RBD was purified from culture supernatant by a single step of metal ion affinity chromatography.

SDS-polyacrylamide gel electrophoresis of elution fractions showed the presence of RBD migrating at the expected molecular weight of around 30 kDa (Fig. 1). The recombinant protein yield was 10 mg/l.
In this study, we used an easy and inexpensive mammalian expression system to produce the RBD domain of S protein of SARS-CoV-2. Starting with the transfection of a cell monolayer in a 9.6 cm² well with the 2 μg of the episomal recombinant plasmid, at the end of the process we purified 2 mg of the RBD protein. The entire protocol avoids the use of expensive reagents or media and any eventual low cell transfection efficiency can be easily resolved by expanding the transfected cells in medium containing puromycin. According to Thermo-fisher official web page, 1 l of transfected cells produced with Exp293 Expression System or other FreeStyle 293 Medium costs $1531 and $548, respectively. Although the production levels vary significantly among culture media, which makes it impossible to make an adequate comparison of production costs, for this study we estimated a value of $2.35 for lipofectamine 3000 (ThermoFisher, the most expensive reagent used in our protocol) to produce 200 ml of semi-stable transfected cells expressing RBD. Thus, the system expression and protocol here described is an attractive option for low budget scenarios.

Interestingly, the results (positive/negative) obtained with the in-house RBD IgG ELISA matched exactly to those of a commercially available ELISA test that employs the full-length S protein of SARS-CoV-2. The results of this study were reproducible in two independent determinations made in a time interval of six months and performed by different operators. The commercial test used here for comparison, called COVIDAR, has been tested in RT-qPCR Sars-CoV-2 positive patients and in a large panel of human sera obtained before 2020 (Ojeda et al., 2021). The sensitivity of COVIDAR reported for IgG detection was 72% to 74% between 2 and 3 weeks from the onset of symptoms and seroconversion increased up to 90.4% after 3 weeks (Ojeda et al., 2021). Based on the high concordance of our test with COVIDAR, we may estimate similar performance in both assays. However, further analysis with a larger panel of serum samples is necessary to precisely determine the sensitivity and specificity of this RBD IgG ELISA test.

As previously reported (Amanat et al., 2020; Stadlbauer et al., 2020), we have found that the antibody reactivity against COVID-19 was stronger against the S protein than against the RBD domain. This was an expected result since more B epitopes are present in the full-length S than in RBD region. However, despite its lower reactivity, RBD is the target of 90% of the neutralizing activity present in SARS-CoV-2 immune sera (Piccoli et al., 2020). Therefore, the use of RBD in serological studies would allow a more direct estimation of neutralizing antibodies in serum samples. This feature makes the test developed in this study a simple and very useful tool for first screening of sera with neutralizing capacity.

Another important outcome of this study is the good performance of the in-house RBD IgG ELISA to identify vaccinated people. All serum samples from vaccinated people (33) were positive for RBD IgG (Fig. 3) and COVIDAR tests. This result indicates that, at least for the analysed samples, the test identified vaccinated people.

4. Discussion

Table 1

| Serum sample # | RBD  | S  |
|----------------|------|----|
| 1              | 10.8 | 41.3|
| 2              | 4.3  | 25.6|
| 3              | 11   | 35  |
| 4              | 7.9  | 29.5|
| 5              | 9.3  | 36.3|
| 6              | 6.5  | 36.2|
| 7              | 7.2  | 36.2|
| 8              | 3    | 14.2|
| 9              | 4.3  | 17.2|
| 10             | 4.1  | 5.6 |

| Ratio of serum sample#/average negative sera. |

Transfected cells were viable after freezing and thawing, with production of equivalent amounts of secreted recombinant protein to that of the non-frozen cells (10 mg/l).

3.2. Evaluation of RBD antigenicity against human sera

In order to assess the antigenicity power of RBD, we developed an in-house ELISA that was subsequently evaluated with a panel of human sera. The serum panel included 53 samples positive for a commercial IgG ELISA based on the entire S protein of SARS-CoV-2 (COVIDAR) and 59 negative sera according to this ELISA test.

The concordance between the commercially available S IgG ELISA test and in-house IgG RBD ELISA was 100% (Fig. 2A). Linear regression analysis showed a very good correlation ($R = 0.84$) between the OD values obtained by the in-house RBD ELISA and COVIDAR test used as a reference (Fig. 2B). However, in general, the ratio of positive serum to the average value of the negative sera was higher for the S-based test than for the RBD-based test (Table 1).

To determine the usefulness of the in-house RBD IgG ELISA test as a tool to estimate the rate of COVID19 vaccination, we subsequently evaluated the reactivity of this test against sera of vaccinated people with different vaccine formulations. All serum samples (33) were positive for RBD IgG (Fig. 3) and COVIDAR tests. This result indicates that, at least for the analysed samples, the test identified vaccinated people.

Fig. 3. Reactivity of human sera to RBD and S proteins.

ELISA for detection of SARS-CoV-2-specific IgG antibodies in serum samples from vaccinated people. Blue dots indicate serum reactivity to RBD and red dots indicate serum reactivity to S. The dotted line indicates ELISA cut-off value = 0.1 (mean of negative sera +3xSD) and 0.25 for IgG RBD ELISA and COVIDAR, respectively. These results represent one of two independent replicates.
virus variants. In this regard, since RBD is a protein of a lower molecular weight compared to S, modifying its gene sequence according to the new adaptive variants would be quick and easy. The optimal size of RBD gene facilitates its PCR amplification and further cloning steps in the multiple cloning site of pEAK 8 CMV episomal plasmid.

In conclusion, the protein expression system and the assay developed and tested in this study is an inexpensive alternative in serological surveillance and vaccine evaluation studies.

Funding

This work was supported by the Instituto Nacional de Tecnología Agropecuaria (INTA) Grant i102 and by ANPCyT PICT-2018-01113 and 2017-1721.

Competing financial and non-financial interests

All authors declare no competing or financial interests.

Author contributions statement

Luciana Villafañe, Lucía Gallo Vauletand Marcelo Rodríguez Fermeipin: serology studies, resources and writing. Laura I. Klepp and Marina A. Forrellad: protein expression and purification; Florencia M. Viere: conceptualization and resources; María M. Bīgi: statistical analysis. María I. Romano: conceptualization and writing; Giovanni Magisstrelli: analysis, resource, review and editing; Fabiana Bīgi: conceptualization, formal analysis, writing the original draft and editing.

Acknowledgements

We thank COVIDAR consortium and F. Krammer very much for providing us with COVIDAR kit and the plasmid expressing RBD domain, respectively. We are grateful to people from the community providing us with COVIDAR kit and the plasmid expressing RDB domain, respectively. We are grateful to people from the community providing us with COVIDAR kit and the plasmid expressing RDB domain, respectively. We are grateful to people from the community providing us with COVIDAR kit and the plasmid expressing RDB domain, respectively.

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