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A rapid, point-of-care red blood cell agglutination assay detecting antibodies against SARS-CoV-2

Robert L. Kruse [a, *], Yuting Huang [b, c], Heather Smetana [a], Eric A. Gehrie [a], Timothy K. Amukele [a], Aaron A.R. Tobian [a], Heba H. Mostafa [a], Zack Z. Wang [d, **]

[a] Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, USA
[b] Department of Medicine, University of Maryland Medical Center Midtown Campus, Baltimore, MD, USA
[c] Division of Gastroenterology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA
[d] Division of Hematology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA

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ABSTRACT

The COVID-19 pandemic has caused significant morbidity and mortality. There is an urgent need for serological tests to detect antibodies against SARS-CoV-2, which could be used to assess past infection, evaluate responses to vaccines in development, and determine individuals who may be protected from future infection. Current serological tests developed for SARS-CoV-2 rely on traditional technologies such as enzyme-linked immunosorbent assays (ELISA) and lateral flow assays, which have not scaled to meet the demand of hundreds of millions of antibody tests so far. Herein, we present an alternative method of antibody testing that depends on one protein reagent being added to patient serum/plasma or whole blood with direct, visual readout. Two novel fusion proteins, RBD-2E8 and B6–CH1-RBD, were designed to bind red blood cells (RBCs) via a single-chain variable fragment (scFv), thereby displaying the receptor-binding domain (RBD) of SARS-CoV-2 spike protein on the surface of RBCs. Mixing mammalian-derived RBD-2E8 and B6–CH1-RBD with convalescent COVID-19 patient serum and RBCs led to visible hemagglutination, indicating the presence of antibodies against SARS-CoV-2 RBD. B6–CH1-RBD made in bacteria was not as effective in inducing agglutination, indicating better recognition of RBD epitopes from mammalian cells. Given that our hemagglutination test uses methods routinely used in hospital clinical labs across the world for blood typing, we anticipate the test can be rapidly deployed at minimal cost. We anticipate our hemagglutination assay may find extensive use in low-resource settings for detecting SARS-CoV-2 antibodies.

1. Introduction

The SARS-CoV-2 coronavirus causing COVID-19 disease represents a growing pandemic, leading to acute respiratory distress syndrome in a portion of patients and ultimately significant mortality [1]. Serologic testing for antibodies against SARS-CoV-2 could detect both recent and past infection, which is crucial for surveillance and epidemiological studies [2]. However, current enzyme-linked immunosorbent assay (ELISA) tests for COVID-19 require a number of steps, washes, and reagents, involving hours of manual time and/or automated machines [3]. Lateral flow immunoassays have been developed for SARS-CoV-2, but still require the manufacturing of strips, plastic holders, and multiple different antibody types and conjugates [4]. There is an urgent need for a low complexity assay that could be performed as a point-of-care test in low-resourced health care settings, without the need for machines.

As an alternative method to detect antibodies, blood banks across the world routinely detect antibodies against blood group antigens as part of the type and screen assay, performed before blood transfusions are given. The readout of the assay is hemagglutination, or the aggregation of red blood cells (RBCs), which can be captured by a camera or easily observed with the naked eye. Furthermore, hemagglutination testing, whether by hand or by an automated machine, can be used to titer antibodies, measuring their levels in the serum [5]. This particular flexibility to range from point-of-care, single patient testing to scalable applications on

* Corresponding author. Carnegie Building, Room 401, Johns Hopkins Hospital, 600N Wolfe St, Baltimore, MD, 21287, USA.
** Corresponding author.
E-mail addresses: robert.kruse@aya.yale.edu (R.L. Kruse), zack.wang@jhu.edu (Z.Z. Wang).

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existing automated platforms in clinical labs is unique among the different serologic testing options.

Hemagglutination has been leveraged in the past to detect antibodies against different pathogens. The first iteration consisted of cross-linking an antibody against RBC antigens with a peptide antigen from human immunodeficiency virus (HIV) [6]. When incubated with whole blood from HIV patients, RBC agglutination could be observed, indicating antibodies specific to that antigen were detected [6]. A comparison of 1800 patient blood specimens found similar sensitivity and specificity between commercial ELISA kits and 2-min autologous RBC agglutination testing [7]. Later studies improved on the technology by building fusion proteins of antibody fragments with antigens from HIV [8,9]. Targeting multiple different RBC antigens at the same time improved the performance characteristics of the assay [10]. Antibodies against West Nile virus have also been detected by autologous RBC agglutination assay [11]. Outside of infectious disease, elevated D-dimer levels could also be determined with a similar RBC agglutination assay, SimpliRED, for point-of-care testing for patients with suspected deep vein thrombosis [12].

Herein, we describe an RBC agglutination assay to detect antibodies against the receptor-binding domain (RBD) of SARS-CoV-2 spike protein in COVID-19 patients, which is the frequent target of neutralizing antibodies against coronaviruses [13]. The assay may find use in low-resource settings as a simple method of testing for current or past SARS-CoV-2 infection.

2. Materials and methods

2.1. Gene construction

Two different fusion proteins were designed. The first fusion protein consisted of SARS-CoV-2 RBD (amino acids 330–524 of the spike protein) of the SARS-CoV-2 spike protein [14], connected via a short linker to a single-chain variable fragment (scFv) derived from the antibody 2E8 that binds to the H antigen on RBCs [15] to form RBD-2E8 (Fig. 1). RBD-2E8 also contained an IgG heavy-chain secretion signal for export from mammalian cells, and a hexa-histidine tag located at *C*-terminus to allow for convenient purification. The RBD-2E8 gene was synthesized (Twist Bioscience) and cloned into a pCMV-IRES-GFP vector.

A second fusion protein, B6–CH1-RBD, was designed, consisting of an scFv binding to RBCs at the *N*-terminus, and the RBD sequence at the *C*-terminus with hexa-histidine tag. B6 is an scFv clone against a high frequency antigen on human RBCs [16]. The human IgG CH1 domain was included as a linker to facilitate additional length for antigen binding [17]. RBD sequence was longer than prior, ranging from 319 to 550 amino acid. B6–CH1-RBD was synthesized with an IgG heavy-chain secretion signal (Twist Bioscience) and cloned into the pTwist vector driven by a CMV promoter. A second B6–CH1-RBD gene was codon-optimized for *E. coli* expression and synthesized by BioBasic (Markham, Ontario, Canada), and subsequently cloned into a pET vector for expression.

Please see the **Supplementary Methods** for description of protein production.

2.2. Patient serum samples

De-identified, discarded serum samples were collected and provided from Johns Hopkins Bayview Hospital, representing blood draws by phlebotomists for other medical testing during hospitalization. The clinical lab had collected and banked recovered COVID-19 patient specimens who were greater than 28 days post COVID-19 symptoms with negative PCR testing at the time. All patients were previously positive by nasopharyngeal swab PCR testing at their admission for COVID-19. Aliquots of these samples were then provided to the investigators without identification. De-identified, discarded patient samples with known ABO typing were also provided by the hospital as control anti-sera for isohemagglutination assays. As non-identifiable human material for the current research, the specimens are not subject to Human Subjects research regulations according to the National Institutes of Health (NIH) regulation 45 CFR Part 46 and comply with the code of ethics outlined in the World Medical Association Declaration of Helsinki.

2.3. Red blood cell agglutination testing

For the RBC agglutination assay, a round-bottom 96-well plate (CoStar) was used. O-type Rh-positive red blood cells suspended in 2–4% solution (Immucor) were obtained.

The assay was carried out in two different conditions. In the first, 20 μL of RBC solution, 10 μL of undiluted COVID-19 patient serum, and 10 μL of RBD-2E8 or B6–CH1-RBD solution were pipetted into each well, following a similar protocol from a previous study [15]. The solution was thoroughly mixed and incubated for 5-min at room temperature; agglutination was then visualized by the naked eye. For testing COVID-19 patient serum, a dilution series of RBD-2E8 was performed to test for optimal levels of protein to induce agglutination in presence of patient anti-RBD antibodies. A series of six 1:1 dilutions were performed from the ~100 μg/mL of RBD-2E8, B6–CH1-RBD (mammalian), and B6–CH1-RBD (bacterial) stocks. A seventh well containing non-infected patient serum, 10 μL of phosphate-buffered saline (PBS) and RBC solution alone was used as a negative control to rule out potential patient alloantibody induced agglutination, or alternatively, cold-reactive IgM autoantibodies.

In a second assay condition, a longer incubation time was utilized to augment a weaker rate of hemagglutination according to a published protocol [19]. 50 μL of RBCs (2–4%) were added to 50 μL of COVID-19 patient serum along with 25 μL of purified mammalian fusion protein solution. For the bacterial protein, a solution of 10 μg/mL of B6–CH1-RBD was prepared and 50 μL added to the reaction. After mixing, the solution was allowed to incubate for 1 h in the 96-well plate. To read the assay, the plate was then tilted to allow the RBC pellet to dislodge; agglutination kept the pellet effectively in place. In other experiments, 50 μL solution of recombinant proteins ACE2-Fc (10 μg/mL), and CR3022 antibody (0.6 μg/mL) (ProMab Biotechnologies) were added in the place of COVID-19 patient serum at the designated amounts listed in the figures, and the assay similarly executed.

3. Results

3.1. Construction of SARS-CoV-2 fusion proteins

We aimed to decorate SARS-CoV-2 antigens on the surface of RBCs (Fig. 1). These decorated RBCs would then yield hemagglutination in the presence of SARS-CoV-2 antibodies (Fig. 1). As a proof of concept, the RBD of the SARS-CoV-2 spike protein, corresponding to amino acids 330–524 of the spike protein [14], was chosen for its small size and stable folding, as well as the fact that the RBD is the target of the majority of neutralizing antibodies against coronaviruses [20]. Any positive test for antibodies binding to RBD would be highly suggestive of the presence of neutralizing antibodies that would be protective against infection [21]. Moreover, the RBD antigen has demonstrated to have the highest specificity toward SARS-CoV-2 to distinguish it from other coronaviruses, and 98% of patients develop RBD antibodies by day 9 of symptoms [13].

Using SARS-CoV-2 RBD as a target, we prepared two different

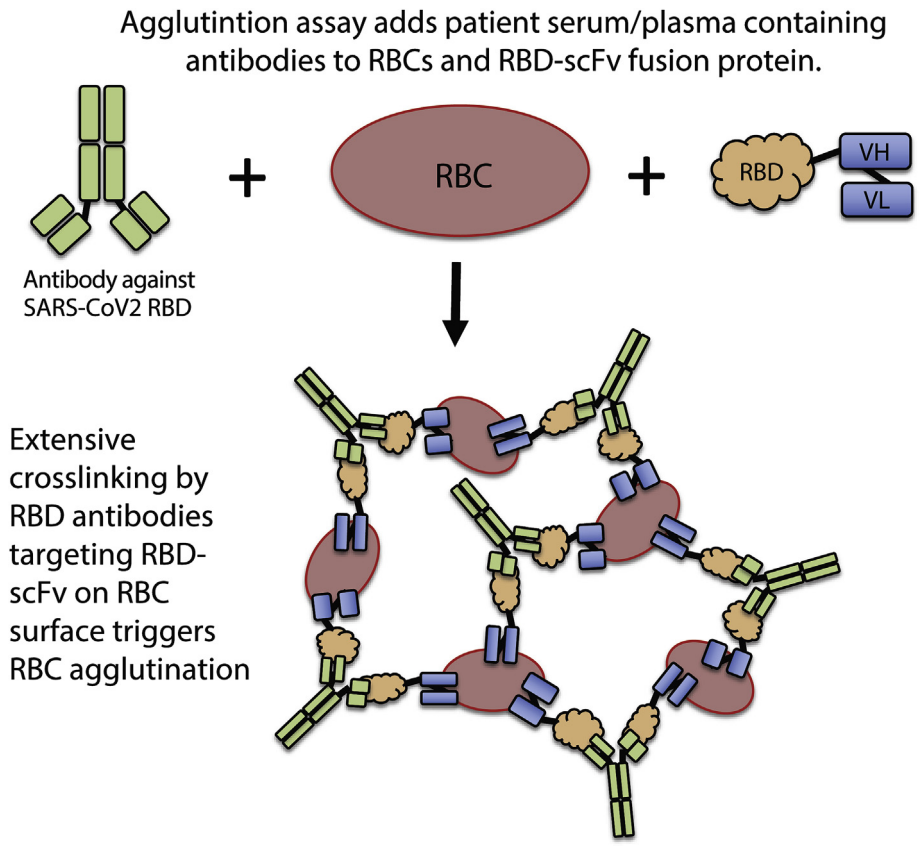

Fig. 1. Mechanism of using fusion proteins to induce hemagglutination to detect SARS-CoV-2 antibodies. A fusion protein, RBD-2E8, was constructed, consisting of the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein at the *N*-terminus connected via a linker to a single-chain variable fragment (scFv, consisting of VH and VL domains connected with a flexible linker) at the *C*-terminus targeting the H antigen on the surface of red blood cells (RBCs). Patient serum/plasma containing antibodies targeting the SARS-CoV-2 RBD are mixed with RBCs and RBD-2E8 fusion protein and allowed to cross-link multiple RBCs in mass, eventually leading to visible agglutination seen with the naked eye. VH = variable heavy; VL = variable light. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

fusion protein designs. The first one had RBD at the *N*-terminus, a short linker, and a *C*-terminal scFv against the H antigen, a carbohydrate antigen located within ABO polysaccharides [22]. The H antigen is ubiquitous in RBCs in the human population, except among Bombay individuals, who are exceptionally rare [23]. A previous study indicated that the scFv 2E8 could successfully bind to RBCs and be used to display HIV gp41 peptides to detect HIV antibodies in a similar RBC agglutination assay [15]. The conditioned medium containing RBD-2E8 fusion protein was harvested from 293T cell culture after 72 h of transfection of expression plasmids. The RBD-2E8 fusion protein was purified with a nickel column via His-tag affinity, and the RBD-2E8 protein was run on a protein gel to confirm the proper size (Fig. 2A).

The second fusion protein had an scFv, B6, on the *N*-terminus against an uncharacterized, ubiquitous RBC antigen [16]. B6 has also been used in fusion protein constructs to detect HIV antibodies in hemagglutination assays [8]. A short CH1 domain linker connects to RBD at the *C*-terminus, which improved agglutination previously by displaying the viral antigens further away from the RBC surface [17]. This fusion protein, B6–CH1-RBD, was prepared in 293T cells according to the same protocol for RBD-2E8. B6–CH1-RBD was also prepared in *E. coli* as in prior studies for this class of diagnostic [10,18] with the thought this could be more scalable for manufacturing the reagents. Following nickel column purification via His-tag affinity, the proper size of B6–CH1-RBD bacterial protein on a protein gel was confirmed (Fig. 2B).

3.2. Testing red blood cell agglutination in a rapid assay to detect SARS-CoV-2 antibodies

We first tested for SARS-CoV-2 antibodies in a rapid, 5-min hemagglutination assay. The mammalian RBD-2E8 and bacterial B6–CH1-RBD fusion proteins were mixed with RBCs in the presence of COVID-19 patient serum in order to detect for agglutination. The assay was carried out in a small total volume, 40 μL, in a U bottom 96-well plate. The RBC solution (2–4% red blood cells) is at a dilution commonly used in manual tube testing for ABO typing in blood banks. We first validated this assay via ABO antibody-mediated hemagglutination, finding visible, though subtle, agglutination already at 5-min (Fig. 3A).

Given that the amount of RBD-2E8 and B6–CH1-RBD fusion proteins necessary to cross-link antibodies and RBCs and trigger agglutination is unknown, a dilution series was performed, starting with a high concentration of the RBD-2E8 and B6–CH1-RBD stock solution (~100 μg/mL) through 5 successive 1:1 dilutions. A negative control contained RBCs and COVID-19 patient serum without fusion proteins. After 5-min of incubation, no agglutination was seen with the bacterial B6–CH1-RBD protein (Fig. 3B). By contrast, moderate but perceptible agglutination was observed in the three higher concentrations of mammalian RBD-2E8 protein, with no agglutination observed in the more dilute RBD-2E8 concentrations (Fig. 3C). Decreasing levels of agglutination were observed along with a decreased concentration of RBD-2E8 (100% > 50% >25%). Incubation of RBD-2E8 and RBCs without patient serum did not

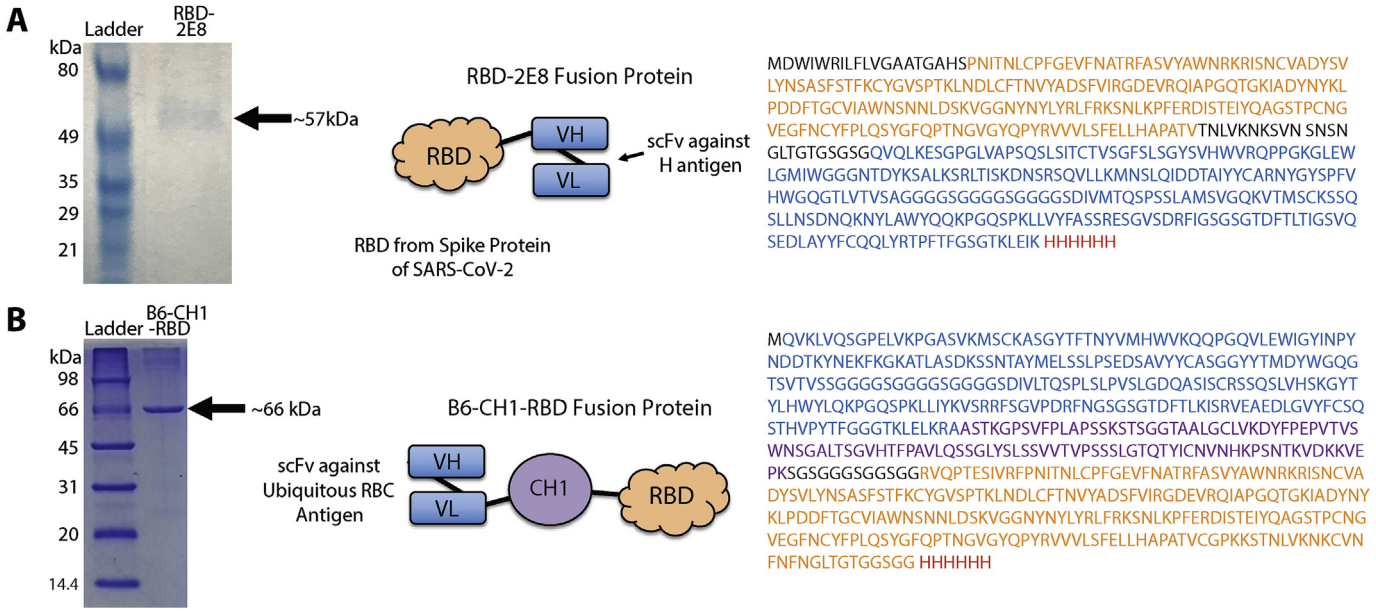

Fig. 2. RBD-antibody fusion proteins were successfully isolated. (A) The size of RBD-2E8 protein expressed in 293T cells was confirmed on protein gel electrophoresis demonstrating expected band size (~57 kDa). A cartoon image of RBD-2E8 is also provided, along with sequence information. (B) The size of B6–CH1-RBD expressed in *E. coli* is confirmed on protein gel electrophoresis demonstrating expected band size (~66 kDa). A cartoon image of B6–CH1-RBD is also provided, along with sequence information. His-tag sequence for purification of fusion proteins is in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

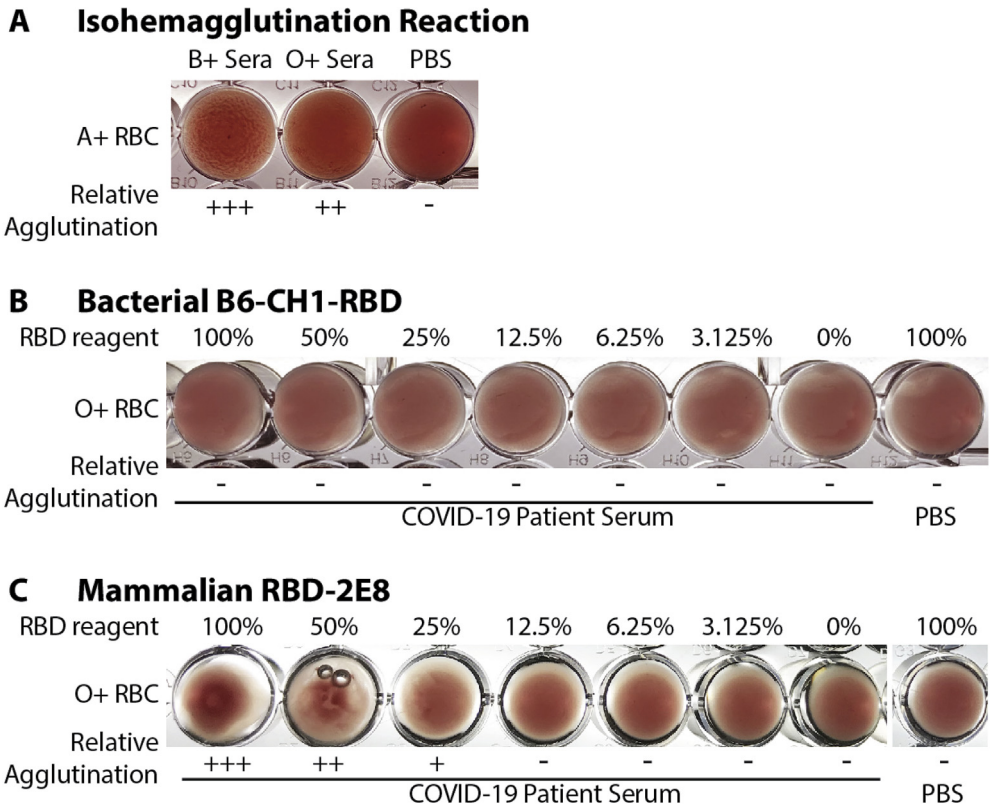

Fig. 3. RBD-2E8 mediates agglutination of red blood cells in the presence of COVID-19 patient serum after 5 min. (A) Control agglutination reaction was performed for 5-min between B and O anti-sera and A cells. (B) Bacterial B6–CH1-RBD and (C) mammalian RBD-2E8 proteins were serially diluted in the presence of fixed concentrations of RBC and undiluted COVID-19 convalescent serum from a single patient. Agglutination was seen in the highest three concentrations of RBD-2E8 after 5-min of incubation, most intense in the highest concentration of RBD-2E8. Negative control contained phosphate-buffered solution (PBS) in the place of patient serum. Bacterial B6–CH1-RBD showed no agglutination at any protein concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

yield any agglutination (Fig. 3B and C). This indicates the presence of antibodies that bind to RBD in patient serum, with agglutination activity reflective of RBD-2E8 concentration.

3.3. An extended assay yields more robust agglutination to detect SARS-CoV-2 antibodies

Because the 5-min assay results were relatively weak making interpretation difficult, we extended the assay to 1 h according to an agglutination protocol developed by another group [19]. The longer incubation would allow all RBCs to effectively come in contact with each other by gravity at the bottom of the well. The plate would then be tilted to observe if the red blood cells held together or fell down by gravity.

As before, we validated the assay via ABO antibody-mediated hemagglutination (Fig. 4A). We first tested control antibodies of ACE2-Fc and CR3022 to mediate agglutination, both of which are known to bind to SARS-CoV-2 RBD at two different, non-overlapping epitopes [24]. For this assay, we produced B6–CH1-RBD by mammalian transfection, given the failure to mediate visible agglutination in the 5-min assay. Testing mammalian RBD-2E8 and B6–CH1-RBD, we found that both antibodies could efficiently mediate agglutination of red blood cells that was visually clear from the PBS control, confirming RBD binding (Fig. 4B). Separately, we tested the bacterial protein for this 1-h assay with ACE2-Fc and CR3022, but again found no agglutination when different concentrations of B6–CH1-RBD were used (Supplemental Fig. 1).

We next sought to test the analytical sensitivity of the assay. We made serial dilutions of CR3022 antibody and found that a level of 12.5 ng of antibody in the well could still trigger agglutination in our assay (Fig. 4C). Recovered COVID-19 patient serum was next tested. We found that mammalian RBD-2E8 and B6–CH1-RBD fusion protein could yield efficient agglutination reactions after 1 h, which were significantly more visually clear than the 5-min incubation time (Fig. 4D). No agglutination was seen without fusion protein (Fig. 4D). Only purified protein could yield agglutination compared to supernatant from transfected 293T cells, emphasizing the increased concentration of protein from nickel column purification (Supplemental Fig. 2).

4. Discussion

In this study, we demonstrated a proof of concept for a rapid, point-of-care RBC agglutination test for SARS-CoV-2 antibodies. We chose the SARS-CoV-2 RBD as our primary target antigen for detection, since antibodies binding to the SARS-CoV-2 RBD have exhibited limited cross-reactivity with other coronaviruses [13]. RBD antibodies were maintained with little decrease through at least 75 days post-infection, correlating with neutralizing antibody titers [25]. Based on available data then, an RBD fusion protein reagent could potentially be used to detect patients who have been infected and likely protected by neutralizing antibodies from infection.

Our experiments demonstrated that COVID-19 patient serum could agglutinate RBCs in the presence of mammalian-derived RBD-2E8 within 5-min of incubation, although longer incubation for 1-h was required for strong agglutination and clear visualization. We observed that the mammalian-derived RBD-2E8 fusion protein was more effective than the bacterial-derived B6–CH1-RBD fusion protein, which did not have any significant agglutination reaction in the 5-min or 1-h assay. A previous study on SARS RBD documented that mammalian-expressed RBD was significantly more reactive on ELISA to antisera from vaccinated mice than *E. coli* RBD protein [26]. It is likely that the native glycosylation and RBD folding in mammalian cells is essential for efficient antibody

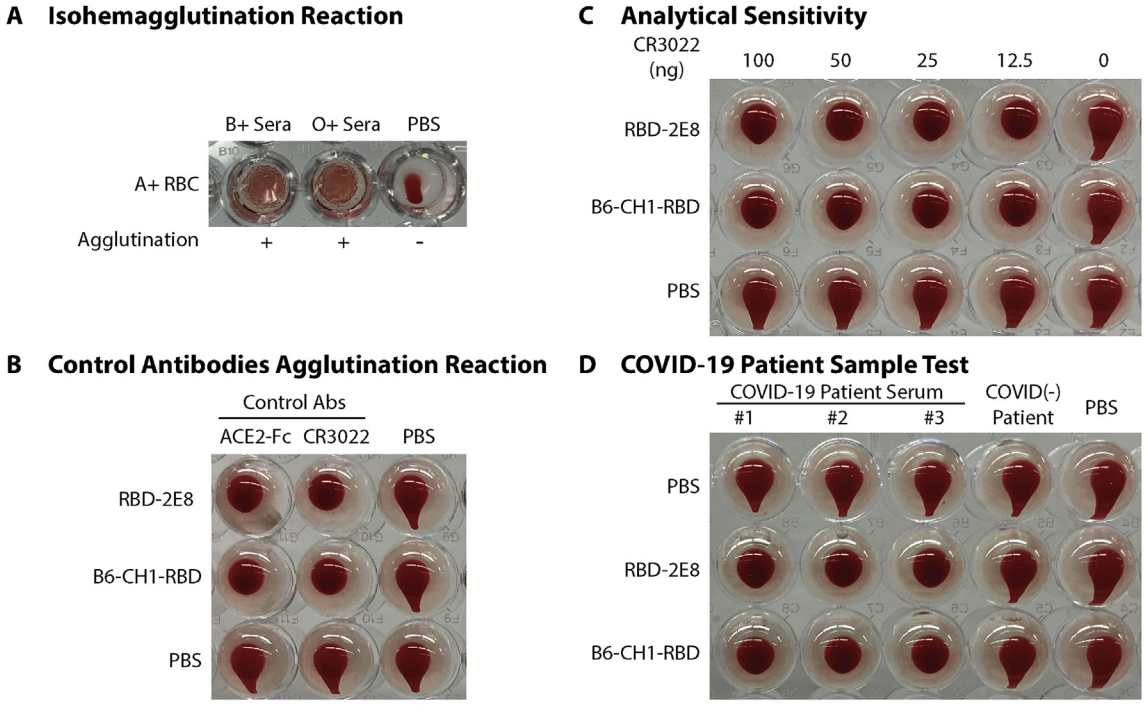

Fig. 4. Prolonged hemagglutination assay times yield strong agglutination. A longer, 1 h incubation was performed, whereafter the plate was tilted; downward motion of RBCs indicates no agglutination, while spreading RBC surface or stable RBC pellet indicates agglutination. (A) Control agglutination reaction was performed between B and O anti-sera and A cells. (B) Mammalian RBD-2E8 and B6–CH1-RBD were mixed with ACE2-Fc (500 ng), CR3022 (30 ng), or PBS and evaluated at 1 h. (C) To test the analytic sensitivity, a dilution series of CR3022 antibody (ng) was prepared and tested to detect the lowest antibody concentration yielding agglutination. (D) Three different COVID-19 patient sera were incubated with B6–CH1-RBD, RBD-2E8, and RBCs. Control conditions had PBS alone and non-infected patient plasma.

recognition. This result differs from previous studies to detect HIV antibodies, where a p24 fusion protein could be expressed in *E. coli* and efficiently cause hemagglutination reactions [9,18].

A key limitation of this assay is that it does not distinguish between IgG, IgA, or IgM against SARS-CoV-2, which may be desired in certain clinical scenarios. IgG subclasses can similarly not be distinguished. While the assay is simple and can be read with the naked eye, there is more subjectivity compared to lateral flow assays or chemiluminescent ELISA's. A negative control test without fusion protein will be important to include during clinical implementation, given that rare patients may have false positives from agglutination-inducing IgM autoantibodies [27]. Ongoing studies will confirm the sensitivity and specificity of the assay with more COVID-19 patient samples. Similar, prior hemagglutination assays for HIV antibodies demonstrated 100% sensitivity (n = 94) and 99.5% specificity (n = 596) [28].

Moving forward, the hemagglutination assay should be feasible to use a drop of whole blood from a patient finger-stick, wherein RBD-2E8 or B6–CH1-RBD fusion protein alone could be added for the assay. The estimated protein reagent cost for the research assay using small scale production and purification was 25 cents (U.S.) per test, which would make the assay financially feasible for low-resource health care settings. While the assay was carried out in 96-well plates similar to previous studies [15], the format is transferrable to a slide agglutination test [10], which is often used for ABO testing in low-resource settings. Point-of-care applications are emphasized in this study, but fusion protein reagents could potentially be employed in other hemagglutination assays used in clinical labs, such as tube testing and gel card testing, as well as on automated solid-phase assay machines. Beyond diagnostic use of COVID-19 patients, the ability to rapidly screen donors for RBD antibodies in the plasma of COVID-19 recovered patients could facilitate the continued deployment and scaling of convalescent plasma as a therapy against COVID-19 [29,30]. Rapid testing would also help validate RBD antibody production induced by SARS-CoV-2 vaccines in trials, and screen for patients in need of the vaccine.

In conclusion, our RBC agglutination assay with cross-linked viral antigen-antibody fusion proteins may represent a simple, cheap, and scalable way to increase our capability of detecting antibodies against SARS-CoV-2. It may find utility, particularly in low-resource settings, in the efforts to combat the COVID-19 pandemic.

Declaration of competing interest

R.L.K. is an inventor on a provisional patent application related to the work described in the manuscript. All other 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.bbrc.2021.03.016.

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