ORIGINAL ARTICLE

Anti-SARS-CoV-2 Nucleoprotein Antibodies Derived from Pig Serum with a Controlled Specificity

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Abstract

Antibodies against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) nucleoprotein (NP) were purified from pig serum through two steps: (1) isolation of anti-NP IgG antibodies using magnetic beads with immobilized human SARS-CoV-2 NP and (2) filtration of anti-spike protein (SP) IgG antibodies using magnetic beads with immobilized human SARS-CoV SP. The enhanced specificity of the purified antibodies to the NP of SARS-CoV-2 was demonstrated using an immunoassay with anti-NP IgG antibodies after the isolation and filtration steps. The binding constants (K_d) of the purified anti-NP IgG antibodies to the NP of SARS-CoV-2 and the SP of SARS-CoV were estimated using a surface plasmon resonance biosensor (SPR). A competitive assay using the two-step purified anti-NP IgG antibodies from pig serum demonstrated (a) the detection of SARS-CoV-2 in viral fluid and (b) the discrimination of SARS-CoV-2 from SARS-CoV, MERS-CoV, and CoV strain 229E in viral fluids.

1 Introduction

Recently, we reported that the antibodies against the nucleoprotein (NP) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) could be isolated from pig serum and the antibodies could be used for the detection of SARS-CoV-2 in viral culture fluid [1]. Coronavirus (CoV) has been classified into four genera, including alpha-, beta-, gamma-, and delta-CoV. The CoV strains causing severe acute respiratory syndromes such as SARS-CoV-2 of COVID-19, SARS-CoV, and MERS-CoV, belong to beta-CoV genera [2, 3]. Alpha- and beta-CoV genera are known to infect humans and other animals [4, 5]. In pigs, several types of CoV infections occur, such as those due to pig epidemic diarrhea virus (PEDV; alpha-CoV), transmissible gastroenteritis virus (TGEV; alpha-CoV), and pig hemagglutinating encephalomyelitis virus (PHEV; beta-CoV) [6, 7]. From homology analysis of the amino acid sequences, the NP of SARS-CoV-2 and the above-mentioned CoVs of pigs show a high similarity (identity) of 40% (27%) to PEDV (Uniprot ID: Q07499), 48% (28%) to TGEV (Uniprot ID: P04134), and 55% (40%) to PHEV (Uniprot ID: Q8BB23). Thus, pig serum is considered to contain antibodies against these CoVs from infection history, and these antibodies in pig serum are considered to have a cross-affinity to the NP of SARS-CoV-2 [1]. Thus, in this study, isolated anti-NP IgG antibodies were a polyclonal mixture and these antibodies could be used for the detection of SARS-CoV-2 as well as other CoV strains such as SARS-CoV, MERS-CoV, and CoV strain 229E in viral culture fluids using a competitive immunoassay configuration [8] .

From the competitive assay, the isolated anti-NP IgG antibodies were estimated to have a similar affinity to SARS-CoV-2 and SARS-CoV, and these antibodies showed a relatively low affinity to MERS-CoV and CoV strain 229E in viral culture fluids. For selective detection

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of SARS-CoV-2, affinity enhancement was required. In this study, the isolation of antibodies against SARS-CoV-2 was accomplished by removing the antibodies with affinity to SARS-CoV from the polyclonal antibody mixture [9]. Antibodies were isolated from pig serum through two steps: (Step 1) isolation of anti-NP IgG antibodies using magnetic beads with immobilized nucleoprotein (NP) of SARS-CoV-2 and (Step 2) filtration to remove antibodies with SARS-CoV affinity using magnetic beads with immobilized SARS-CoV spike protein (SP) (Fig. 1a) [10]. The enhanced specificity of the isolated antibodies to the NP of SARS-CoV-2 was observed using an immunoassay with anti-NP IgG antibodies after Step 1 and after Step 1 + Step 2. The binding constants (K_d) of anti-NP IgG antibodies

to the NP of SARS-CoV-2 and SP of SARS-CoV after Step 1 and after Step 1 + Step 2 were estimated using a surface plasmon resonance biosensor. A competitive assay using anti-NP IgG antibodies obtained from pig serum through two-step purification demonstrated (a) detection of SARS-CoV-2 in viral fluid and (b) discrimination of SARS-CoV-2 from SARS-CoV, MERS-CoV, and CoV strain 229E in viral fluids.
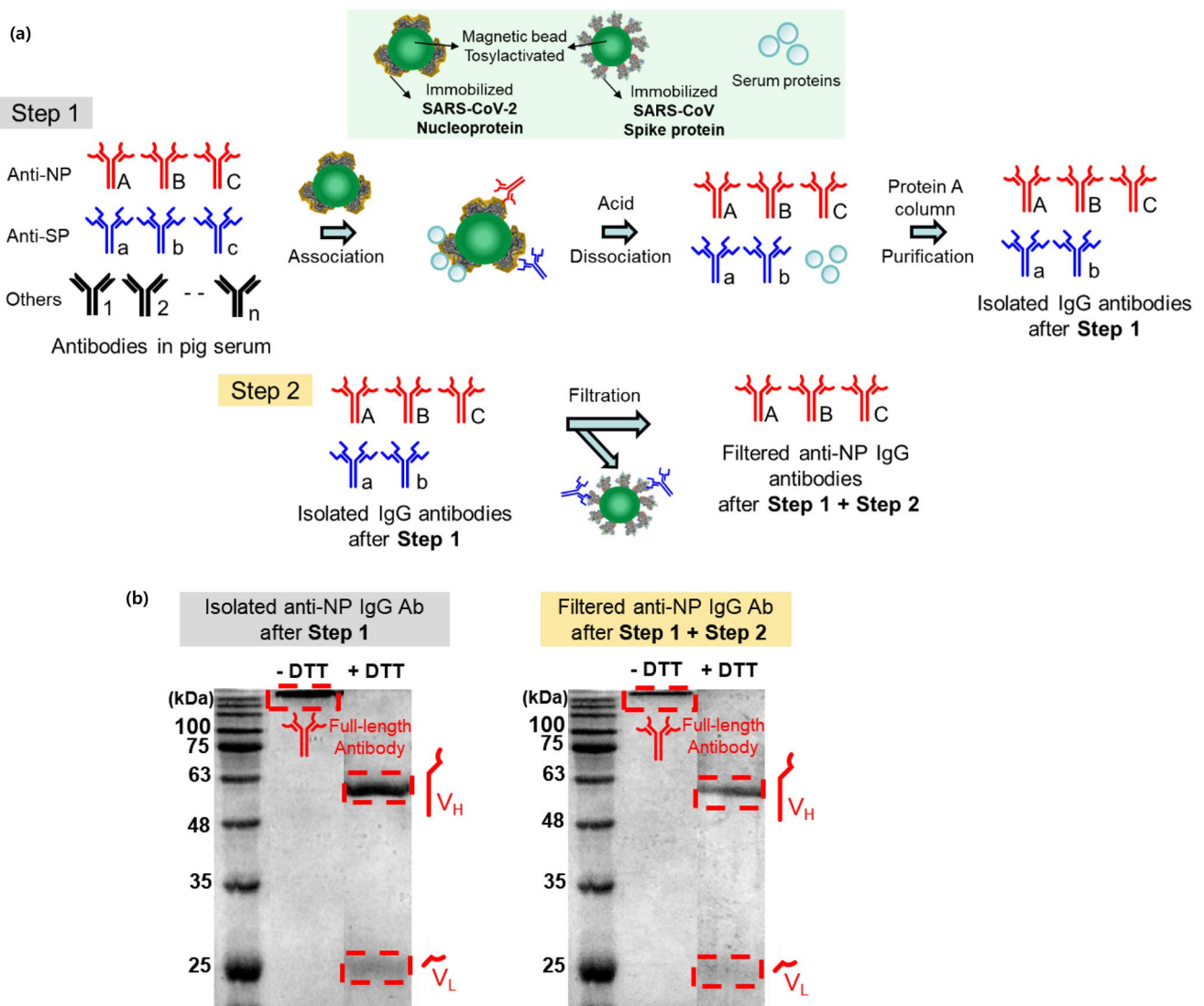

Fig. 1 Isolation of anti-nucleoprotein of SARS-CoV-2 (NP) IgG antibodies from pig serum. **a** The anti-NP IgG antibodies were first isolated using magnetic beads with immobilized NP (Step 1), and then the filtration was carried out using magnetic beads with immobilized SP (Step 2). **b** SDS-PAGE of the isolated antibodies after Step 1 and after Step 1 + Step 2 under non-reducing/reducing conditions without/with dithiotreitol (DTT)

2 Results and Discussion

2.1 Purification of Anti-NP IgG Antibodies

As previously mentioned, pig serum is known to contain antibodies against proteins from various CoVs, because pigs have been infected by several kinds of alpha- and beta-CoVs, such as PEDV (alpha-CoV), TGEV (alpha-CoV), and PHEV (beta-CoV) [6]. The amino acid sequences of the NPs of these CoVs known to infect pigs were analyzed and showed significant similarities of more than 40–55% compared with the nucleoprotein (NP) of human SARS-CoV-2 [1]. Thus, anti-NP IgG antibodies were isolated from pig serum, and the isolated antibodies were reported to have a specificity to NP of human SARS-CoV-2. However, the pig-serum-derived anti-NP IgG antibodies also showed a similar specific binding affinity to the viral culture fluids of human SARS-CoV. CoVs are known to produce four types of major proteins, including envelope proteins, spike proteins, membrane proteins, and nucleoproteins. Among these proteins, nucleoprotein and spike proteins are known to be produced most abundantly, and these proteins have been frequently used for vaccine development and assays [11–15]. In this study, the isolation of anti-NP IgG antibodies specific to SARS-CoV-2 was enhanced using a two-step purification method. A precondition for this method is that the isolated anti-NP IgG antibodies must be part of a polyclonal mixture of antibodies. As shown in Fig. 1a, anti-NP IgG antibodies were first isolated using magnetic beads with immobilized NP, which contained anti-NP IgG antibodies as well as anti-SP IgG antibodies. Among the isolated antibodies, the antibodies with affinity to SP were filtered out using magnetic beads with immobilized SP. Finally, anti-NP IgG antibodies with enhanced specificity to the SARS-CoV-2 NP could be obtained, because the anti-SP IgG antibodies were removed by the filtering step.

The isolated antibodies are shown in Fig. 1b. SDS-PAGE showed that the isolated antibodies from Step 1 and Step 1 + Step 2 were immunoglobulin G (IgG) comprising heavy chains (50 kDa) and light chains (25 kDa). Moreover, the area of antibody protein bands decreased by as much as 32% based on densitometric analysis [16, 17]. The yield of antibody isolation was estimated to be 8.37 ± 0.53 µg per 1 ml of pig serum after Step 1 and 5.71 ± 0.18 µg per 1 ml of pig serum after Step 1 + Step 2.

2.2 Binding Properties of Purified Anti-NP IgG Antibodies

The binding properties of anti-NP IgG antibodies after Step 1 and after Step 1 + Step 2 were analyzed using an immunoassay. The binding properties of anti-NP IgG antibodies after Step 1 were analyzed separately using microplates with immobilized NP of SARS-CoV-2 and immobilized SP of SARS-CoV (Fig. 2a). The quantity of bound antibodies was analyzed using horseradish peroxidases (HRP)-labeled secondary antibodies and the chromogenic reaction of 3,3′,5,5′-tetra-methylbenzidine (TMB) [18]. The relative binding intensity of antibodies after Step 1 was estimated to be 43.1% to SP of SARS-CoV compared with 100% to NP of SARS-CoV-2 (Fig. 2b). The same immunoassay was performed using anti-NP IgG antibodies after Step 1 + Step 2 using magnetic beads with immobilized SP. The relative binding intensity of antibodies after Step 1 + Step 2 was estimated to be 9.2% to SP of SARS-CoV compared with 100% to NP of SARS-CoV-2 (Fig. 2c). From the comparison of relative binding intensities, the anti-NP IgG antibodies collected after the filtration step showed that the binding affinity to the SP of SARS-CoV decreased from 43.1% (Step 1) to 9.2% (Step 1 + Step 2) compared with the binding affinity to the NP of SARS-CoV-2 (100%). These results showed that the isolation step (Step 1) followed by the filtration step (Step 2) could enhance the specificity of the anti-NP IgG antibodies by decreasing the relative amount of anti-SP IgG antibodies.

The binding properties of anti-NP IgG antibodies after Step 1 and after Step 1 + Step 2 were analyzed separately using an Au-SPR chip with immobilized NP of SARS-CoV-2 and immobilized SP of SARS-CoV (Fig. 3a). The quantity of bound antibodies was analyzed at a range of 37–1000 ng/mL antibody concentration from the difference in baselines between initial and after dissociation of antibodies from the Au-SPR chip. The binding constant (K_d) of antibodies after Step 1 was estimated to be 2.3×10^{-9} M to the SP of SARS-CoV and 1.5×10^{-9} M to the NP of SARS-CoV-2 (Fig. 3b). The same immunoassay was performed using anti-NP antibodies after the filtration step (Step 2). The binding constant (K_d) of antibodies after Step 1 + Step 2 was estimated to be 3.4×10^{-9} M to the SP of SARS-CoV and 9.9×10^{-10} M to the NP of SARS-CoV-2 (Fig. 3c). Based on the binding constants, the anti-NP IgG antibodies after the filtration step showed that the binding affinity to the SP of SARS-CoV decreased by as much as 43%, whereas that to the NP of SARS-CoV-2 increased by as much as 63% compared with the antibodies after Step 1. These results also showed that the isolation step (Step 1) followed by the filtration step (Step 2) could enhance the specificity of the anti-NP IgG antibodies by decreasing the relative amount of anti-SP IgG antibodies.

Fig. 2 Binding properties of the isolated anti-NP IgG antibodies after Step 1 and after Step 1 + Step 2. **a** Configuration for the binding activity test using immobilized NP on microplate. Comparison of binding affinities of the isolated anti-NP IgG antibodies after **b** Step 1 (*n* = 3) and **c** Step 1 + Step 2 (*n* = 3) against nucleoprotein (NP) of SARS-CoV-2 and spike protein (SP) of SARS-CoV
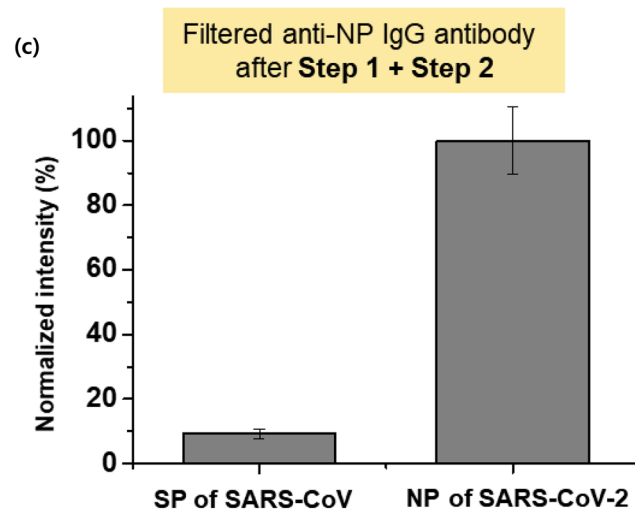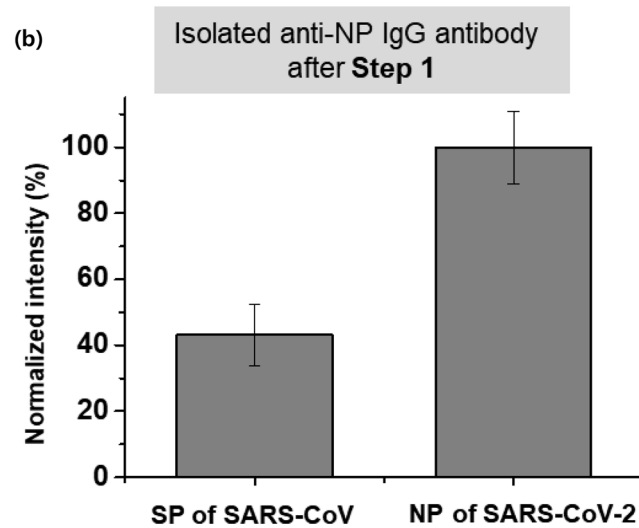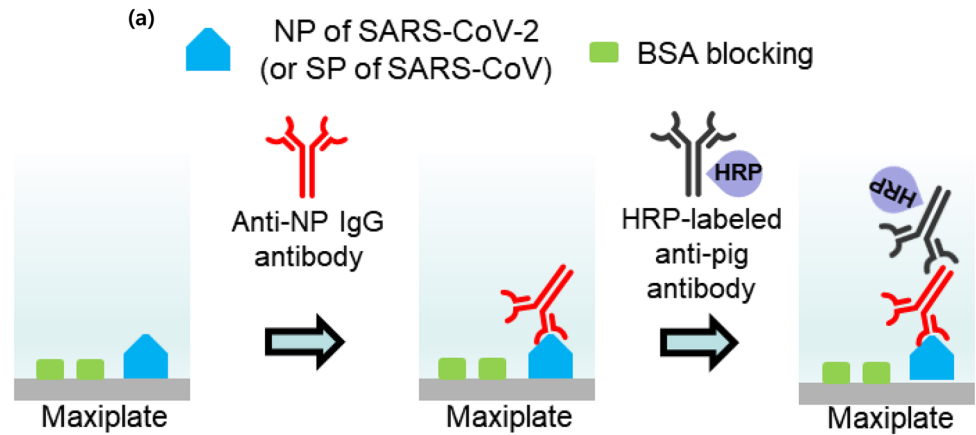
2.3 Competitive Assay Using Purified Anti-NP Antibodies

Anti-NP IgG antibodies from the two-step purification were applied in a competitive assay of various CoVs in viral culture fluids. The competitive assay was performed by mixing a sample with an assay solution comprising anti-NP IgG antibodies and magnetic beads with immobilized
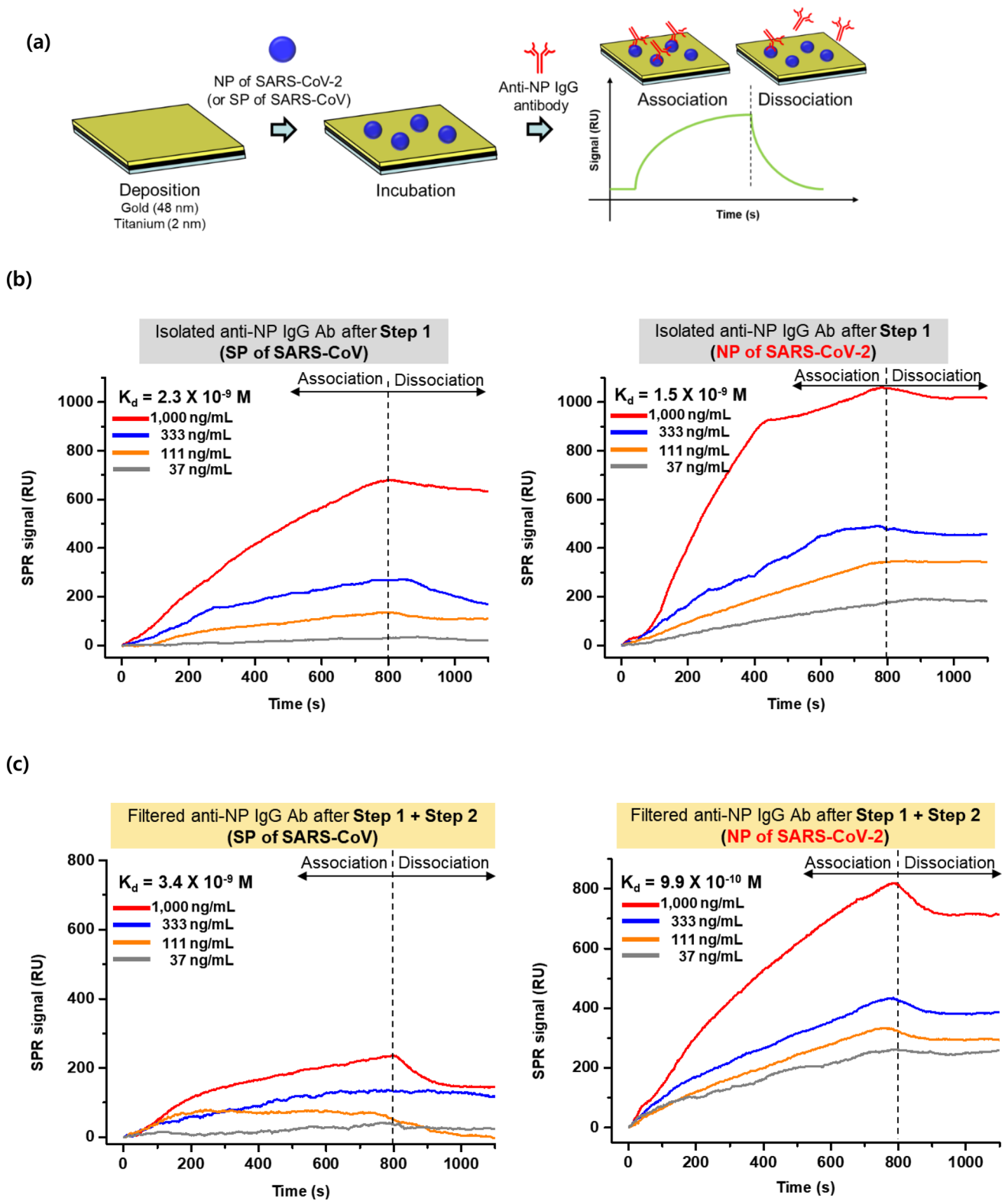
Fig. 3 Estimation of binding constants (K_d) of the isolated anti-NP IgG antibodies using SPR biosensor. **a** Configuration for the SPR measurement using immobilized SP of SARS-CoV and NP of SARS-CoV-2 on Au-chip. Binding constants (K_d) of the isolated anti-NP IgG antibodies after **b** Step 1 and **c** Step 1 + Step 2 against NP of SARS-CoV-2 and SP of SARS-CoV
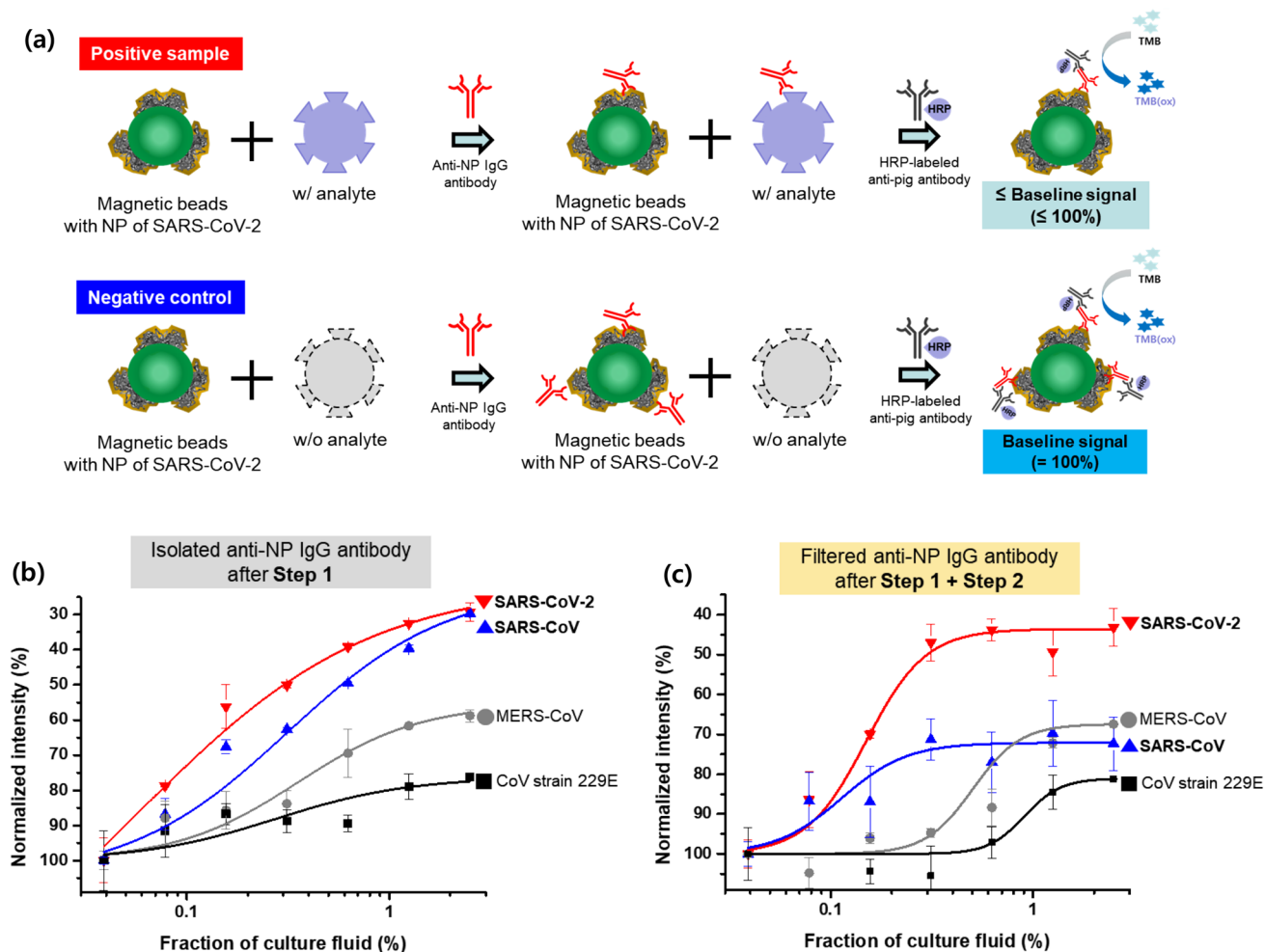
Fig. 4 Competitive assay of CoV viral culture fluids of SARS-CoV-2, SARS-CoV, MERS-CoV, CoV strain 229E using the isolated anti-NP IgG antibodies. **a** Configuration for the competitive assay using magnetic beads with immobilized NP and the isolated anti-NP IgG antibodies at a known concentration. Results of the competitive assay for four kinds of CoV viral culture fluids using the isolated anti-NP IgG antibodies after **b** Step 1 ($n = 3$) and **c** Step 1 + Step 2 ($n = 3$)

NP at known concentrations (Fig. 4a). In the negative control without NP of SARS-CoV-2, the mixed anti-NP IgG antibodies in the assay solution were bound only to the magnetic beads with immobilized NP. The quantity of bound antibodies on the magnetic beads was evaluated using secondary antibodies labeled with HRP and TMB as chromogenic substrates. In the case of positive samples with NP of SARS-CoV-2, the NP in the sample reacted with the anti-NP IgG antibodies and the remaining antibodies bound to the magnetic beads. Because of competition with the mixed anti-NP IgG antibodies, the quantity of bound anti-NP IgG antibodies on the magnetic beads was less than that of the negative control. From the chromogenic reaction of secondary antibodies labeled with HRP and TMB, the optical density at 450 nm (OD_{450nm}) decreased according to the amount of NP in the sample, as shown in Fig. 4b and c. The amount of NP in the sample

could be estimated based on the difference between the OD_{450nm} of each sample and the negative control.

When the anti-NP IgG antibodies from the isolation step (Step 1) were used for the competitive assay, standard curves were obtained for four viral culture fluids including SARS-CoV-2, SARS-CoV, MERS-CoV, and CoV strain 229E (Fig. 4b). The viral culture fluids were diluted from 40-fold (fraction of 2.5%) to 1280-fold (fraction of 0.08%) with PBS. As the fraction of culture fluid increased or as the NP concentration increased, the normalized intensity decreased for all four viral culture fluids. In particular, the difference in signal was significantly greater for SARS-CoV-2 and SARS-CoV samples than for the other two culture fluids, demonstrating the high specificity of the antibodies to NP in SARS-CoV-2 and SARS-CoV samples. Additionally, the signals from the competitive assays for SARS-CoV-2 and SARS-CoV samples were quite similar, demonstrating the

Table 1 Comparison of amino acid sequence of nucleoprotein (NP) of CoV strains

Compared viral species	Similarity (%)	Identity (%)
SARS-CoV-2 NP versus SARS-CoV NP	94	91
SARS-CoV-2 NP versus MERS-CoV NP	63	48
SARS-CoV-2 NP versus CoV 229E NP	40	28

low specificity of the antibodies in discriminating between the NP of SARS-CoV-2 and SARS-CoV in samples. As summarized in Table 1, such similarities in the assay results were considered to be because of the homology of amino acid sequences between the NP of SARS-CoV-2 and the three other CoV strains.

Anti-NP IgG antibodies after the two-step purification were used for the competitive assay, and standard curves were obtained for four viral culture fluids including SARS-CoV-2, SARS-CoV, MERS-CoV, and CoV strain 229E, as shown in Fig. 4c. When the viral culture fluids were diluted using the same range as the previous experiment, the difference in normalized intensity was also decreased for all four viral culture fluids. The difference in signal was significantly greater for SARS-CoV-2 samples than for the other three culture fluids using the two-step purified anti-NP antibodies, demonstrating a high specificity of the antibodies to the NP of SARS-CoV-2. Additionally, the signals from the competitive assays for SARS-CoV-2 and SARS-CoV samples were at a significantly level, demonstrating the high specificity of the antibodies in discriminating between the NPs of SARS-CoV-2 and SARS-CoV.

3 Conclusions

Specific anti-NP IgG antibodies against SARS-CoV-2 were isolated from pig serum. The antibodies were purified using a two-step method: (Step 1) isolation of anti-NP IgG antibodies using magnetic beads with immobilized NP and (Step 2) filtration of anti-SP IgG antibodies from previously isolated antibodies using magnetic beads with immobilized SP. The yield of antibody isolation was estimated to be 8.37 ± 0.53 µg per 1 ml of pig serum after Step 1 and 5.71 ± 0.18 µg per 1 ml of pig serum after Step 1+Step 2. Analysis of the binding properties of anti-NP IgG antibodies following the two-step purification showed enhanced specificity of the anti-NP IgG antibodies to SARS-CoV-2 by decreasing the relative amount of anti-SP IgG antibodies. The binding constant (K_d) of antibodies after Step 1 was estimated to be 2.34×10^{-9} M to the SP of SARS-CoV and 1.54×10^{-9} M to the NP of SARS-CoV-2 using an SPR biosensor. Additionally, the binding constant (K_d) of antibodies

after Step 1+Step 2 was estimated to be 3.36×10^{-9} M to the SP of SARS-CoV and 9.91×10^{-10} M to the NP of SARS-CoV-2. From the comparison of the binding constants, the anti-NP antibodies after the filtration step showed that the binding affinity to the SP of SARS-CoV was reduced by 43% and that to the NP of SARS-CoV-2 was increased by 63% relative to the antibodies from Step 1. Anti-NP IgG antibodies were applied in a competitive assay of various CoVs in culture fluids: SARS-CoV-2, SARS-CoV, MERS-CoV, and CoV strain 229E. For the competitive assay with the anti-NP IgG antibodies from the two-step purification process, the difference in signal was significantly greater for SARS-CoV-2 samples than for the other three culture fluids, demonstrating the high specificity of the antibodies to the NP in SARS-CoV-2 samples. Additionally, the signals from the competitive assays for SARS-CoV-2 and SARS-CoV samples were at a significant level, demonstrating a high specificity of the antibodies in discriminating between the NPs of SARS-CoV-2 and SARS-CoV. The results show that isolated anti-NP IgG antibodies from this two-step purification process could be used for the detection of SARS-CoV-2 and the discrimination of SARS-CoV-2 from other CoV strains in viral fluids.

4 Materials and Methods

4.1 Materials

HRP-labeled anti-pig IgG antibody (ab102135) was purchased from Abcam (Cambridge, UK). Tosyl-activated magnetic beads (diameter, 2.8 µm) were purchased from Invitrogen Co. (Carlsbad, CA, USA). Bicinchoninic acid (BCA) assay kit, Maxisorp microplates, pig serum, and TMB reagent were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Other chemicals were purchased from Sigma-Aldrich Korea (Seoul, Korea). Culture fluids of SARS-CoV-2, SARS-CoV, MERS-CoV, and CoV strain of 229E were purchased from Zeptometrix (Buffalo, NY, USA). NP of SARS-CoV-2 and SP of SARS-CoV were supplied by Optolane (Seongnam, Korea) and Genbody (Cheonan, Korea).

4.2 Purification of Anti-NP Antibodies

Magnetic beads with immobilized NP of SARS-CoV-2 and SP of SARS-CoV were prepared by mild mixing of magnetic beads (165 µL) at 5 mg/mL and NP or SP solution (100 µL) at 1 mg/mL for 18 h at 37 °C. Anti-NP IgG antibodies were isolated from pig serum as follows (Fig. 1). Step (1) Pig serum (1 mL) was reacted to magnetic beads with immobilized NP of SARS-CoV-2 by mild shaking with a wheel-rotating mixer (RT-10, Daehan Scientific Co, Wonju,

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