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Development of monoclonal antibodies against SARS-CoV-2 nucleocapsid protein for COVID-19 antigen detection

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Abstract

Background The coronavirus disease 2019 (COVID-19) pandemic underscored the global need for reliable diagnostic tools with quick turnaround time for effective patient management and mitigation of virus spread. This study aimed to express severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) nucleocapsid protein and produce monoclonal antibodies (mAbs) against the expressed protein.

Methods Following successful expression and purification of His-tagged SARS-CoV-2 N protein using a wheat germ cell-free protein expression system (WGCFS), BALB/c mice were immunized, and generated hybridomas screened for mAb production. Indirect and sandwich ELISA were used to screen the reactivity of the monoclonal antibody against both our recombinant antigen and commercial antigen. The mAbs were also assessed for their performance using RT-PCR confirmed positive samples with varying cycle threshold (CT) values and their specificity screened using virus isolates of other respiratory viruses.

Results Our mAb demonstrated high reactivity against our recombinant antigen, commercial antigen, SARS-CoV-2 Beta and Omicron variants. There was no significant difference in the binding affinity of our mAb and commercial mAb against the study recombinant ($p=0.12$) and commercial ($p=0.072$) antigens. Our mAb detected SARS-CoV-2 from clinical samples with varying CT values and exhibited no cross-reactivity against other respiratory viruses.

Conclusions We successfully expressed SARS-CoV-2 N protein leveraging WGCFS in a resource-limited setting. Our mAb had a high binding affinity to the recombinant antigen, making it a suitable candidate for antigen detection kit development. Beyond diagnostics, the mAb holds potential for therapeutic applications as well as use in clinical and environmental surveillance platforms.

Keywords Monoclonal antibodies, SARS-CoV-2, Nucleocapsid protein, COVID-19, Diagnostics

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Introduction

The severe acute respiratory syndrome coronavirus 2 (SARSCoV2) genome encodes four structural proteins, namely, Spike (S), Membrane (M), Envelope (E) and Nucleocapsid protein (N) [1]. The N and S proteins are the key targets for diagnosis and serological surveillance [2]. The N protein serves as an antigen regulating RNA replication and mRNA transcription, while the S protein plays a key role in viral entry into the host cell by binding to the human angiotensin-converting enzyme 2 (ACE-2) to initiate infection [3]. While the S protein has more than 30 mutations in the currently circulating Omicron variant of concern [4], the N protein remains relatively conserved and stable and, therefore, a key target for diagnostic testing [5]. Furthermore, anti-N antibodies can be used to differentiate between SARS-CoV-2 in natural infection and vaccine-induced antibody response via the S protein [5].

Reverse transcription polymerase chain reaction (RT-PCR) is the gold standard for COVID-19 diagnosis and the most used technique for detecting active infection [6]. RT-PCR accuracy depends on several factors including timing of sample collection, sample processing, technical expertise, long turn-around time and sample storage [7]. Enzyme-linked immuno-assays (ELISA) such as IgM and IgG are based on antibody production after infection and, therefore, not suitable for acute detection as antibody generation takes time [8]. Antigen detection kits are reliable serological tests that detect active infections, even in individuals who are asymptomatic, and are suitable for mass testing as they are affordable, have a quick turnaround time, and can be used in resource-limited settings [9]. The effectiveness of an antigen-detection kit primarily depends on the use of high-quality monoclonal antibodies (mAbs) that accurately target specific viral antigens [10].

Muromonab-CD3 was the first mAb to be approved for clinical use by the US Food and Drug Administration (FDA) in 1986 [11]. Since then, mAbs have been licensed yearly with an estimated US\$75 billion sales as of 2021 [11]. mAbs are highly specific and reliable reagents that have been applied in many molecular and immunological investigations [10]. They have become essential reagents for detection [12–14] as well as treatment [15, 16] of various diseases owing to their specificity and versatility, as they are produced from a stable cloned hybridoma [14]. mAbs are produced in human form for immunotherapy to avoid adverse reactions, while for immunodiagnosics, mAbs are produced in mice using hybridoma technology [17].

Neutralizing mAbs (NMABs) have been produced against different coronaviruses. During the SARS-CoV-1 outbreak in 2002/2003, CR3014 and CR3022 NMABs

were identified which, when combined, effectively neutralized multiple SARS-CoV-1 variants. In addition, B1, the first S2-targeting mAb, was discovered from convalescent patient antibody libraries, providing a strategy for targeting conserved viral regions [18]. NMABs m336, m337, m338, 3B11, MERS-4, and MERS-27 were identified from phage- and yeast-displayed libraries from healthy donors and effectively blocked Middle East Respiratory Syndrome Coronavirus (MERS-CoV) RNA binding domain (RBD) binding to Dipeptidyl Peptidase 4 (DPP4). In addition, MCA1, derived from MERS-CoV-infected patients, also targeted the RBD, inhibiting viral invasion [19, 20]. NMAB S309 recovered from the memory B cells of SARS-CoV-1 an infected patient during the 2003 outbreak has shown cross-neutralization reactivity against both SARS-CoV-1 and SARS-CoV-2 [18]. In addition, COVID-19 convalescent sera exhibited cross-reactivity to the MERS-CoV S2 subunit, likely due to structural similarities, suggesting that convalescent sera could aid in developing mAbs targeting multiple coronaviruses [21]. Bamlanivimab and etesevimab are among the anti-spike SARS-CoV-2 mAbs approved for early therapy, while tixagevimab and cilgavimab were approved for pre-exposure prophylaxis in 2021 by FDA during the COVID-19 pandemic [11].

As COVID-19 becomes endemic and continues to cause periodic outbreaks, alongside other circulating respiratory viruses with overlapping symptoms, the use of accurate diagnostic tools is essential for effective patient management and reliable disease surveillance. In response to the COVID-19 pandemic, monoclonal antibodies against different SARS-CoV-2 proteins were used to develop antigen kits for detection of SARS-CoV-2 [10, 22]. The quality of antigen used in immunization plays a critical role in determining the precision of mAbs produced [10]. Cell-free protein expression provides a rapid and efficient approach to producing high-quality proteins while maintaining their structure and functionality [23].

Wheat germ cell free system (WGCFS) is an excellent alternative to mammalian cell expression for proteins requiring rapid and scalable production [24]. WGCFS has been demonstrated to produce near native and functionally active proteins while negating posttranslational protein modifications that reduce antigenicity. In addition, the system can express membrane proteins in liposomes, which are extremely difficult to express in classical systems and have previously been used to express more than 2000 proteins from the complex *P. falciparum* genome [25, 26]. In addition, a study that expressed RNA binding domain protein for SARS-CoV-2 using WGCFS confirmed the protein retained proper conformation

and receptor-binding capabilities, making it suitable for therapeutic research and serological assays [23].

In this study, we leveraged the WGCFS to synthesize viral antigens to produce mAbs against SARS-CoV-2 N protein in a resource-limited setting. Using the generated mAbs, we detected the N-antigen generated from commercial antigen in a concentration-dependent manner. In addition, our mAbs were able to detect both the beta and omicron variant.

Materials and methods

Target gene amplification

Clinical samples confirmed positive for SARS-CoV-2 by real time PCR (RT-qPCR) were used for N gene amplification. Briefly, cDNA synthesis was performed using the ReverTra Ace[®] qPCR RT Kit (Toyobo) following the manufacturer's instructions. The resulting cDNA (Wuhan-Hu-1, GenBank MN908947.3) was subjected to PCR amplification using specific primers targeting the N gene: (forward primer: 5'-GAGAGAGACTCGAGCGAG GACAAGGCGTTCCAATTAAC-3' and reverse primer: 5'-GAGAGAGAGCGGCCGCTTATTTTTCAAACT GCGGATGGCTCCAGGCCTGAGTTGAGTCAGCAC TGC-3'). KOD-Plus-Ver.2 high-fidelity DNA polymerase kit (Toyobo) was used for amplification according to the manufacturer's protocol. Amplicons were visualized by 1.2% agarose gel electrophoresis and purified using the Wizard[®] SV Gel and PCR Clean-Up System (Promega) following the manufacturer's instructions.

Recombinant protein expression and purification

The purified PCR amplicons were digested with restriction enzymes (NotI and XhoI, New England Biolabs[®]), and ligated into a pEU-E01-His-TEV-MCS, a WGCFS-specific protein expression plasmid. Positive clones were confirmed by colony PCR and Sanger sequences. The sequenced fragments were blasted against non-redundant databases in NCBI blast to confirm the cloned N gene and aligned against the SARS-CoV-2 N gene reference (Wuhan-Hu-1, GenBank MN908947.3) genome using MAFFT to identify any mutations that might introduce stop codons within the coding region of the antigen. This ensured that the expressed protein retained its complete amino acid sequence. The sequences were also aligned against Africa and Kenya specific sequences.

Protein expression was performed using WGCFS as previously described [27]. Briefly, plasmid-derived mRNA was synthesized using SP6 RNA polymerase and translated via the bilayer method [23, 28]. For transcription, a total mixture of 200 µl containing 40 µl of 5X transcription buffer, 20 µl of 25 mM nucleotide triphosphates mixture, 2.5 µl of SP6 polymerase (80U/

µl), 2.5 µl of RNase inhibitor (80U/µl), 20 µl of plasmid DNA and 115 µl of nuclease-free water was incubated at 37 °C for 18 h. The reaction was centrifuged at 15,000 rpm for 15 min and the supernatant containing the mRNA subjected to translation. Using bilayer translation, a lower layer consisted of 250 µl of 240 OD/ml wheat germ extract (Cell Free Sciences, Yokohama, Japan), mRNA and 20 mg/mL of creatine kinase, while the upper layer contained 5.5 ml of Sub-AMIX buffer made up of 30 mM HEPES, pH 7.8, 100 mM potassium acetate, 2.7 mM magnesium acetate, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, 0.4 mM spermidine, 0.3 mM of each amino acid, 24 units of RNase inhibitor, and 60 µg of liposomes. The upper layer was poured into a plate well, while the lower layer was drawn into a micropipette tip, ensuring no air remained at the tip's end. The tip was then carefully inserted to the bottom of the well containing the upper layer. The mixture was gently dispensed under the buffer without mixing, avoiding the formation of bubbles, so that the upper and lower layer formed a bilayer. The samples were not mixed, and the plate was handled without shaking and was incubated at 16 °C for 72 h. The resulting protein was purified using affinity chromatography. Briefly, a column with Ni-Sepharose Fast Flow beads (Cytiva, Waukesha, WI, USA) was used as stationary phase in the presence of imidazole. Our target protein was bound to the Ni-Sepharose ligand, while the other wheat germ proteins were washed using phosphate buffer and the pure protein eluted using elution buffer. The protein purity and molecular weight were assessed through SDS-PAGE (Coomassie staining, CBB Plus) and western blot.

Production and characterization of monoclonal antibodies against SARS-CoV-2 N protein

BALB/c mice were immunized with purified recombinant SARS-CoV-2 N protein (50 µL subcutaneously and 100 µL intraperitoneally) with Ribi (sigma) adjuvant after 2 week interval. To generate hybridomas, spleen cells were fused with SP 2/0 myeloma cell line (ATCC#CRL-1581) with polyethylene glycol 1500 (PEG 1500) and cultured in HAT-1640 medium containing Hypoxanthine (H), aminopterin (A), and thymine (T). To select stable hybridomas, we employed indirect ELISA against our recombinant N protein. Briefly, recombinant SARS-CoV-2 N protein (1 µg/ml) was used as the coating antigen for 96-well plates and incubated overnight at 4 °C. After blocking using 2% bovine serum albumin (BSA), 20 hybridomas were diluted from 1:10¹ to 1:10⁸ starting with 1 mg/ml and incubated for 1 h at 37 °C. After three washes with PBS-T, the wells were incubated with 100 µl of HRP-anti-mouse IgG antibody (1:5,000) for 1 h. After three washes with PBS-T, 100 µl of OPD

substrate (O-phenyl diamine) was added to the wells and incubated in the dark for 30 min. 100 μ l of 1 N sulfuric acid was used to stop the reaction and absorbance read at 492 nm. Five hybridomas with the highest absorbance indicating strong antibody binding were selected for sub-cloning to select stable hybridoma. Indirect ELISA was used to select the most reactive hybridoma, which was cultured in-vitro for mAbs production. The produced mAbs were purified using affinity chromatography and characterized using ELISA. The summary of mAbs production is illustrated (Fig. 1).

Indirect ELISA for reactivity screening

A 96-well plate was coated with our recombinant SARS-CoV-2 N antigen (CoVAT) and commercial recombinant SARS-CoV-2 N antigen (Nagasaki University, DIA-5028) concurrently except for blank. Following blocking and washing to prevent nonspecific binding, our mAb and commercial mAb (2G4-H1-B2-C3, Cat No. 130-10836) were added to the wells to separately with varying concentration from 2 μ g/ml to 0.03125 μ g/ml and incubated at 37 °C for 1 h. 100 μ l of HRP-conjugated anti-mouse IgG was added (1:5,000) into the wells and incubated at 37 °C for 1 h. The reaction was visualized using an OPD substrate and absorbance read at 492 nm. This method assessed the binding specificity and strength of our mAb against the recombinant and commercial antigens. It also compared the binding affinity of our mAb and commercial mAb.

Sandwich ELISA for reactivity screening against SARS-CoV-2 variants

The mAb reactivity with SARS-CoV-2-infected fluid culture (ICF) derived from the Beta and Omicron variants was evaluated. Plate-1 was coated with our mAb in two folds serial dilutions and incubated overnight at 4 °C. After blocking and washing as previously described. Virus isolates (ICF) of 3.45×10^5 plaque forming unit/ml (PFU/ml) of Beta and Omicron variants were added to the plate in duplicates and incubated at 37 °C 1 h. Concurrently, Plate-2 was prepared by forming a complex of commercial mAb with HRP-conjugated anti-mouse IgG, which was then transferred to Plate-1, incubated at 37 °C for 1 h. Culture media with no viruses were used as a negative control. After incubation and washing with PBST, the reaction was detected using an OPD substrate and absorbance read at 492 nm. This approach minimized cross-reactivity between the coated mAbs and the secondary antibody without SARS-CoV-2 ICF, ensuring specificity in detecting the target antigen.

Screening of mAb for clinical performance using ELISA

A 96-well plate was coated with our mAb (1 μ g/ml) except for the blank well and incubated overnight at 4 °C. Following blocking and washing as previously described, RT-PCR SARS-CoV-2 confirmed nasopharyngeal swabs with varying CT values ranging from 15.45 to 31.18 were added to respective wells and incubated at 37 °C for 1 h. A known SARS-CoV-2 negative sample was used as a negative control. Following washing with PBST, a mouse anti-SARS-CoV-2 HRP conjugated IgG secondary

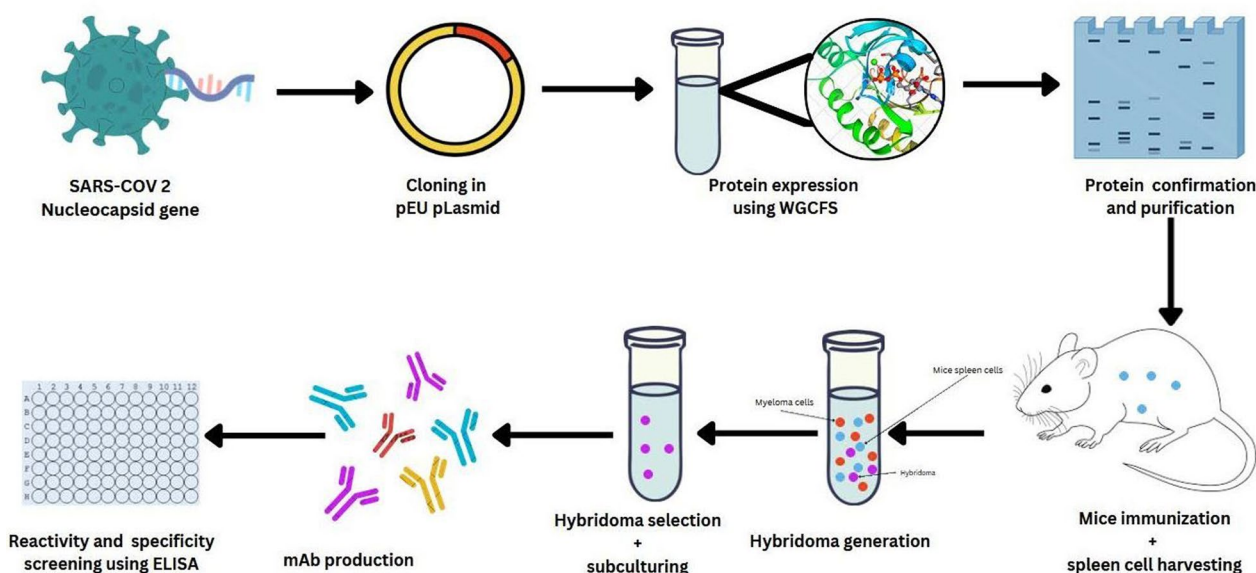


Fig. 1 Summary of monoclonal antibody production against SARS-CoV-2 NP using wheat-germ cell free system

antibody was added to all wells except the blank. After incubation and washing, OPD substrate and stop solution were added as previously described and absorbance read at 492 nm.

Screening the mAbs specificity using ICF of respiratory viruses

Sandwich ELISA was used to screen for mAbs specificity. Briefly, our mAb (1 µg/ml) was coated in a plate and incubated overnight at 4 °C. After blocking and washing, virus isolates of Human parainfluenza virus (HPIV-1,2,3), Adenovirus (Adv), A-pH1 N1—influenza A virus type H1 N1, A-H3—influenza A virus type H3 N2, Influenza B virus (BS), B/Yamagata lineage, Influenza B virus (BB), B/Victoria lineage, Respiratory syncytial virus (RSV), Herpes simplex virus (HSV type 1/2), SARS-CoV-2 Beta and Omicron mutants were added in triplicates to the coated plate in designated wells. SARS-CoV-2 N antigen was used as a positive control, while culture medium with no virus was used as negative control. After 1 h of incubation and washing at 37 °C, a complex of commercial mAb with HRP-conjugated anti-mouse IgG was added and incubated for 1 h. After washing, the reaction was detected using OPD substrate and absorbance determined at 492 nm. The mean absorbance of three independent assays was calculated.

Statistical analysis

All plots and statistical analyses were conducted using R software v4.4.1. Inferential statistical methods (student *T* test or Kruskal–Wallis test) compared whether there was a significant difference in the reactivity of the study mAb versus a commercial mAb with a *p* value <0.05 showing statistical significance.

Results

Wheat germ cell free system allows for generation of high-quality recombinant N-protein

The amplification of the N gene using specific primers by RT-PCR resulted in a 1200 bp fragment that was cloned into an expression vector. Sanger sequencing confirmed the correct insertion and orientation of the antigen sequence within the plasmid clones. The protein was confirmed using SDS–PAGE analysis and western blotting at the expected size of 46 kDa (Fig. 2).

Hybridoma selection for mAbs production

Among the 20 stable hybridomas generated, five clones (5, 6, 7, 12, and 15) were subcloned to select the most reactive hybridoma for mAb production. This selection was based on their binding affinities to the target antigen. The average OD values at 492 nm ranged from approximately 0.1 to 2.5 across serial dilutions (Fig. 3).

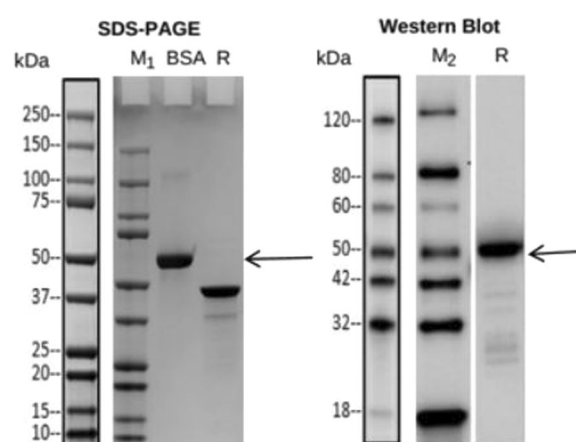


Fig. 2 Expression of SARS-CoV-2 N antigen using wheat germ cell free system confirmed by SDS–PAGE and Western blot (anti-His tag, Abcam ab18184); M₁: protein marker, Bio-Rad, Cat No. 1610374S; M₂: protein marker, GenScript, Cat. No. M00673; BSA: 2.00 µg; R: reducing condition. The expected band size is ~46 kDa

Clone 12 exhibited the strongest binding affinity, maintaining higher OD values across the dilutions, with a peak OD of 2.5 at the initial dilution (10¹). Clone 15 followed with slightly lower binding strength, while clones 5, 6, and 7 demonstrated progressively weaker affinities (Fig. 3). Based on these findings, clone 12 (3H1–2) was selected for monoclonal antibody production due to its superior performance in antigen binding.

Reactivity screening of the study mAb and commercial mAb

We evaluated the reactivity of this study and commercial mAb against both commercial and our antigen, CoVAT. Our mAb showed a higher reactivity against both antigens, but there was no statistical difference in the binding affinities of both mAbs (Fig. 4A, b). Using sandwich ELISA, we checked the reactivity of our mAb against Beta (B. 1.351) and Omicron (B.1.1.529) SARS-CoV-2 variants obtained from Kenya and Japan, respectively. The mAb effectively detected both variants, with the OD remaining high and stable across dilutions, suggesting strong cross-variant binding (Fig. 4C).

Clinical performance of the study mAb

We further evaluated the ability of this study mAb to detect SARS-CoV-2 using clinical nasopharyngeal samples with confirmed SARS-CoV-2 infection across a range of RT-PCR Ct values. A clear inverse correlation was observed between the Ct values and ELISA absorbance at 492 nm (Fig. 4D), indicating higher antigen detection in samples with lower Ct values. The detected Ct values ranged between 15.5 and 31.14 and

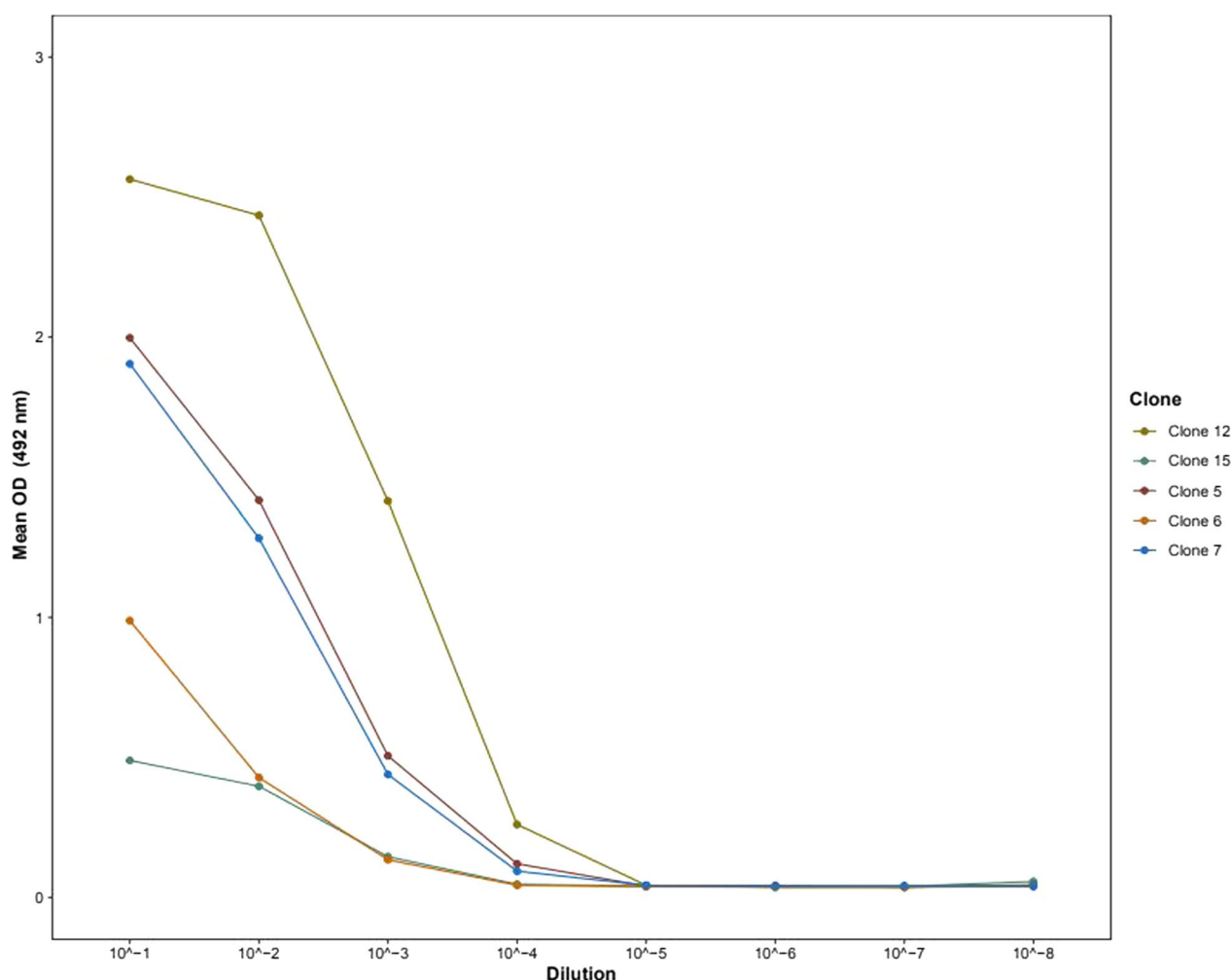


Fig. 3 Evaluation of antigen binding affinities of selected hybridoma clones using indirect ELISA. Clone 12 (3H1-2) exhibited the highest binding affinity among the five clones

explained 91.5% of the data between the Ct values and absorbance measurements, indicating the study mAb performs optimally in samples with higher viral loads but also detect samples with lower viral loads. The findings underscore the utility of the study mAb in antigen detection-based platforms, especially in identifying SARS-CoV-2 infection samples with either high or low viral loads during any phase of an outbreak.

mAb specificity screening against other respiratory viruses

The mean absorbance values for Human parainfluenza virus (HPIV-1,2,3), Adenovirus (Adv), A-pH1 N1—influenza A virus type H1 N1, A-H3—influenza A virus type H3 N2, Influenza B virus (BS), B/Yamagata lineage, Influenza B virus (BB), B/Victoria lineage, Respiratory syncytial virus (RSV), Herpes simplex virus (HSV type 1/2), SARS-CoV-2 Beta and Omicron mutants was obtained. The positive control was our recombinant N

antigen which exhibited the highest absorbance. The non-target viruses showed low absorbance, as shown in Fig. 5. SARS-CoV-2 beta and omicron variants showed higher reactivity compared to the other respiratory viruses.

Discussion

In this study, we leveraged WGCFS to generate SARS-CoV-2 N recombinant protein which could be used for the active detection of SARS-CoV-2. WGCFS is a powerful tool that surpasses the traditional prokaryotic translation system. It can be utilized for large-scale production of proteins that are soluble and retain their native conformation, mimicking the structure of the target antigen encountered by the immune system [28–30]. These benefits highlight the suitability of WGCFS to produce quality antigens that can be used to immunize animals for production of precise and

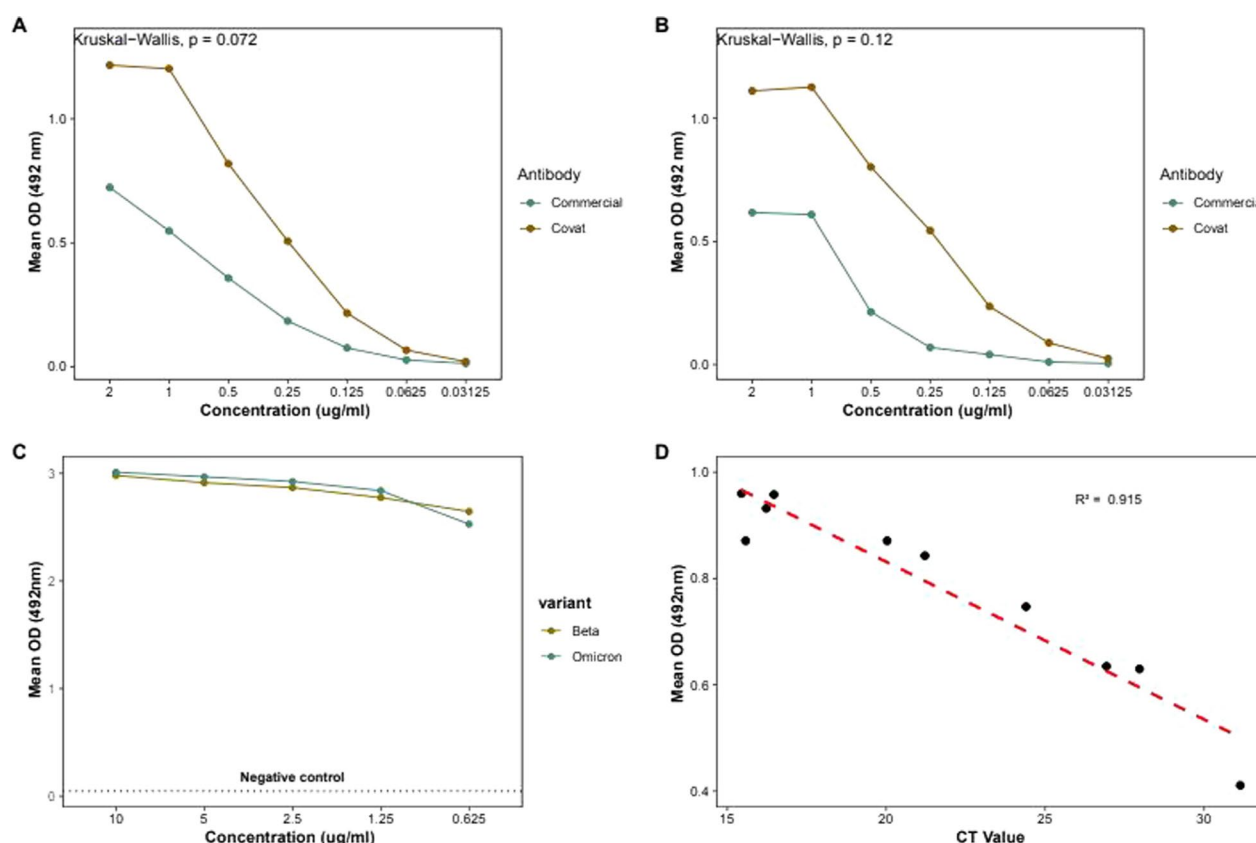


Fig. 4 **A, B** Titration curves showing the binding efficiency of two antibodies; Covat (study mAb from clone 12–3H1–2) and Commercial mAb (2G4–H1–B2–C3, Cat No. 130–10836) against commercial antigen (Nagasaki, DIA-5028) and study recombinant antigen, respectively. Antibody concentrations range from 2.0 to 0.03125 $\mu\text{g/ml}$. Statistical comparison using the Kruskal–Wallis test showed no significant difference in binding ($p = 0.072$ and $p = 0.12$ for panels A and B, respectively) **C** Binding activity of varying concentrations of our monoclonal antibody against SARS-CoV-2 beta and omicron variants from Kenya and Japan, respectively. The monoclonal antibody with decreasing concentrations (10 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$, 2.5 $\mu\text{g/ml}$, 1.25 $\mu\text{g/ml}$ and 0.625 $\mu\text{g/ml}$) showed strong and consistent signal against the virus infected culture fluid (ICF) both Beta (3.45×10^5 PFU/mL) and Omicron (3.45×10^5 PFU/mL) SARS-CoV-2 variants, with minimal reduction in optical density. **D** Correlation between RT-qPCR cycle threshold (CT) values and ELISA absorbance at 492 nm. An inverse relationship was observed ($R^2 = 0.915$), indicating high assay sensitivity.

reliable mAbs. This system has been used to express proteins for drug development [23], vaccine targets [26], as well as for monoclonal antibody production [10]. Our mAb demonstrated high reactivity against our SARS-CoV-2 N antigen as well as the commercial N protein (Fig. 4A, B). The higher reactivities observed in our SARS-CoV-2 antigen may be attributed to similarities in conformational epitopes recognized from the mAbs, while the reactivities in the commercial antigen are likely associated with the conserved nature of conformational epitopes present in the N protein.

SARS-CoV-2 evolution is primarily driven by point mutations and recombination resulting in new lineages and variants of concern defined by their reduced effectiveness to public health interventions [5]. The major VOCs are Beta (B.1.315), Alpha (B.1.1.7), Delta (B.1.617.2), Gamma (P.1), and Omicron (B.1.1.529) [31]. While the spike protein has been undergoing changes due

to immune pressure, mutations have also been reported in the nucleocapsid protein [5]. This raises a fundamental question on whether the currently generated monoclonal antibodies will be able to detect active infections or result in false negative cases. These mutations have impacted both molecular and serological diagnosis of SARS-CoV-2. S gene mutations have necessitated regular validation of commercial RT-PCR kits to prevent false negatives, while mutations in N and RdRp genes have shown to reduce assay sensitivity. Mutations that alter epitope structure prevent mAbs from binding effectively. This can lead to reduced sensitivity making polyclonal antibodies a more reliable option [32]. However, monoclonal antibodies targeting conserved regions of the N gene could help minimize false negatives by maintaining their ability to bind despite viral mutations. By focusing on stable regions of the N gene, mAbs could provide more

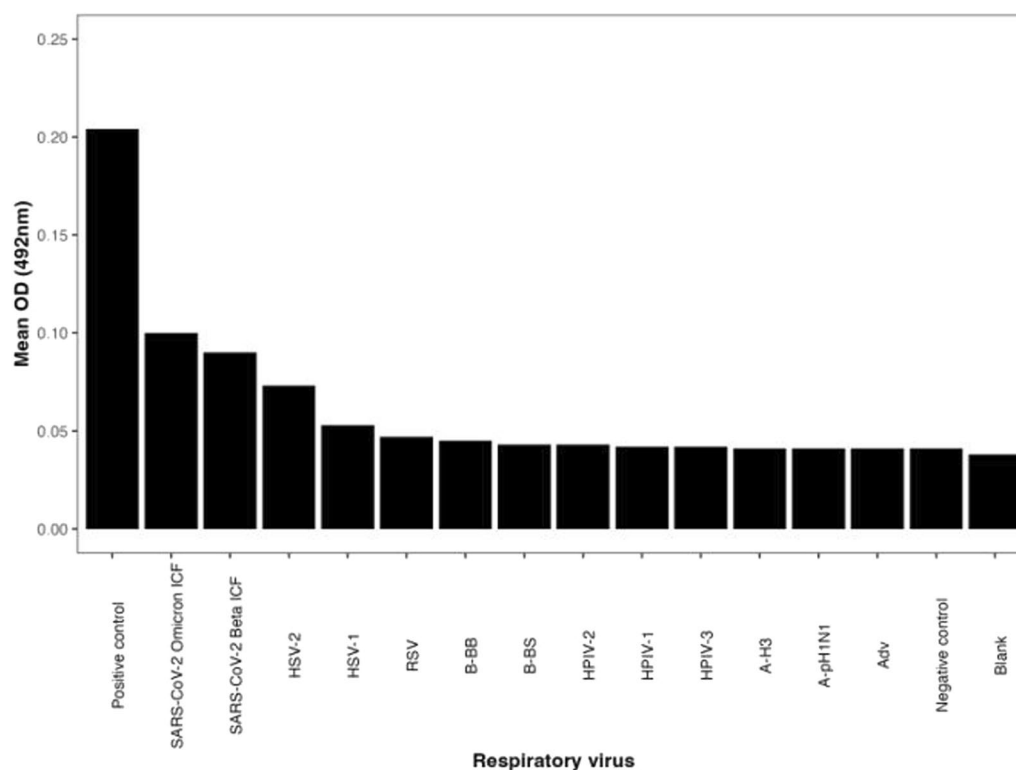


Fig. 5 Specificity of monoclonal antibodies against other respiratory viruses: B-BB; influenza B virus/Yamagata lineage, B-BS; influenza B virus/Victoria lineage, A-H3; influenza A virus type H3 N2, RSV; respiratory syncytial virus, HSV-1/2; herpes simplex virus types 1 and 2, A-pH1 N1; influenza A virus type H1 N1, Adv; adenovirus; HPIV-1,2,3; human parainfluenza virus, PC; positive control (recombinant SARS-CoV-2 N protein)

consistent results across various SARS-CoV-2 variants, enhancing diagnostic accuracy.

Our generated monoclonal antibody was able to detect both the Beta (B.1.315) and Omicron (B.1.1.529) variants with the omicron variant showing better reactivity values to the beta variant from ICF (Fig. 4C). The detection of both variants reinforces the dependability of using the N antigen for SAR-CoV-2 diagnostic test. Previous developed monoclonal antibody using the N protein were able to detect both the alpha variant with mutations D3L, R203 K, G204R, and S235 F and the delta variant with mutations D63G, R203M, D377Y, and R385 K as well as the wild type, beta and omicron variant [33]. These results indicate that the presence of these mutations may not prevent the epitopes from binding on the monoclonal antibody. Our mAb demonstrated the ability to detect SARS-CoV-2 antigen from nasopharyngeal swab clinical samples across a range of Ct values in ELISA-based detection (Fig. 4D). This capability is important considering the phases of any infection are associated with varying levels of viral loads, with the early and late phase of the SARS-CoV-2 wave being linked with high Ct values. By enabling timely detection throughout the

course of infection, our mAb could contribute to public health interventions.

We evaluated the specificity of our mAb against other respiratory viruses. The binding affinity of our recombinant N antigen, which was used as the positive control exhibited the highest absorbance, confirming the validity of the assay. The other non-target viruses including Human parainfluenza virus (HPIV-1,2,3), Adenovirus (Adv), A-pH1 N1—influenza A virus type H1 N1, A-H3—influenza A virus type H3 N2, Influenza B virus (BS), B/Yamagata lineage, Influenza B virus (BB), B/Victoria lineage, Respiratory syncytial virus (RSV), and Herpes simplex virus (HSV type 1), did not indicate cross-reactivity with our monoclonal antibody. Although we noted some potential reactivity between our mAb and HSV-2, this may be attributed to epitope cross-reactivity. A study reported that mAbs produced against SARS-CoV-2 N antigen showed cross-reactivity with parts of SARS-CoV but not on other coronaviruses [34]. Notably, in this study, our monoclonal antibody successfully bound to SARS-CoV-2 beta and omicron variants. This indicates that the selected epitope remains partially conserved across different SARS-CoV-2 lineages, supporting its potential use in variant-inclusive diagnostics. A similar

study reported low to negative reactivity of the study mAb to other coronaviruses including SARS-CoV-1 but strong reaction towards omicron lineages [35].

The limitation of this study is that we did not evaluate the mAb against other coronaviruses, such as SARS-CoV-1, MERS-CoV, and common cold coronaviruses, such as OC43, 229E, NL63, and HKU1. Assessing cross-reactivity with these viruses would provide a more comprehensive understanding of our mAbs specificity and potential limitations in distinguishing SARS-CoV-2 from related coronaviruses. In addition, while a limited number of clinical samples were tested to assess the diagnostic performance of the mAbs, broader evaluation using a larger and more diverse set of clinical specimens is still needed. This would help establish the sensitivity, specificity, and the clinical applicability of the antibodies across different populations and viral variants.

Despite these limitations, the high reactivity pair of our mAb and recombinant antigen suggests potential for their use in development of lateral flow antigen (LFA) kits for point of care use, which are currently under development. Furthermore, our mAbs can be applied in therapeutic development and environmental or clinical surveillance platforms.

Conclusion

This study demonstrated the successful use of the WGCFS to express SARS-CoV-2 N antigen in resource-limited setting. We generated mAb against SARS-CoV-2 in mice which showed high reactivity against our recombinant protein, commercial (Nagasaki) antigen, SARS-CoV-2 beta and omicron variants. Our mAb showed comparable reactivity to that of a commercially available antibody, reinforcing its diagnostic potential. While no cross-reactivity was observed with common respiratory viruses, further validation is needed to assess potential cross-reactivity with other coronaviruses and to confirm performance in large-scale clinical testing. These findings support its potential for use in the development of LFA kit which is ongoing as well as therapeutic development and clinical or environmental surveillance platforms.

Abbreviations

ACE-2	Angiotensin-converting enzyme 2
Adv	Adenovirus
A-H3	Influenza A virus
A-pH1 N1	Influenza A virus type H1N1
ATP	Adenosine triphosphate
BB	Influenza B virus/Victoria lineage
BS	Influenza B virus/Yamagata lineage
BSA	Bovine serum albumin
COVID-19	Coronavirus 2019
CT	Cycle threshold
DPP4:	Dipeptidyl peptidase 4
ELISA	Enzyme linked immuno-assays

FDA	Food and drug Administration
GTP	Guanosine triphosphate
HAT	Hypoxanthine aminopterin and thymine
HPIV	Human parainfluenza virus
HSV	Herpes simplex virus
ICF	Infected fluid culture
LFA	Lateral flow antigen
mAb	Monoclonal antibody
MAFFT	Multiple alignment using Fast Fourier Transform
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
NCBI	National centre for biotechnology information
NMAbs	Neutralizing monoclonal antibodies
OPD	O-phenyl diamine
PBS-T	Phosphate buffered saline with tween 20
PEG	Polyethylene glycol
RBD	RNA binding domain
RSV	Respiratory syncytial virus
RT-qPCR	Reverse transcription real-time polymerase chain reaction
SARS-CoV-1	Severe acute respiratory syndrome coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus-2
SDS-PAGE	Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis
WGCFS	Wheat germ cell-free system

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Author contributions

Conceptualization: E.A.W., B.N.K., J.G., and M.M.M.; Data curation, M.M.M., M.M., and E.A.W.; Formal analysis, M.M.M., S.M., and E.A.W.; Investigation, M.M.M., and E.A.W.; Methodology, M.M.M., M.M., E.A.W., and B.N.K.; Supervision, S.G.N., B.N.K., and E.A.W.; Resources, M.M.M., B.N.K., S.I., S.M., S.A.K., J.G., and E.W.; Funding acquisition, E.A.W., B.N.K., and J.G.; Writing—original draft, M.M.M., Writing—review and editing, M.M.M., S.M., B.N.K., S.G.N., M.M., S.I., S.A.K., J.G., and E.A.W. All authors have read and agreed to the published version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the institutional review board at Mount Kenya University REF: MKU/ERC/1926 and the National Commission of Science and Technology (NACOSTI) under reference number NACOSTI/P/23/24347.

Informed consent

Patient consent was not required, because this investigation used archived material that had already received informed consent.

Competing interests

The authors declare no competing interests.

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