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1 **Single-Domain SARS-CoV-2 S1 and RBD Antibodies Isolated from Immunized Llama**
2 **Effectively Bind Targets of the Wuhan, UK, and South African Strains in vitro**
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20 **Key Words:** COVID-SARS-2; Single Domain Antibody; Nanoantibody; Nanobody, Variants; UK
21 Strain; South African Strain; Antigen Test; mNeonGreen.

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22 **Abstract**

23 The spreading of SARS-CoV-2 variants has become a major challenge of the current fight against
24 the pandemic. Of particular concerns are the strains that have arisen from the United Kingdom
25 (UK) and South Africa. The UK variant spreads rapidly and is projected to overtake the original
26 strain in the US as early as in March 2021, while the South African variant appears to evade some
27 effects of the current vaccines. Potential false-negative diagnosis using currently available antigen
28 kits that may not recognize these variants could cause another wave of community infection.
29 Therefore, it is imperative that antibodies used in the detection kits are validated for binding
30 against these variants. Here we report that the nanoantibodies (nAbs in our terminology, also
31 referred to as VHH fragments, single domain antibodies, nanobodies^{TM}) that we have developed
32 for rapid antigen detection test bind the receptor binding domain (RBD) of the S1 protein from the
33 original COVID-SARS-2 virus as well as those from the UK and South African variants. This
34 finding validates our antibodies used in our assay for the detection of these major variant strains.

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36 **Main Text**

37 We immunized a llama by repeated injections of recombinant S1 and RBD proteins. Using our
38 nanoantibody (nAb) development pipeline that resulted in dozens of drug candidate nAbs over a
39 decade [1], we isolated multiple families of anti-RBD nAbs that showed strong binding affinity and
40 specificity against the RBD of the clinical strain Wuhan-Hu-1. We then characterized the
41 properties of these nAbs through various experiments that will be published separately. We
42 further developed a prototype rapid antigen test in the format of lateral flow assay with these nAbs
43 using clinical samples before the spread of the current major variants.

44

45 The UK variant, also referred to as SARS-CoV-2 B.1.1.7 lineage, contains ~20 amino acid
46 changes in total, one of which is in the RBD (N501Y) and several in the N-terminus of the S1
47 protein. The South Africa variant, also known as B.1.351 lineage, contains N501Y and two more
48 changes in RBD (K417N, E484K). These alterations might impact the epitope binding of
49 conventional monoclonal antibody (mAb) pairs selected for existing antigen detection tests.
50 Nanoantibodies, on the other hand, bind their antigens through distinct mechanism than IgG
51 antibodies. With a size that is approximately 10x smaller than IgG, the VHH single-domain derived
52 nAbs can insert its epitope-contacting amino acids into crevices on their antigens [2] instead of
53 "wrapping around" exposed epitopes by a heavy chain/light chain arm pair of IgG, affording a
54 possibility that the epitope recognition of nAbs might be less affected by individual amino-acid
55 mutations of the variants than that of mAbs. In this report, we show that our nAbs indeed bind
56 RBDs of both the UK and the South Africa lineages.

57

58 Recombinant RBDs of Wuhan-Hu-1 (WT), B.1.1.7 (UK), and B.1.351 (South Africa) were obtained
59 from BEI and conjugated to Spherotech particles before flow cytometry was performed. We tested
60 the binding ability of nAb1 and nAb2, representing two of several families of anti-SARS-CoV-2

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61 nAbs that we isolated. We previously determined their dissociation constant (Kd) toward WT RBD
62 through serial dilutions as a measure of binding affinity and found that nAb1 and nAb2 showed
63 Kds between 6 nM to 15nM when conjugated to different fluorescent proteins. We then set to test
64 the binding affinities of nAb1 and nAb2 towards variant RBDs as described below. To facilitate
65 the detection of nAbs in the flowcytometry assay as well as our rapid antigen diagnostic kit, we
66 genetically fused each nAb with mNeonGreen, the brightest monomeric fluorescent protein that
67 we previously developed [3]. We also produced a genetic fusion protein that contains two nAb1
68 domains linked via a linker (referred to as nAb1 dimer).

69

70 As shown in Figure 1, we found that both mNeonGreen-fused nAb1 and nAb2 bind to RBDs from
71 all three strains above the background binding to bovine serum albumin (BSA). Note that these
72 three RBDs when conjugated to the Spherotech beads as carrier particles have not been
73 normalized for quantitative cross-comparison, therefore we could not conclude relative binding
74 affinity for each RBD. More quantitative and direct comparison will be performed with additional
75 experiments. As a control, we used the monoclonal antibody CR3022, which was originally
76 isolated for its binding to the S protein of SARS-CoV [4] and crossreacts to SARS-CoV-2 S
77 protein. CR3022 we obtained from R&D Systems binds to the WT RBD but not to UK or South
78 African RBDs in our hand, despite the computational prediction that mutations on South African
79 RBD might minimally affect CR3022 binding [5]. Interestingly but not surprisingly, nAb1 dimer
80 binds all RBDs with affinity seemingly higher (after adjusting different fluorescent labelling) than
81 the nAb1 monomer, indicating that the dimerization of nAb1 produces cooperative binding
82 perhaps to RBD proteins juxtaposed on the same bead. Since individual SARS-CoV-2 virions
83 contain 24 ± 9 S protein trimers [6], such cooperative binding by the nAb dimer (and potentially
84 trimer, data not shown) would likely increase the detection sensitivity of virus particles in antigen
85 test kit. Incidentally, the benefit of creating nAb1 linear multimers have been independently
86 demonstrated in their increased ability to neutralize SARS-CoV-2 infection using our human iPSC-

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87 derived lung epithelial cell infection experiments, to be published elsewhere.

88

89 Ongoing testing of at least three more families of nAbs selected from immunized llama should
90 yield additional nAbs that are potential detectors of not only the two variants tested in this study
91 but also other variants in circulation or will arise in the future. Finally, Allele Biotech's COVID
92 antigen rapid test, designed for both point-of-care clinical and over-the-counter home use,
93 integrates multiple nAbs' capabilities of recognizing hundreds of binding pockets of the spike
94 protein on the surface of virus particles, enabling detection of current and future variants and
95 much improved sensitivity over the existing test kits.

96

97 **Methods**

98 **nAb production.**

99 The nAbs that bind to SARS-CoV-2 Wuhan RBD/S1 proteins were isolated by methods that we
100 described in detail [1]. Briefly, a llama was repeatedly immnunized with recombinant Wuhan RBD
101 and S1 proteins obtained from BEI Resources. Several weeks after the initial injection, the blood
102 from the llama was harvested and mRNAs isolated from an enriched lymphocyte population.
103 Reverse transcriptase (RT)-PCR was performed using a set of primers that specifically amplify
104 VHH domains and amplified cDNAs cloned into an M13 vector. Phage display based on enzyme-
105 linked immunosorbent assay (ELISA) was performed in 96-well plates to isolate VHH cDNAs that
106 specificially bind to Wuhan RBD and S1 proteins. The positivie cDNAs are subcloned into an E.
107 coli expression vector for the production of RBD/S1 nAbs.

108

109 **Flow cytometry using protein-conjugated beads.**

110 Bovine serum albumin (BSA) and three recombinant RBDs (Wuhan, UK, and South African) were
111 covalently conjugated to Speherotech bead particles and flow cytometry was performed after the
112 beads were mixed with three fluorescent labeled nAbs that we generated; S-nAb1, S-nAb2 and

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113 S-nAb1 Dimer. For fluorescent labeling, S-nAb1 and S-nAb2 are genetically fused with
114 mNeonGreen [3]. S-nAb1 Dimer is HA-tagged and detected through anti-HA mAb (Cat# IC6875G,
115 Lot# AFFY0119021, Alexa Fluor 488, Novus Biologicals). In two control experiments (1 and 2),
116 the secondary antibody anti-HA mAb alone and the commercially available anti-SARS-CoV-2
117 Spike mAb (CR3022, CAT# NBP2-90980F, Lot#T2022B04-110920-F, FITC, R&D Biosystems)
118 were tested for their ability to bind the four beads described above. Fluorescence intensity (a.u.)
119 was adjusted per labeling methods and chromophores used in each experiment.
120

121 **Acknowledgment**

122 The following reagents were obtained through BEI Resources, NIAID, NIH: Spike Glycoprotein
123 S1 Domain from SARS-Related Coronavirus 2, Wuhan-Hu-1 with C-Terminal Histidine Tag,
124 Recombinant from HEK293 Cells, NR-53798; Spike Glycoprotein Receptor Binding Domain
125 (RBD) from SARS-Related Coronavirus 2, Wuhan-Hu-1 with C-Terminal Histidine Tag,
126 Recombinant from HEK293 Cells, NR-53800; Spike Glycoprotein Receptor Binding Domain
127 (RBD) from SARS-Related Coronavirus 2, United Kingdom Variant with C-Terminal Histidine
128 Tag, Recombinant from HEK293 Cells, NR-54004; Spike Glycoprotein Receptor Binding
129 Domain (RBD) from SARS-Related Coronavirus 2, South Africa Variant with C-Terminal
130 Histidine Tag, Recombinant from HEK293 Cells, NR-54005.
131

132 **Author Contributions Statement**

133 DY, IL, WT, LR carried out the experiments; JCC designed the solubilized version of the nAbs
134 and nAb multimers; NCS, NN and JW designed and together with AH supervised the nAb
135 generation experiments; ED conducted and supervised flowcytometry experiment; JW wrote the
136 main manuscript; JCC prepared the figure; NN and JW edited the draft; JW secured and provided
137 funding.

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138

139 **Competing Interest**

140 DY, IL, WT, LR, AH, NCS, NN and JW are current or former employees of Allele Biotech, a US
141 small business that commercializes nAb-based and fluorescent protein-based products, among
142 other for-profit activities. Contribution from Scintillon to this work was paid through contracts and
143 conducted on behalf of Allele Biotech.

144

145 **Data Availability**

146 Additional, related data of the present report is available from the corresponding author upon
147 request.

148

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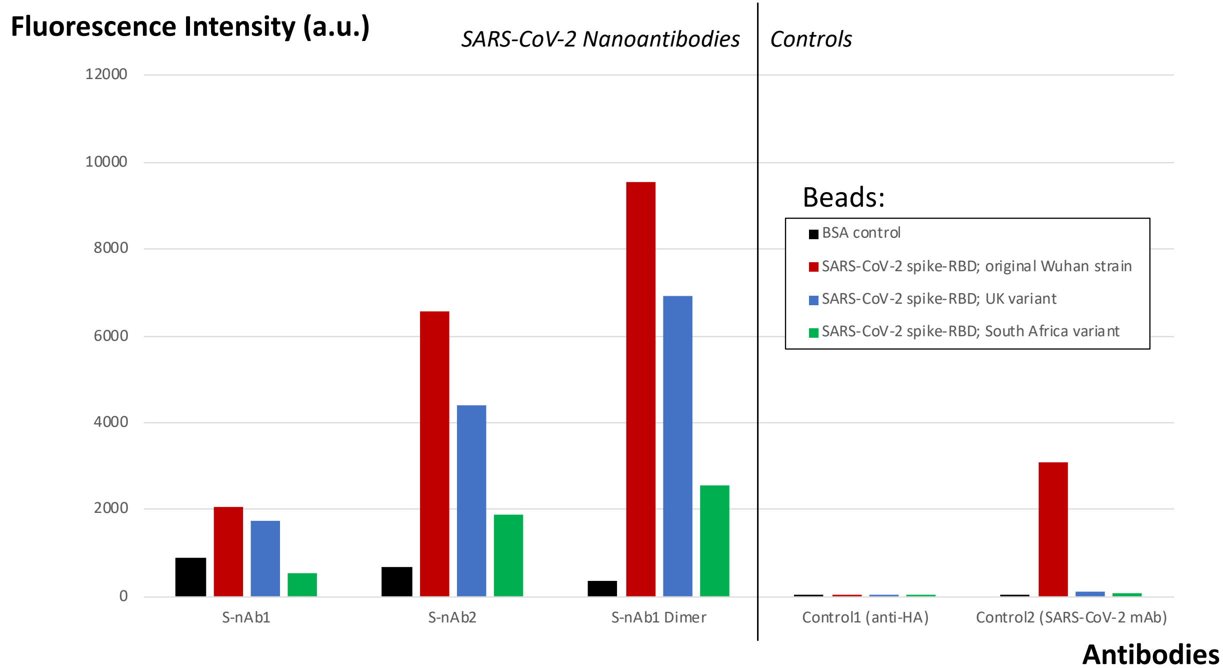

174
175 **Figure 1**. **Graphical representation of binding assay results obtained via flow cytometry.**
176 We evaluated the ability of nAbs to bind spike Receptor Binding Proteins (RBPs) from SARS-
177 CoV-2 Wuhan, UK and South African strains via . The results presented in the left panel indicate
178 that S-nAb1 binds to Wuhan and UK RBPs, but not to South African RBP; while S-nAb2 and S-
179 nAb1 Dimer bind to all three (Wuhan, UK, South Africa) RBPs. The right panel of the figure shows
180 two control experiments. In control 1 (negative control), the anti-HA mAb alone resulted in low or
181 no binding to BSA- or RBP- conjugated beads (within a range of 5 to 28 a.u.). In ctonrol 2, the
182 commercially avaialbe SARS-CoV-2 mAb CR3022 binds to Wuhan RBD, but not to UK or South
183 African RBPs.