Title: Deletion disrupts a conserved antibody epitope in a SARS-CoV-2 variant of concern

Running Title: Transmission of an unexpected SARS-CoV-2 RBD deletion variant

Authors: Linda J. Rennick^{1,2}, Lindsey R. Robinson-McCarthy^{3,4}, Sham Nambulli^{1,2}, W. Paul Duprex^{1,2*} and Kevin R. McCarthy^{1,2*}

Affiliations: ¹ The Center for Vaccine Research, University of Pittsburgh School of Medicine, Pittsburgh PA ² The Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh PA ³ Hillman Cancer Center, Pittsburgh PA and ⁴ The Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh PA.

Abstract:

Multiple SARS-CoV-2 variants with altered antigenicity have emerged and spread internationally. In one lineage of global concern, we identify a transmitted variant with a deletion in its receptor binding domain (RBD) that disrupts an epitope which is conserved across sarbecoviruses. Overcoming antigenic variation by selectively focusing immune pressure on this conserved site may, ultimately, drive viral resistance.

Main Text:

The SARS-CoV-2 spike (S) glycoprotein is the target of protective antibodies and sole antigen delivered in widely deployed vaccines ¹⁻⁴. Emergent variants (B.1.1.7, B.1.351, P.1) have independently acquired a common series of mutations that confer resistance to therapeutic antibodies and serum from infected or vaccinated individuals. Variant spread has been rapid. Reformulated therapies and vaccines are in development. Directing antibodies to conserved sites therapeutically, as a consequence of immune recall or via designed immunogens can, in

theory, overcome antigenic variation. These strategies rely upon sites that slowly, if ever, accrue diversity. We identify a transmitted B.1.1.7 variant with a deletion in a site of pan-sarbecoviruses conservation. This deletion disrupts the binding of an antibody that engages both SARS-CoV and SARS-CoV-2. The acquisition of antigenic novelty in S glycoprotein has been recurrent and convergent. By extension conservation at this site may not persist.

Using sequences deposited in the GISAID database⁵, we have monitored variants of concern for the acquisition of additional epitope-altering mutations. We identified a transmission cluster of six identifiably different individuals that share a nine-nucleotide deletion within the S gene encoding the RBD. These viruses belong to the B.1.1.7 lineage, which had already acquired two independent deletions in recurrent deletion region ⁶ (RDR) 1 ($\Delta 69-70$) and RDR2 ($\Delta 144/145$). Spread via human-to-human transmission is likely given the timing of sample collection, geographic proximity and the clustering of sequences within a phylogeny of contemporaneously circulating B.1.1.7 isolates (Fig. 1).

The deletion removes residues 375-377 of the RBD. All three have been conserved for the duration of the pandemic. We examined their conservation among divergent sarbecoviruses, including SARS-CoV, bat and pangolin sequences (Fig. 2a). Among these isolates the three codons differ only by synonymous nucleotide substitutions suggesting selective pressures to preserve the identity of each amino acid. Residues 375-377 contribute to an extended surface that is broadly conserved among sarbecovirus (Fig. 2b) . This site is distal from the interface between RBD and its receptor angiotensin-converting enzyme 2 (ACE2) ⁷ . In the “three RBD” down state of the S glycoprotein trimer, residues 375-377 form a β -strand which is recessed and facing inward to the trimer 3-fold axis of symmetry. Sampling of “RBD-up” conformations and receptor engagement exposes this site.

Despite its transient exposure, the conserved surface is immunogenic. A number of reported antibodies, isolated from different donors, engage this site and can bind/neutralize other sarbecoviruses. Antibody CR3022, elicited by a SARS-CoV infection during the 2003-2004 outbreak, initially defined this antibody class and their common epitope⁸⁻¹⁰ (Fig. 2b). These antibodies inhibit viral replication but have limited neutralization potency in single round infection assays¹¹⁻¹³. CR3022, like others makes direct contacts with residues 375-377. We introduced $\Delta 375-377$ into an S glycoprotein expression construct and detected expression by indirect immunofluorescence using an N-terminal domain binding antibody, 4A8¹⁴. The formation of multinucleated, syncytia demonstrates S- $\Delta 375-377$ remains a functional membrane fusogen (Fig. 2c). However, the deletion abolishes CR3022 binding. While evolutionary conserved, this site is mutable and a single mutation event results in antibody escape.

SARS-CoV-2 only recently crossed into humans. Mounting evidence suggests that specific variants of concern have evolved some resistance to dominant humoral responses. Specifically how SARS-CoV-2 will adapt to immune pressures imposed by a human antibody repertoire is to be determined. This transmitted variant, with a deletion at an otherwise conserved site demonstrates that antigenic stability in animal species may not always extend to humans.

Focused genetic surveillance has not identified additional $\Delta 375-377$ -linked cases. The virus was sufficiently fit to transmit between at least five individuals and to define their viral consensus sequences. This early period of S evolution has been defined by recurrent, convergent evolution. Many defining mutations in current variants of concern are identical or functionally equivalent. The alteration of a conserved epitope by $\Delta 375-377$ not only represents an additional antigenic step in a variant of concern (B.1.1.7), but also demonstrates a capacity of this site to acquire antibody resistance rapidly.

The emergence of variants of concern and their continued evolution demonstrate that S is not as antigenically stable as initially hypothesized^{15,16}. Second generation vaccines may selectively focus antibodies to conserved epitopes, either by design or as a consequence of recalling responses from immunologic memory. The conserved site tolerates both S-Δ375-377 and mutations about its periphery¹⁷⁻²⁰. It may not be a suitable target for therapeutic antibodies or immune focusing immunogens.

Acknowledgements:

We gratefully acknowledge the authors from the originating laboratories and the submitting laboratories, who generated and shared via GISAID genetic sequence data on which this research is based (Table 1). We sincerely apologize to the many authors we could not cite due to limitations on references. This work was supported by The University of Pittsburgh, the Center for Vaccine Research (WPD and KRM), The Richard King Mellon Foundation, the Henry L. Hillman Foundation and the Commonwealth of Pennsylvania, Department of Community and Economic Development (WPD)

Author contributions:

L.J.R., L.R.R-M, S.N, W.P.D and K.R.M designed the experiments; L.J.R., L.R.R-M, S.N and K.R.M. performed the experiments; L.J.R., L.R.R-M, S.N, W.P.D and K.R.M. analyzed data and L.J.R., L.R.R-M, S.N, W.P.D and K.R.M wrote the manuscript.

Methods

Materials and Methods

Sequence analysis:

Sequences were obtained from the publicly available GISAID database⁵ and acknowledged in supporting Table 1. Sequence analysis was performed in Geneious (Biomatters, New Zealand). To identify deletion variants in S gene, sequences were mapped to NCBI reference sequence MN985325 (SARS-CoV-2/human/USA/WA-CDC-WA1/2020), the S gene open reading frame was extracted, remapped to reference and parsed for deletions using a search for gaps function.

All sequences were aligned in MAFFT^{21,22} and adjusted manually for consistency. To evaluate the phylogenetic relationships between S-Δ375-377 variants and isolates from contemporaneously circulating B.1.1.7 we obtained sequences of B.1.1.7 variants from the United Kingdom from samples that were obtained from Mid-late December 2020. The first sequenced B.1.1.7 isolate hCoV-19/England/MILK-9E2FE0/2020 (EPI_ISL_581117), was included. FastTree²³ was used to generate a preliminary phylogeny. Branches from a node containing S-Δ375-377 variants were extracted and along with hCoV-19/England/MILK-9E2FE0/2020 (EPI_ISL_581117) were re-aligned. The final Maximum- Likelihood phylogenetic trees were calculated using RAxML²⁴ using a general time reversible model with optimization of substitution rates (GTR GAMMA setting), starting with a completely random tree, using rapid Bootstrapping and search for best-scoring ML tree with 10,000 bootstraps of support performed. The phylogeny of sarbecoviruses used the indicated sequences and were produced using RAxML²⁴ with the same parameters as above.

Cell lines:

Human 293F cells were maintained at 37° Celsius with 5% CO₂ in FreeStyle 293 Expression Medium (ThermoFisher) supplemented with penicillin and streptomycin. Vero E6 cells were maintained at 37° Celsius with 5% CO₂ in high glucose DMEM (Invitrogen) supplemented with 1% (v/v) Glutamax (Invitrogen) and 10% (v/v) fetal bovine serum (Invitrogen).

Recombinant IgG expression and purification:

The heavy and light chain variable domains of 4A8¹⁴ and CR3022⁸ were synthesized by Integrated DNA Technologies (Coralville, Iowa) and cloned into a modified human pVRC8400 expression vector encoding for full length human or mouse IgG1 heavy chains and human kappa light chains²⁵. IgGs were produced by polyethylenimine (PEI) facilitated, transient transfection of 293F cells that were maintained in FreeStyle 293 Expression Medium. Transfection complexes were prepared in Opti-MEM and added to cells. Five days post-transfection supernatants were harvested, clarified by low-speed centrifugation, adjusted to pH 5 by addition of 1 M 2-(N-morpholino)ethanesulfonic acid (MES) (pH 5.0), and incubated overnight with Pierce Protein G agarose resin (Pierce, ThermoFisher). The resin was collected in a chromatography column, washed with a column volume of 100 mM sodium chloride 20 mM (MES) (pH 5.0) and eluted in 0.1 M glycine (pH 2.5) which was immediately neutralized by 1 M TRIS(hydroxymethyl)aminomethane (pH 8). IgGs were then dialyzed against phosphate buffered saline (PBS) pH 7.4.

Cloning and transfection of SARS-CoV-2 spike protein deletion mutants:

S-Δ375-377 was generated in HDM_SARS2_Spike_del21_D614G²⁶ a plasmid containing SARS-CoV-2 S protein lacking the 21 C-terminal amino acids. HDM_SARS2_Spike_del21_D614G was a gift from Jesse Bloom (Addgene plasmid # 158762; <http://n2t.net/addgene:158762>; RRID:Addgene_158762). The region containing amino acids

375-377 was excised using appropriate restriction enzymes and replaced by a synthetically generated gBlock (Integrated DNA Technologies) with amino acids 375-377 deleted. The Gibson Assembly reaction, transformations, clone screening, plasmid DNA preparation and transfections were performed as described previously ⁶

Indirect immunofluorescence:

Indirect immunofluorescence was performed as previously reported ²⁷. Cells were transfected with the SARS-CoV-2 S-Δ375-377 protein deletion mutant and controls. Primary monoclonal antibodies were 4A8 (mouse Fc; 1 µg/ml) and CR3022 (human Fc; 4 µg/ml), and secondary antibodies were goat anti-mouse Alexa Fluor-568, Invitrogen, and goat anti-human Alexa Fluor-488, Invitrogen, both used at 1:400 dilution.

Structure visualization:

Structural figures were rendered in Pymol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

References

- 1 Baden, L. R. *et al.* Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N Engl J Med* **384**, 403-416, doi:10.1056/NEJMoa2035389 (2021).
- 2 Logunov, D. Y. *et al.* Safety and efficacy of an rAd26 and rAd5 vector-based heterologous prime-boost COVID-19 vaccine: an interim analysis of a randomised controlled phase 3 trial in Russia. *Lancet* **397**, 671-681, doi:10.1016/S0140-6736(21)00234-8 (2021).
- 3 Polack, F. P. *et al.* Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N Engl J Med* **383**, 2603-2615, doi:10.1056/NEJMoa2034577 (2020).
- 4 Voysey, M. *et al.* Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK. *Lancet* **397**, 99-111, doi:10.1016/S0140-6736(20)32661-1 (2021).
- 5 Shu, Y. & McCauley, J. GISAID: Global initiative on sharing all influenza data - from vision to reality. *Euro Surveill* **22**, doi:10.2807/1560-7917.ES.2017.22.13.30494 (2017).
- 6 McCarthy, K. R. *et al.* Recurrent deletions in the SARS-CoV-2 spike glycoprotein drive antibody escape. *Science*, eabf6950, doi:10.1126/science.abf6950 (2021).

- 7 Lan, J. *et al.* Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature* **581**, 215-220, doi:10.1038/s41586-020-2180-5 (2020).
- 8 ter Meulen, J. *et al.* Human monoclonal antibody combination against SARS coronavirus: synergy and coverage of escape mutants. *PLoS Med* **3**, e237, doi:10.1371/journal.pmed.0030237 (2006).
- 9 Tian, X. *et al.* Potent binding of 2019 novel coronavirus spike protein by a SARS coronavirus-specific human monoclonal antibody. *Emerg Microbes Infect* **9**, 382-385, doi:10.1080/22221751.2020.1729069 (2020).
- 10 Yuan, M. *et al.* A highly conserved cryptic epitope in the receptor binding domains of SARS-CoV-2 and SARS-CoV. *Science* **368**, 630-633, doi:10.1126/science.abb7269 (2020).
- 11 Huo, J. *et al.* Neutralization of SARS-CoV-2 by Destruction of the Prefusion Spike. *Cell Host Microbe* **28**, 445-454 e446, doi:10.1016/j.chom.2020.06.010 (2020).
- 12 Wrobel, A. G. *et al.* Antibody-mediated disruption of the SARS-CoV-2 spike glycoprotein. *Nat Commun* **11**, 5337, doi:10.1038/s41467-020-19146-5 (2020).
- 13 Finkelstein, M. T. *et al.* Structural Analysis of Neutralizing Epitopes of the SARS-CoV-2 Spike to Guide Therapy and Vaccine Design Strategies. *Viruses* **13**, doi:10.3390/v13010134 (2021).
- 14 Chi, X. *et al.* A neutralizing human antibody binds to the N-terminal domain of the Spike protein of SARS-CoV-2. *Science* **369**, 650-655, doi:10.1126/science.abc6952 (2020).
- 15 Dearlove, B. *et al.* A SARS-CoV-2 vaccine candidate would likely match all currently circulating variants. *Proc Natl Acad Sci U S A* **117**, 23652-23662, doi:10.1073/pnas.2008281117 (2020).
- 16 Rausch, J. W., Capoferri, A. A., Katusiime, M. G., Patro, S. C. & Kearney, M. F. Low genetic diversity may be an Achilles heel of SARS-CoV-2. *Proc Natl Acad Sci U S A* **117**, 24614-24616, doi:10.1073/pnas.2017726117 (2020).
- 17 Greaney, A. J. *et al.* Comprehensive mapping of mutations to the SARS-CoV-2 receptor-binding domain that affect recognition by polyclonal human serum antibodies. *bioRxiv*, 2020.2012.2031.425021, doi:10.1101/2020.12.31.425021 (2021).
- 18 Greaney, A. J. *et al.* Complete Mapping of Mutations to the SARS-CoV-2 Spike Receptor-Binding Domain that Escape Antibody Recognition. *Cell Host Microbe* **29**, 44-57 e49, doi:10.1016/j.chom.2020.11.007 (2021).
- 19 Starr, T. N. *et al.* Deep Mutational Scanning of SARS-CoV-2 Receptor Binding Domain Reveals Constraints on Folding and ACE2 Binding. *Cell* **182**, 1295-1310 e1220, doi:10.1016/j.cell.2020.08.012 (2020).
- 20 Liu, Z. *et al.* Identification of SARS-CoV-2 spike mutations that attenuate monoclonal and serum antibody neutralization. *Cell Host Microbe*, doi:10.1016/j.chom.2021.01.014 (2021).
- 21 Katoh, K., Misawa, K., Kuma, K. & Miyata, T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* **30**, 3059-3066, doi:10.1093/nar/gkf436 (2002).
- 22 Katoh, K. & Standley, D. M. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* **30**, 772-780, doi:10.1093/molbev/mst010 (2013).
- 23 Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol* **26**, 1641-1650, doi:10.1093/molbev/msp077 (2009).
- 24 Stamatakis, A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312-1313, doi:10.1093/bioinformatics/btu033 (2014).

233 25 Watanabe, A. *et al.* Antibodies to a Conserved Influenza Head Interface Epitope Protect
234 by an IgG Subtype-Dependent Mechanism. *Cell* **177**, 1124-1135 e1116,
235 doi:10.1016/j.cell.2019.03.048 (2019).
236 26 Crawford, K. H. D. *et al.* Protocol and Reagents for Pseudotyping Lentiviral Particles with
237 SARS-CoV-2 Spike Protein for Neutralization Assays. *Viruses* **12**,
238 doi:10.3390/v12050513 (2020).
239 27 Klimstra, W. B. *et al.* SARS-CoV-2 growth, furin-cleavage-site adaptation and
240 neutralization using serum from acutely infected hospitalized COVID-19 patients. *J Gen*
241 *Viol*, doi:10.1099/jgv.0.001481 (2020).
242 28 Ashkenazy, H. *et al.* ConSurf 2016: an improved methodology to estimate and visualize
243 evolutionary conservation in macromolecules. *Nucleic Acids Res* **44**, W344-350,
244 doi:10.1093/nar/gkw408 (2016).
245 29 Celniker, G. *et al.* ConSurf: Using Evolutionary Data to Raise Testable Hypotheses
246 about Protein Function. *Israel Journal of Chemistry* **53**, 199-206,
247 doi:<https://doi.org/10.1002/ijch.201200096> (2013).
248

249 **Figures:**

250

251

252

253 **Fig. 1. Transmission cluster within the B.1.1.7 lineage with a novel deletion in RBD. a.**

254 SARS-CoV-2 genome sequences with a nine-nucleotide deletion at codons 375-377 (red

255 branches) cluster together among contemporaneously circulating B.1.1.7 isolates (black

256 branches). The maximum likelihood phylogenetic tree is, rooted on EPI_ISL_581117 and was

266 6M0J). CR3022 (6W41) (yellows) has been docked in by aligning ACE2 RBDs. The RBD
 267 surface is colored by conservation among the coronavirus isolates above using ConSurf^{28,29}
 268 and residues 375-377 are circled in cyan. S glycoprotein distribution in Vero E6 cells at 24 h
 269 post-transfection with plasmids encoding SARS-CoV-2 S-Δ375-377 or wild-type S glycoprotein,
 270 visualized by indirect immunofluorescence in permeabilized cells. A monoclonal antibody
 271 against the N-terminal domain of SARS-CoV-2 S protein (4A8; red) detects wild-type and
 272 mutant protein. CR3022 monoclonal antibody (CR3022; green) does not detect the S-Δ375-377
 273 mutant. Overlay images (4A8/CR3022/DAPI) depict co-localization of the antibodies; nuclei
 274 were counterstained with DAPI (blue). The scale bars represent 100 μm.