

# The SARS-CoV-2 variants associated with infections in India, B.1.617, show enhanced spike cleavage by furin

Authors: Thomas P. Peacock<sup>1#</sup>, Carol M. Sheppard<sup>1#</sup>, Jonathan C. Brown<sup>1#</sup>, Niluka Goonawardane<sup>1</sup>, Jie Zhou<sup>1</sup>, Max Whiteley<sup>2</sup>, PHE Virology Consortium<sup>3</sup>, Thushan I. de Silva<sup>2,4</sup>, Wendy S. Barclay<sup>1\*</sup>

<sup>1</sup>Department of Infectious Disease, Imperial College London, UK, W2 1PG

<sup>2</sup>The Florey Institute for Host-Pathogen Interactions and Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield, UK, S10 2RX

<sup>3</sup>Public Health England, UK

<sup>4</sup>South Yorkshire Regional Department of Infection and Tropical Medicine, Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield, UK, S10 2JF

#These authors contributed equally to this work.

\*Corresponding author: tel: +44 (0)20 7594 5035, email: [w.barclay@imperial.ac.uk](mailto:w.barclay@imperial.ac.uk)

## 17 Abstract

18           The spike (S) glycoprotein of the SARS-CoV-2 virus that emerged in 2019 contained a  
19 suboptimal furin cleavage site at the S1/S2 junction with the sequence <sub>681</sub>PRRAR/S<sub>686</sub>. This cleavage  
20 site is required for efficient airway replication, transmission, and pathogenicity of the virus. The  
21 B.1.617 lineage has recently emerged in India, coinciding with substantial disease burden across the  
22 country. Early evidence suggests that B.1.617.2 (a sublineage of B.1.617) is more highly transmissible  
23 than contemporary lineages. B.1.617 and its sublineages contain a constellation of S mutations  
24 including the substitution P681R predicted to further optimise this furin cleavage site. We provide  
25 experimental evidence that virus of the B.1.617 lineage has enhanced S cleavage, that enhanced  
26 processing of an expressed B.1.617 S protein in cells is due to P681R, and that this mutation enables  
27 more efficient cleavage of a peptide mimetic of the B.1.617 S1/S2 cleavage site by recombinant furin.  
28 Together, these data demonstrate viruses in this emerging lineage have enhanced S cleavage by furin  
29 which we hypothesise could be enhancing transmissibility and pathogenicity.

## 30 Introduction

31           Unlike its closest known relatives, the SARS-CoV-2 spike (S) protein contains a furin cleavage  
32 site at the S1/S2 junction that enhances SARS-CoV-2 replication in airway cells and contributes to virus  
33 pathogenicity and transmissibility (1-6). Pre-cleavage of the S protein in producer cells allows SARS-  
34 CoV-2 to enter target cells at the cell surface avoiding endosomal restriction factors (4, 7). However,  
35 the cleavage site of the early SARS-CoV-2 isolates that emerged in late 2019 are suboptimal, leaving  
36 the potential for evolution of variants with increased transmission as a result of an optimised cleavage  
37 site (4).

38           Towards the end of 2020 the SARS-CoV-2 pandemic entered a new phase with repeated  
39 emergence of ‘variants of concern’ lineages with altered viral properties such as transmissibility,  
40 pathogenicity, and antigenicity (8). The most widespread and best characterised of these variants is  
41 the B.1.1.7 lineage, first found in the UK, which has increased transmissibility and pathogenicity

42 compared to other circulating strains (9-11). We and others have previously described that the S1/S2  
43 cleavage site of B.1.1.7 S contains a P681H mutation that enhances post-translational S1/S2 cleavage  
44 during virus budding (12, 13). Other widely circulating variants that arose around the same time  
45 include the B.1.351 and P.1 lineages, first found in South Africa and Brazil, respectively, that show  
46 antigenic escape but do not contain alterations at the furin cleavage site (14). As of May 2021, an  
47 increasing number of variant lineages have been described, one of which is the B.1.617 lineage. The  
48 emergence of this lineage in India coincided with a period of record disease burden across the country,  
49 leading to partial collapse of its health infrastructure (15). Early evidence from the UK suggests one  
50 B.1.617 sublineage (B.1.617.2) likely has enhanced transmissibility, comparable to, or greater than  
51 B.1.1.7 (16). B.1.617 and its sublineages contain several S mutations, some shared with other variants  
52 and associated with antigenic escape (see Table 1). One S substitution shared by all B.1.617  
53 sublineages is P681R which we hypothesise further optimises the furin cleavage site (<sub>681</sub>PRRAR/S<sub>686</sub> to  
54 <sub>681</sub>RRRAR/S<sub>686</sub>, Figure 1a). In this report we characterise the impact of P681R on the S1/S2 cleavage  
55 site.

## 56 Results and discussion

57 To investigate whether the S protein of B.1.617 undergoes a higher degree of post-  
58 translational cleavage at S1/S2 than previously circulating strains, we isolated several B.1.617 lineage  
59 viruses (1 x B.1.617.1 and 2 x B.1.617.2) and compared their S1/S2 cleavage to that of a previously  
60 circulating strain of lineage B.1.238, which contains only D614G. The B.1.617 lineage S proteins were  
61 all more highly cleaved ( $\geq 50\%$  cleaved), with a higher proportion of cleaved S2 and a lower proportion  
62 of full-length S detectable than the control virus ( $\sim 33\%$  cleaved) (Figure 1b, c).

63 To characterise which amino change in the B.1.617 S is responsible for its enhanced cleavage,  
64 we generated pseudovirus containing the SARS-CoV-2 full B.1.617.1 S and compared it to pseudovirus  
65 with D614G spike (WT). As we had previously observed, SARS-CoV-2 spike expressed on pseudovirus  
66 contains a larger proportion of cleaved spike (4). While WT S displayed both full length ( $\sim 20\%$ ) and

67 cleaved (~80%) S, B.1.617.1 S showed significantly enhanced cleavage (~95%), with an almost  
68 complete lack of full-length protein (Figure 1d,e). P681R alone (on a D614G backbone) was sufficient  
69 to convey this phenotype (~96% cleaved), with cleavage enhanced to a similar level as for a previously  
70 described S protein carrying the fully optimised furin cleavage site from an H5N1 avian influenza virus  
71 haemagglutinin (~97% cleaved) (4). This suggests P681R alone is responsible for the enhanced S  
72 cleavage seen in the B.1.617 lineages viruses.

73 We then performed assays to determine whether the optimised cleavage site found in the  
74 B.1.617 S enables better cleavage directly by furin. We measured the ability of recombinant furin to  
75 cleave fluorescently labelled peptides corresponding to the S1/S2 cleavage site of SARS-CoV-2 testing  
76 peptides containing 681P (WT), 681R, or a monobasic mutant (monoCS) whereby two of the arginines  
77 are substituted to non-basic residues (see Figure 1a) (4). As expected, monoCS was poorly cleaved by  
78 recombinant furin compared to the WT peptide which was efficiently cleaved by furin as previously  
79 described (Figure 1f)(2). P681R significantly enhanced the ability of furin to cleave the peptide  
80 confirming that the arginine substitution is responsible for the enhanced cleavage of the B.1.617 S  
81 protein.

82 To conclude, we speculate that enhanced S1/S2 cleavage seen in B.1.617 and B.1.1.7 (which  
83 contains P681H (12)) may be contributing to the enhanced transmissibility of these SARS-CoV-2  
84 variants. As well as B.1.1.7 and B.1.617, several other emerging lineages contain mutations in the furin  
85 cleavage site (8). We would advise that these lineages be kept under close monitoring for any early  
86 evidence of more rapid transmission or higher pathogenesis.

87 Materials and methods

#### 88 **Cells and viruses**

89 Vero E6-ACE2-TMPRSS2 (Glasgow University)(17), were maintained in DMEM, 10% FCS, 1x  
90 non-essential amino acids, 200 µg/ml hygromycin B (Gibco) and 2mg/ml G418 (Gibco). Cells were kept  
91 at 5% CO<sub>2</sub>, 37°C.

92 Upper respiratory tract swabs used to isolate viruses were collected for routine clinical  
93 diagnostic use and sequenced using the ARTIC network protocol (<https://artic.network/ncov-2019>) to  
94 confirm the presence of B.1.617 lineage virus, under approval by the Public Health England Research  
95 Ethics and Governance Group for the COVID-19 Genomics UK consortium (R&D NR0195). Virus was  
96 isolated by inoculating 100 µL of neat swab material onto Vero cells, incubating at 37°C for 1 h before  
97 replacing with growth media supplemented with 1x penicillin/streptomycin and 1x amphotericin B.  
98 Cells were incubated for 5-7 days until cytopathic effect was observed. Isolates were passaged a  
99 further two times in Vero E6-ACE2-TMPRSS2 cells (17), the supernatant clarified by centrifugation and  
100 concentration for western blot analysis viruses by centrifuging in an Amicon® Ultra-15 Centrifugal  
101 Filter Unit followed by an Amicon® Ultra-0.5 Centrifugal Filter Unit with 50 kDa exclusion size.

## 102 **Plasmids and Pseudovirus**

103 The B.1.617.1 plasmid was generated from a previously described codon-optimised SARS-  
104 CoV-2 spike plasmid (Wuhan-hu-1)(18), using the QuikChange Lightning Multi Site-Directed  
105 Mutagenesis kit (Agilent). Pseudovirus was generated and concentrated as previously described (4).  
106 All spike expression plasmids used in this study contain D614G and K1255\*STOP (that results in  
107 deletion of the C-terminal cytoplasmic tail of spike containing the endoplasmic retention signal, aka  
108 the Δ19 spike truncation).

## 109 **Western Blotting**

110 Virus or pseudovirus concentrates were lysed in 4x Laemmli buffer (Bio-rad) with 10% β-  
111 mercaptoethanol and run on SDS-PAGE gels. After semi-dry transfer onto nitrocellulose membrane,  
112 samples were probed with mouse anti-p24 (abcam; ab9071), rabbit anti-SARS spike protein (NOVUS;  
113 NB100-56578), or rabbit anti-SARS-CoV-2 nucleocapsid (SinoBiological; 40143-R019). Near infra-red  
114 (NIR) secondary antibodies, IRDye® 680RD Goat anti-mouse (abcam; ab216776) and IRDye® 800CW  
115 Goat anti-rabbit (abcam; ab216773) were subsequently used to probe membranes. Western blots  
116 were visualised using an Odyssey Imaging System (LI-COR Biosciences).

## 117 **Peptide cleavage assays**

118           The peptide cleavage assay was adapted from the protocol by Jaimes et al (2, 19). Briefly  
119   fluoregenic peptides were synthesised (Cambridge research biochemicals) with the sequences  
120   TNSPRRARSVA (WT), TNSRRRARSVA (P681R) and TNSPSLARSVA (monoCS) and, N-terminally  
121   conjugated with the fluorophore 5-Carboxyfluorescein (FAM) and the C-terminal quencher 2,4-  
122   Dinitrophenyl.

123           Each peptide was tested for its ability to be cleaved by recombinant furin (10 U/mL; NEB;  
124   P8077) in a buffer of 100 mM HEPES, 0.5% Triton X-100, 1mM CaCl<sub>2</sub>, 1 mM β-mercaptoethanol, pH  
125   7.5. Assays were performed in triplicate at 30°C and fluorescence intensity was measured at  
126   wavelengths of 485 and 540 nm every 1 minute for 1 hour using a FLUOstar Omega plate reader (BMG  
127   Labtech). Vmax was then calculated.

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184

185 Tables

186 **Table 1. Spike Mutational profiles of B.1.617 sublineages and B.1.1.7**

Lineage	Spike mutations
B.1.617.1	T95I*, G142D, E154K, L452R, E484Q, D614G, P681R, Q1071H
B.1.617.2	T19R, G142D, Δ156-157/R158G, L452R, T478K, D614G, P681R, D950N
B.1.617.3	T19R, Δ156-157/R158G, L452R, E484Q, D614G, P681R, D950N
B.1.1.7	Δ69-70, Δ144, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H

187 \*Mutation found in most, but not all isolates of this sublineage

188 Figure legends

189 **Figure 1 – P681R results in enhanced furin cleavage of the SARS-CoV-2 B.1.617 spike protein.**

190 (a) Primary sequences of SARS-CoV-2 S1/S2 cleavage sites used throughout this study. Basic  
191 residues shown in bold and red, changes from 'WT' shown in italics. Numbers indicate spike  
192 residues in primary sequence (equivalent to Wuhan-Hu-1 reference sequence).

193 (b) Western blot analysis of spike cleavage of concentrated B.1.238 (D614G) and B.1.617 (P681R  
194 containing) SARS-CoV-2 isolates. Levels of nucleocapsid (N) protein shown as loading control.

195 (c) Densitometry analysis of the western blot from part (b). Densitometry measured using  
196 ImageJ. Points indicate two technical repeats from the same concentrated virus stocks.

197 (d) Western blot analysis of concentrated pseudovirus containing different SARS-CoV-2 spike  
198 mutants. Levels of lentiviral p24 antigen shown as loading control. Representative blot shown  
199 of N=3 independent repeats.

200 (e) Densitometry analysis of pseudovirus spike cleavage (from part d). Each dot indicates one  
201 completely independently produced and concentrated pseudovirus preparation (N=3). Data  
202 plotted as mean with individual repeats shown. The band corresponding to uncleaved Spike



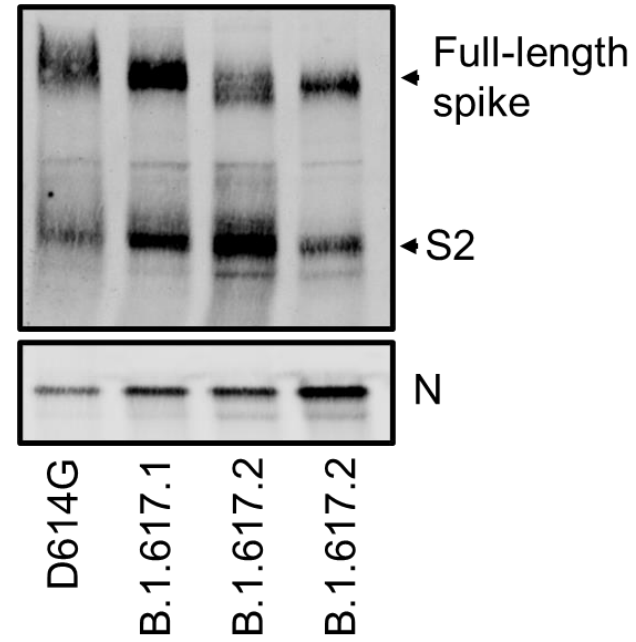
203 was determined by comparing the size to  $\Delta$ CS which is unable to be cleaved by furin as  
204 previously described (4). Statistics performed with one-way ANOVA with multiple  
205 comparisons against the WT. \*\*\*\* $P \leq 0.0001$ .

206 (f) Cleavage of SARS-CoV-2 spike S1/S2 fluorogenic peptide mimetics by recombinant furin.  
207 Plotted as maximum enzymatic activity ( $V_{max}$ ). All assays performed in technical triplicate  
208 (N=3) with a representative repeat from three completely independent repeats (N=3) shown.  
209 Graph plotted as mean + Standard deviation. One-way ANOVA with multiple comparisons  
210 against the WT plotted on the graph. \*\* $0.01 \geq P > 0.001$ ; \*\*\*\* $P \leq 0.0001$ .

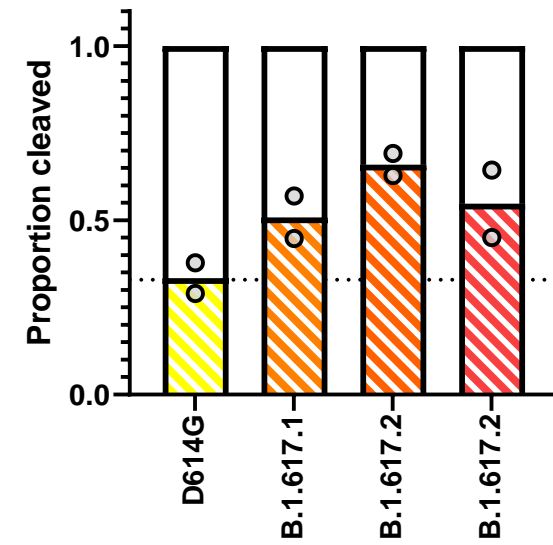
a

		680												690						
SARS-CoV-2	WT	Q	T	N	S	P	R	R	A	R	/	S	V	A	S	Q				
SARS-CoV-2	B.1.617	Q	T	N	S	R	R	R	A	R	/	S	V	A	S	Q				
SARS-CoV-2	B.1.1.7	Q	T	N	S	H	R	R	A	R	/	S	V	A	S	Q				
SARS-CoV-2	MonoCS	Q	T	N	S	P	S	L	A	R	/	S	V	A	S	Q				
SARS-CoV-2	$\Delta$ CS	Q	T	-	-	-	-	-	-	-	/	-	I	A	S	Q				
SARS-CoV-2	H5CS	Q	T	N	S	P	R	E	R	R	R	K	K	R	/	S	V	A	S	Q

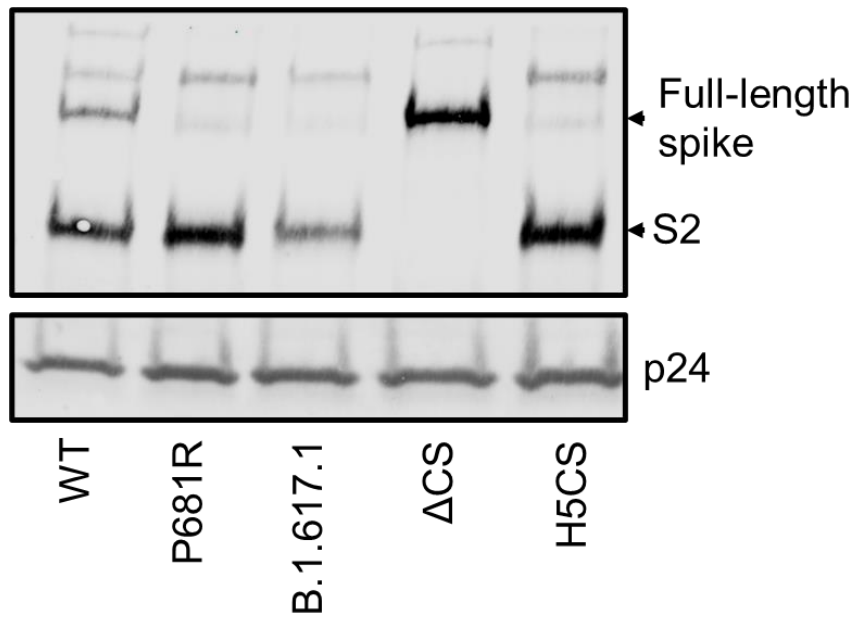
b



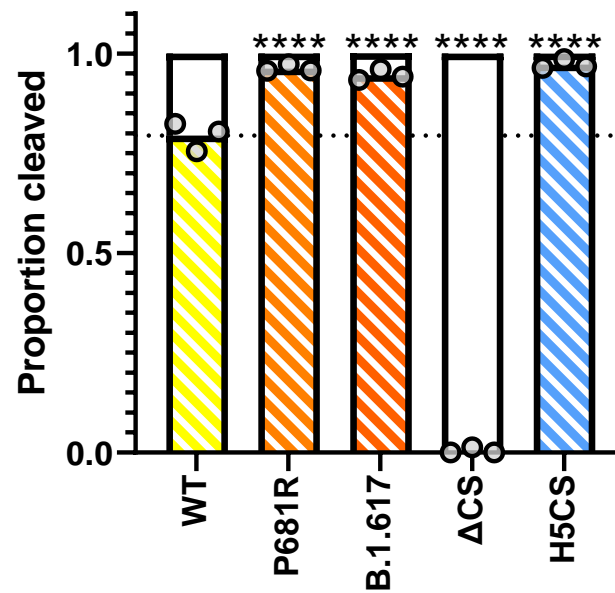
c



d



e



f

