

1 **SARS-CoV-2 variants of concern Alpha, Beta, Gamma and Delta have extended ACE2**
2 **receptor host-ranges**

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21 **Abstract**

22 Following the emergence of SARS-CoV-2 in China in late 2019 a number of variants have
23 emerged, with two of these – Alpha and Delta – subsequently growing to global prevalence.
24 One characteristic of these variants are changes within the Spike protein, in particular the
25 receptor binding domain (RBD). From a public health perspective these changes have
26 important implications for increased transmissibility and immune escape; however, their
27 presence could also modify the intrinsic host-range of the virus. Using viral pseudotyping we
28 examined whether the variants of concern (VOCs) Alpha, Beta, Gamma and Delta have
29 differing host ACE2 receptor usage patterns, focusing on a range of relevant mammalian

30 ACE2 proteins. All four VOCs were able to overcome a previous restriction for mouse ACE2,
31 with demonstrable differences also seen for individual VOCs with rat, ferret or civet ACE2
32 receptors, changes which we subsequently attribute to N501Y and E484K substitutions within
33 the Spike RBD.

34

35 **Main text**

36 SARS-CoV-2, the β -coronavirus responsible for the Covid-19 pandemic, is thought to have
37 emerged from a bat reservoir, potentially via an as yet unidentified intermediate mammalian
38 host. These conclusions are supported by knowledge of the origins of SARS-CoV-1 as well
39 as the sequence data of related viruses isolated in bats e.g., the RaTG13 isolate [1]. Recently,
40 Temmam et al., identified sarbecoviruses in bats that are nearly identical within the Spike
41 RBD, which adds further weight to this conclusion and highlights that coronaviruses with high
42 human ACE2 affinity are actively circulating in wild reservoir populations [2]. Interestingly,
43 experimental infection of ferrets, mice, bats, primates and other animals, together with natural
44 infections in a range of species including cats, dogs and mink indicate these viruses may have
45 a broader host-range than bats and humans [3-6]. Understanding SARS-CoV-2 infection in
46 animals is important for three main reasons. Firstly, to assess the direct risk to livestock,
47 companion animals and wildlife. Secondly, to examine whether these animals can act as
48 secondary reservoirs for SARS-CoV-2. And lastly, to help establish and validate animal
49 models for Covid-19 which can then be used for the development of therapeutics and
50 vaccines, and to better understand the mechanisms of viral pathogenesis. To support these
51 research endeavours, we and others have shown that SARS-CoV-2 Spike has a broad tropism
52 for mammalian ACE2 proteins [7-9]. Using lentiviral pseudotyping combined with live virus
53 experiments we showed that SARS-CoV-2 can use a wide range of mammalian ACE2s
54 including dog, cat, cattle, sheep, pangolin and rabbit, but is restricted with rat, ferret and a
55 subset of bat and bird receptors [7, 10].

56 Importantly, the continued evolution of SARS-CoV-2 in human populations has led to the
57 emergence of variants, a natural result of RNA virus replication. Informed by epidemiological,
58 virological and immunological data, public health bodies such as the World Health
59 Organisation (WHO) and Public Health England (PHE) have assigned some as variants of
60 concern (VOCs). Over the course of the pandemic two of these VOCs (B.1.1.7 [Alpha] and
61 B.1.617.2 [Delta]) have independently risen to prominence, rapidly replacing the previously
62 circulating strain across multiple regions (G614) [11]. The mechanisms underpinning this
63 replacement appear to correlate with continued evolution to the human host, through
64 increases in particle infectivity, replicative capacity, transmission potential, innate immune

65 antagonistic properties and potentially immune escape [12-16]. However, whether these
66 variants have expanded host-ranges and the consequences for the ongoing pandemic, in
67 terms of the factors described above, are not well characterised.

68 Using a lentiviral (HIV-1) pseudotyping approach for SARS-CoV-2 (described previously, [7,
69 17]) we investigated whether the Spike from four different VOCs (Alpha [Spike mutation profile:
70 L18F, Δ69-70, Δ144, N501Y, A570D, D614G, P681H, T716I, S928A, D1118H], Beta [L18F,
71 D80A, D215G, Δ242-244, K417N, E484K, N501Y, D614G, A701V], Gamma [L18F, T20N,
72 P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I, V1176F] and Delta
73 [T19R, G142D, Δ156-157, R158G, L452R, T478K, D614G, P681R, D950N]; Figure 1 A-D; left
74 panel) had altered ACE2 receptor usage across multiple hosts, when compared to a sequence
75 derived earlier in the pandemic (WT/ D614/ Wuhan). Expression constructs for the various
76 Spike proteins were constructed in a background lacking the final 19 amino acids of the C-
77 terminal cytoplasmic tail (Δ19; equivalent to K1255*STOP) to enhance spike incorporation and
78 facilitate increased pseudotyping efficiency, either by site directed mutagenesis or gene
79 synthesis [18]. In addition to human ACE2, other host ACE2s were chosen based on their
80 relevance to previous sarbecovirus emergence (civet), their continued use as animal models
81 for SARS-CoV-2 pathogenesis and transmission studies (ferret, hamster, mouse), as well as
82 a perceived potential to act as a reservoir for human-derived viruses (rat [through excreted
83 virus in sewage], pig [established reservoir for Nipah and influenza]). Briefly, pseudotyped
84 virus for WT D614 and the four VOCs was generated in HEK293s and used to infect BHK-21s
85 over-expressing the various ACE2s. Luciferase activity, a quantitative read-out proportional to
86 pseudo-viral entry, was then assayed at 2 days post-infection. These values were then
87 normalised based on the results of product-enhanced reverse transcriptase (PERT) assays
88 for each pseudovirus preparation, a method used to standardise results when comparing
89 different preparations of lentiviral pseudotypes. Experiments were repeated three times and
90 the data for each VOC collated and compared to WT D614 (Figure 1). For human ACE2, the
91 only statistically significant difference (t-test) in entry was observed for the Beta VOC, which
92 showed a small increase when compared to WT D614 (Figure 1B). Usage of mouse ACE2 by
93 all four VOCs was significantly increased when compared to WT D614 (Figure 1 A-D), in
94 keeping with previous findings that early SARS-CoV-2 isolates were restricted with this
95 specific receptor [7]. Of note, the increase in mouse ACE2 usage was less notable for Delta,
96 the only non-N501Y-containing variant in our study. Similarly, the SARS-CoV-2 Spike – rat
97 ACE2 restriction we reported previously [7] was only overcome by N501Y-containing VOC
98 Spikes (Alpha, Beta, Gamma), with no significant increase seen with Delta (Figure 1). For
99 civet ACE2 significant increases in entry were observed only with the Beta VOC, while for
100 ferret ACE2 Beta and Gamma were both significantly higher than WT D614. Interestingly, no

101 significant differences were observed when hamster or pig ACE2 were used as receptors
102 although in our 2020 study neither of these receptors were restrictive to early isolates of
103 SARS-CoV-2 entry, unlike rat, mouse, civet and ferret ACE2 [7]. Analysis of the same data on
104 radar plots highlights the extended host-range of the N501Y-containing VOCs (Alpha, Beta
105 and Gamma), while simultaneously demonstrating the similar host-range profiles of WT D614
106 and Delta (Figure 1 A-D right panels).

107 To examine the role of individual amino acid changes in Spike in overcoming host-range
108 restrictions we subsequently focused on the mouse, rat and civet ACE2 interactions. Plasmids
109 expressing SARS-CoV-2 Spike mutations found in various VOCs were constructed in a WT
110 $\Delta 19$ background and used to generate pseudotyped viruses. Subsequent infection of mouse
111 ACE2 expressing cells confirmed that a single N501Y change was sufficient to overcome the
112 WT D614 restriction with this receptor, allowing entry equivalent to the Alpha VOC (Figure
113 2A). Changes to the furin cleavage site (P681H, found in Alpha) or deletions in the N-terminal
114 domain (NTD; $\Delta 69-70$, $\Delta 144$), however, appeared to have little appreciable effect on entry,
115 with similar results being observed for rat ACE2 (Figure 2B). Of note the $\Delta 69-70$ mutations
116 were performed in a N501Y-containing background, which could potentially obscure
117 synergistic effects between these two mutations. Interestingly for civet ACE2, the N501Y or
118 K417N changes were inhibitory, with E484K, in contrast, increasing entry efficiency of the
119 respective SARS-CoV-2 pseudotype (Figure 2C). The small, but repeatable, increase in civet
120 ACE2 usage with the Beta VOC Spike (Figure 1B) may therefore be a combinatorial effect of
121 these mutations, with E484K compensating for the inhibitory properties of the 501 and 417-
122 specific changes. Accordingly, an Alpha + E484K Spike-based pseudotype was able to rescue
123 the defect in civet ACE2 usage seen with Alpha alone (Figure 2C; left panel).

124 The continuing evolution of SARS-CoV-2 in human populations raises significant concerns.
125 Principally, these relate to the human pandemic and human disease, e.g., antigenic escape
126 from vaccine- or natural infection-derived immunity, or the acquisition of enhanced
127 transmission potential or increased pathogenicity. Separately, however, the question of
128 whether SARS-CoV-2 will develop enhanced reverse zoonotic potential is also important. The
129 emergence of both the Alpha and Delta VOCs provides clear evidence that a relatively small
130 number of changes within the genome can have far reaching epidemiological consequences
131 for the human pandemic. To our knowledge these VOCs have, however, not yet been linked
132 to any significant increase in spill-over back into livestock, companion animals or wildlife; for
133 which there may be various interpretations. This may reflect a lack of sampling, improved
134 understanding and enhanced biosecurity at the human-animal interface, a consistently low
135 frequency of spill-over or simply the absence of any dramatic change in disease phenotype
136 following infection of animals with VOCs.

137 Regarding infection, our data show that from an ACE2 receptor usage perspective the host-
138 range of Alpha, Beta and Gamma (and to a lesser extent Delta) is broader than the virus that
139 initially emerged in China (WT D614). Several host ACE2 proteins that were previously
140 refractory to viral entry (mouse, rat and ferret) are used more efficiently by VOC Spikes (Figure
141 1). This change in tropism is attributable to specific amino acid changes in the RBD of Spike,
142 in particular N501Y (Figure 2). This is consistent with previous work showing amino acid
143 substitutions within the RBD overcome restriction with murine ACE2. These mutations were
144 identified by structure-led approaches [19] or following passage of SARS-CoV-2 directly in
145 mice [20], results which have been subsequently confirmed *in vitro* [21, 22]. Although these
146 changes are not identical, they fit within an overall pattern that small adaptations in Spike are
147 needed to overcome specific incompatibilities between the viral attachment protein and non-
148 cognate receptors. We observed a similar trend during adaptation of SARS-CoV-2 in ferrets,
149 with Spike mutations Y453F, F486L and N501T leading to increased ferret ACE2 usage [10].
150 This mirrors the situation with natural infections in mink, a related mustelid, where a similar
151 array of mutations has repeatedly arisen [23, 24] leading to severe restrictions on the farming
152 of mink and the mass culling of animals. Importantly, these ferret or mink acquired mutations
153 are unlikely to pose an increased threat to human populations as these changes either inhibit
154 human ACE2 usage (Y453F) and/or have little antigenic impact [10].

155 Whether SARS-CoV-2 VOCs are more pathogenic in rodents is an area of ongoing
156 investigation. Montagutelli et al., showed that the Beta and Gamma VOCs replicated to higher
157 titres than a B.1 virus in the lungs of young adult mice [25] with Horspool et al., showing similar
158 results in K18-human ACE2 transgenic mice with Alpha and Beta VOC infected animals
159 showing higher cumulative clinical scores when compared to the first wave WA-1 strain [26].
160 Similar results were also reported by Shuai et al in mice and rats [27]. This has contributed to
161 speculation that rodents could play an important role in the transmission of SARS-CoV-2 to
162 people [28]; however, these data also have important ramifications beyond reverse zoonosis.
163 For example, significant weight is given to animal model data on SARS-CoV-2 pathogenicity
164 during antiviral screening or vaccine development [6, 29]. If, for example, N501Y containing
165 VOCs cause increased pathogenicity because they are now able to use mouse ACE2 more
166 efficiently, this could complicate or potentially invalidate comparisons between WT D614 and
167 VOC-based mouse data and more broadly correlations with human data, where the difference
168 in human ACE2 usage is marginal. In addition, those animal models that endogenously
169 express restricted ACE2s (ferret, mouse and rat) are likely to drive in-animal adaptation, as
170 we witnessed with ferrets [10], which further complicates conclusions on viral phenotypes such
171 as transmission potential. Fortunately, in hamsters which have emerged as a highly tractable

172 model for SARS-CoV-2, the situation appears less convoluted as differences in hamster and
173 human ACE2 usage between WT D614 and VOC pseudotypes look comparable (Figure 1).

174 While in ferrets and mice the increased capacity for SARS-CoV-2 VOCs and other variants to
175 replicate in these hosts correlates with changes in the Spike protein, it is difficult to make a
176 broader set of conclusions. Why certain hosts develop disease and others don't and how this
177 could impact the Covid-19 pandemic is still a relatively unanswered question. A relevant
178 example is pigs, which we have established encode a cognate receptor for SARS-CoV-2 entry
179 (Figure 1). Pigs have proven time and again to be an adequate reservoir for human tropic
180 viruses (e.g. influenza and Nipah), yet are apparently refractory to SARS-CoV-2 infection [30,
181 31]. Recently, we demonstrated tissue-specific differences in ACE2 expression between
182 various animals, e.g. ACE2 was present in the nasal mucosa epithelium of *Eptesicus serotinus*
183 (serotine bat) but not in pigs (*Sus scrofa domestica*) [32] which may provide some mechanistic
184 insight into the varying susceptibility of hosts to SARS-CoV-2. Beyond entry, various species-
185 specific restrictions, for example at the level of the innate immunity response, may also be
186 playing a role.

187 Nevertheless, the fundamental question that remains is whether existing or emerging SARS-
188 CoV-2 variants can establish themselves in an animal reservoir in a way that is consequential
189 to management of the human pandemic. Aside from mink infections, where the virus likely had
190 to adapt in order to use the mustelid receptor, other relevant examples include spill-over and
191 onward transmission in free-living and captive white tailed deer [33] as well as emerging
192 evidence of cryptic SARS-CoV-2 lineages in wastewater that may be linked to establishment
193 of a rodent reservoir [34]. Whilst this evidence and that of human-companion animal
194 transmission, e.g. for cats and dogs [35], seems strong the reverse scenario, i.e. infection of
195 humans by SARS-CoV-2 infected animals appears much less common, albeit not totally
196 absent [23, 24]. The conceivable 'worst case scenario' for SARS-CoV-2 reverse zoonosis is
197 that the virus establishes itself in a new reservoir and at such a level that antibody selection
198 pressure takes place and/or prolonged antigenic drift leading to escape mutants that are
199 relevant to immune human populations. Our previous data indicated that SARS-CoV-2 was a
200 'generalist' with a broad host-range [7]. Significantly, the VOCs have even broader host ranges
201 and, in the majority of cases, the amino acid changes involved e.g., N501Y, which enhances
202 human ACE2 binding (providing evidence of ongoing human adaptation), do not lead to a loss
203 of activity with other host ACE2s. In other words, the 'generalist' nature of SARS-CoV-2 is
204 being maintained and extended over time. Whether this trend will continue and whether this
205 increases the probability of human-relevant reverse zoonosis events remains to be
206 determined.

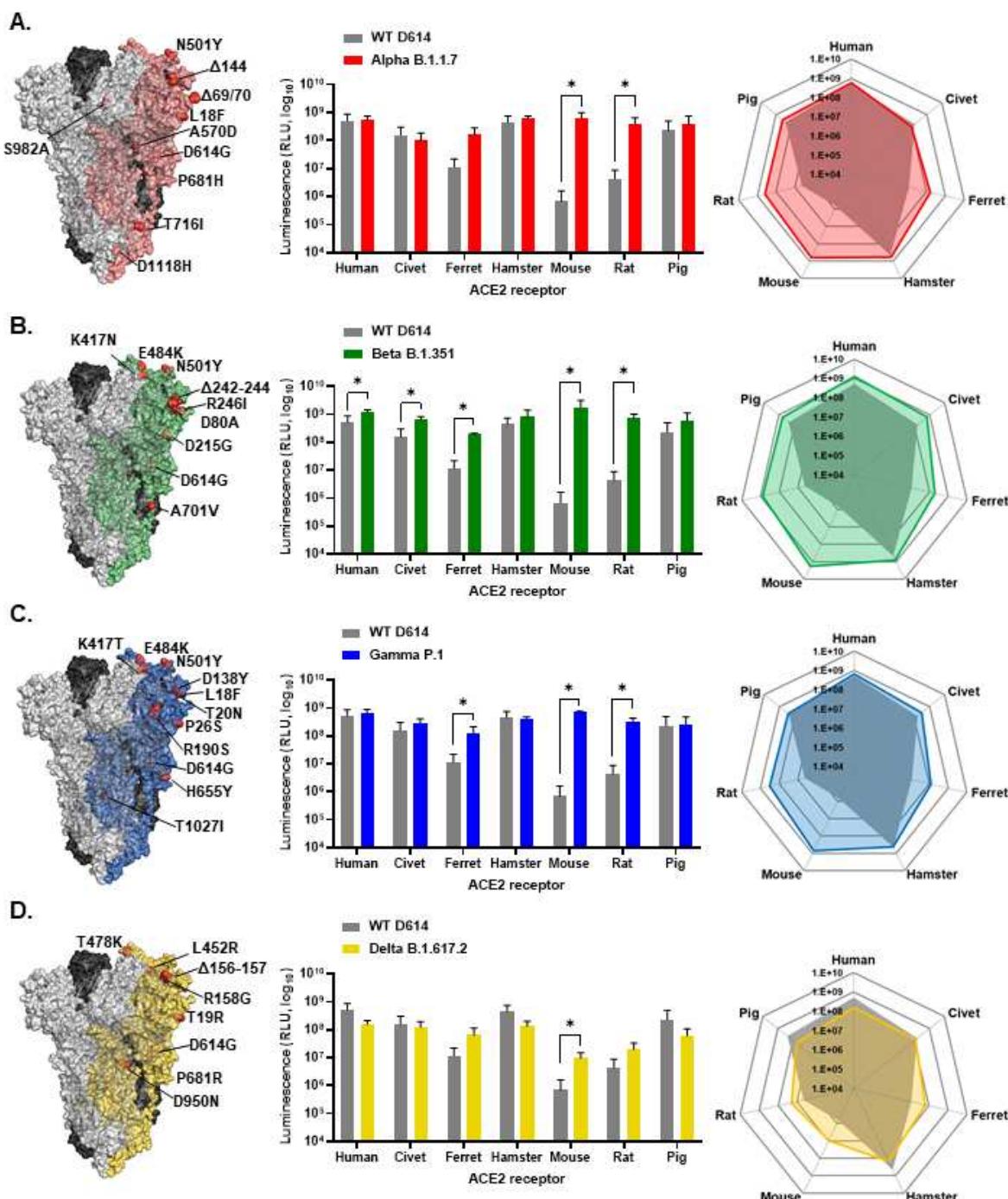
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214 **Conflict of interest:**

215 The authors have no conflicts of interest to declare.

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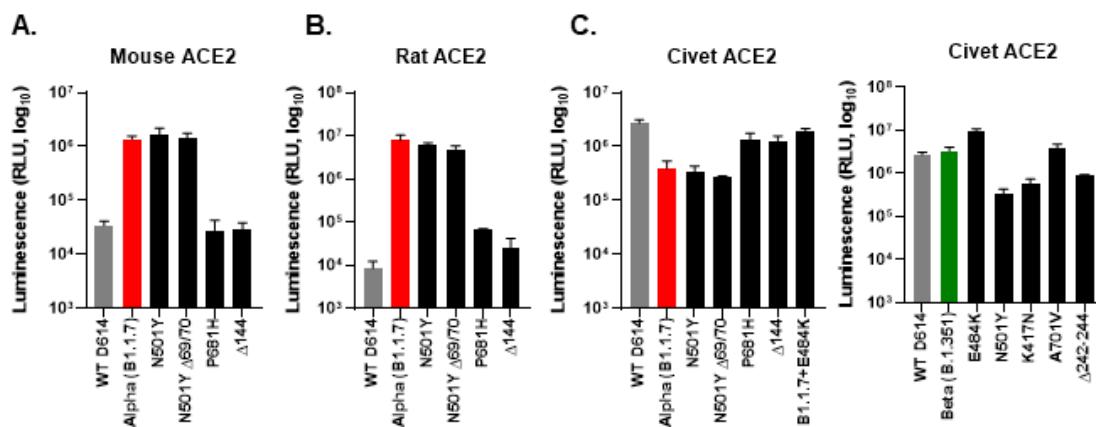


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218 **Figure 1: ACE2 receptor usage screening of SARS-CoV-2 variants of concern (VOC)**

219 **Spike proteins.** Structural maps and receptor usage analysis are shown for SARS-CoV-2
 220 VOCs (A) Alpha (B.1.1.7), (B) Beta (B.1.351), (C) Gamma (P.1) and (D) Delta (B.1.617.2),
 221 compared to WT D614 (Wuhan). **Left panels:** Amino acid differences between the VOC and
 222 WT D614 were highlighted on a trimeric surface structure of SARS-CoV-2 Spike using Pymol
 223 (PDB: 6XM4). Spike monomers are coloured according to the indicated VOC; red (Alpha),

224 green (Beta), blue (Gamma), yellow (Delta), with substitutions identified on the coloured
225 structure only. **Middle panels:** Receptor usage was screened using HIV-1 lentiviral
226 pseudotypes bearing the indicated Spike proteins with HEK293 target cells expressing the
227 indicated ACE2 protein. Viral entry was measured by assaying luciferase activity (RLU) using
228 the BrightGlo reagent (Promega). **Right panels:** The same data was replotted on radar plots
229 to illustrate the broadening host-range of the Alpha (red), Beta (green) and Gamma (blue)
230 VOCs, as opposed to Delta (yellow). In each plot the signal for WT D614 is provided in grey
231 (transparent polygon). For entry assays, BHK-21 cells were plated at 5×10^5 /well in a 6-well
232 dish and transfected with the indicated ACE2 expression plasmids (human, pig, rat, hamster,
233 ferret, civet, mouse) 24 hours later. The next day, cells were harvested using 2mM EDTA-PBS
234 and diluted to 2×10^5 /ml with 100 μ L being seeded into white-bottomed 96-well plates and
235 incubated for 24 hrs at 37°C, 5% CO₂. The media was removed from plated cells and infected
236 with 100 μ L of the indicated pseudotyped virus and incubated for 48 hours. Firefly luciferase
237 was quantified on a GloMax Multi+ Detection System using Bright-Glo (Promega). Individual
238 experiments were performed in biological triplicates. These experiments were then repeated
239 on three separate occasions (on different days). PERT-standardised RLFs were normalised
240 between experiments based on the average RLU calculated across the whole plate, with the
241 mean RLU for each condition then calculated and plotted. To facilitate interpretation and
242 comparison of the dataset the same WT D614 data has been plotted for each VOC. Statistical
243 significance was measured by Student's t-test.



244
245 **Figure 2: Mutational analysis of the RBD identifies N501Y as a critical amino acid for**
246 **extending the host-range of SARS-CoV-2 Spike to rodents.** The entry efficiency of
247 pseudotypes bearing SARS-CoV-2 Spikes with individual or combinations of mutations found
248 in the Alpha and Beta VOCs was assayed in mouse (A), rat (B) or civet (C) ACE2 expressing
249 cells and compared to Alpha, Beta or WT D614. Viral entry was measured by assaying
250 luciferase activity (RLU) using the BrightGlo reagent (Promega). Individual experiments were

251 performed in biological triplicate, with the mean RLU for each condition then calculated and
252 plotted.

253

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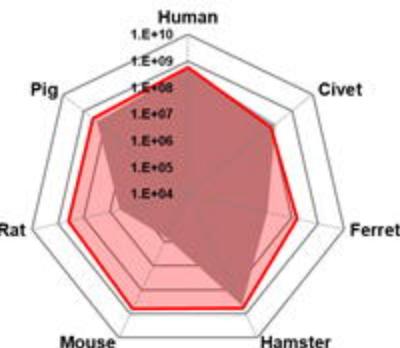
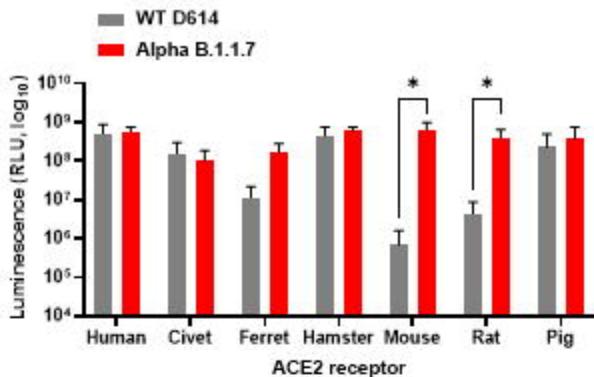
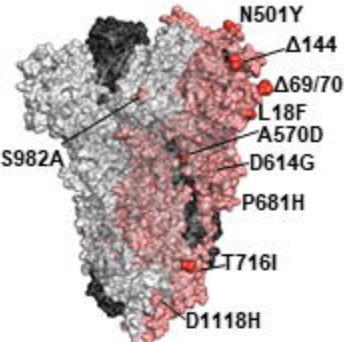
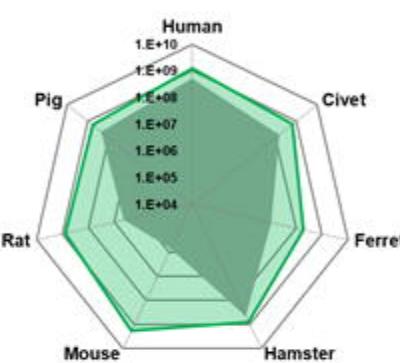
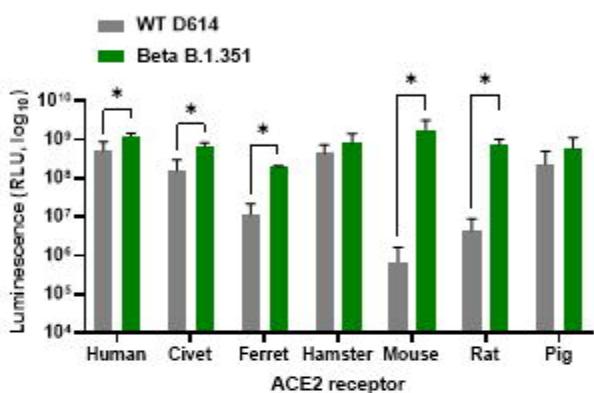
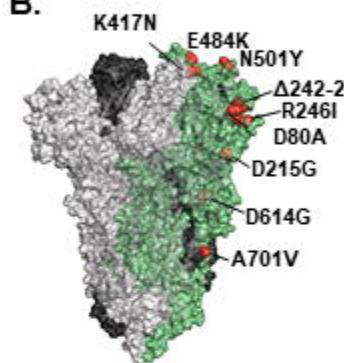
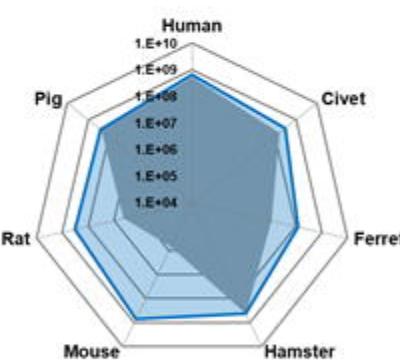
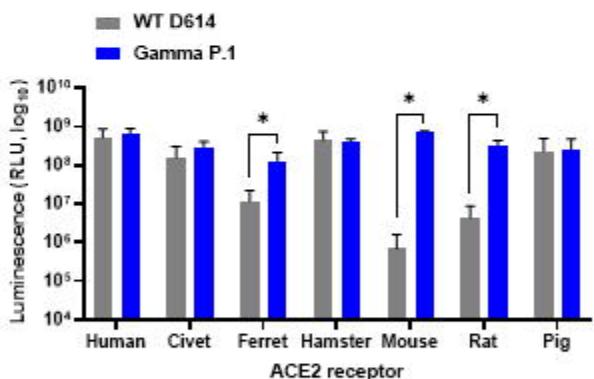
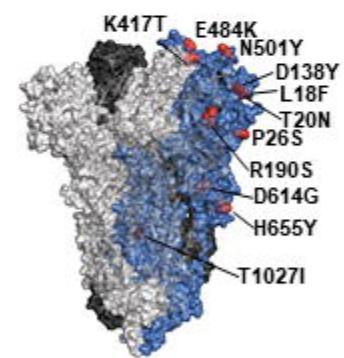
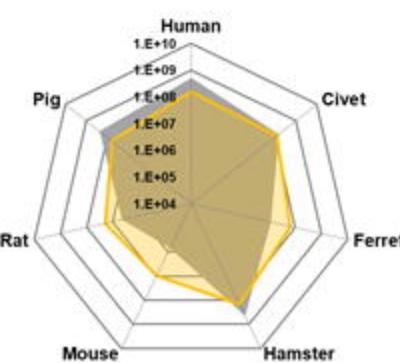
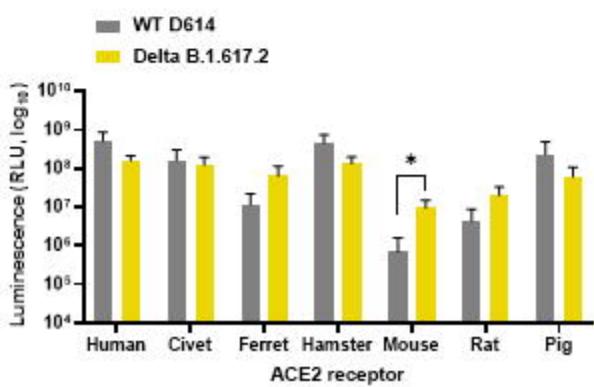
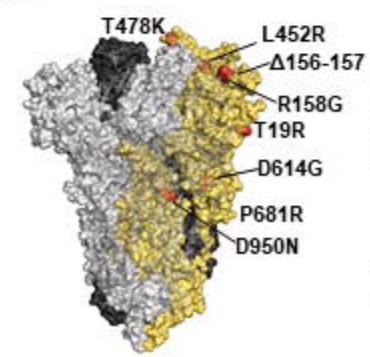
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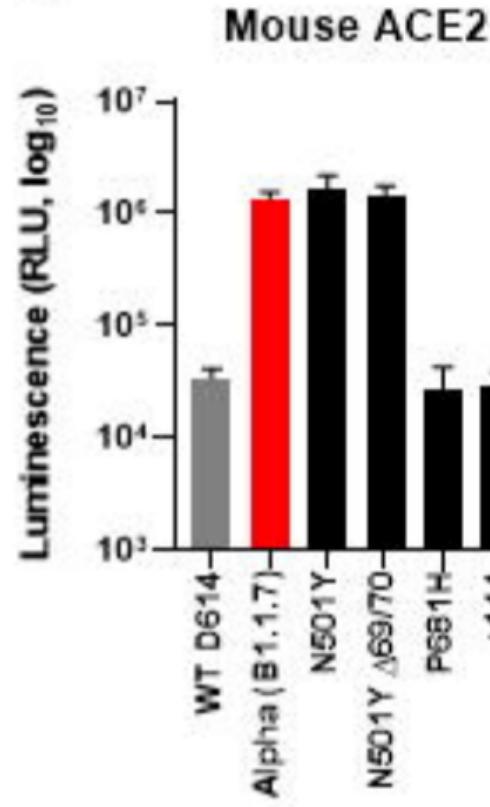
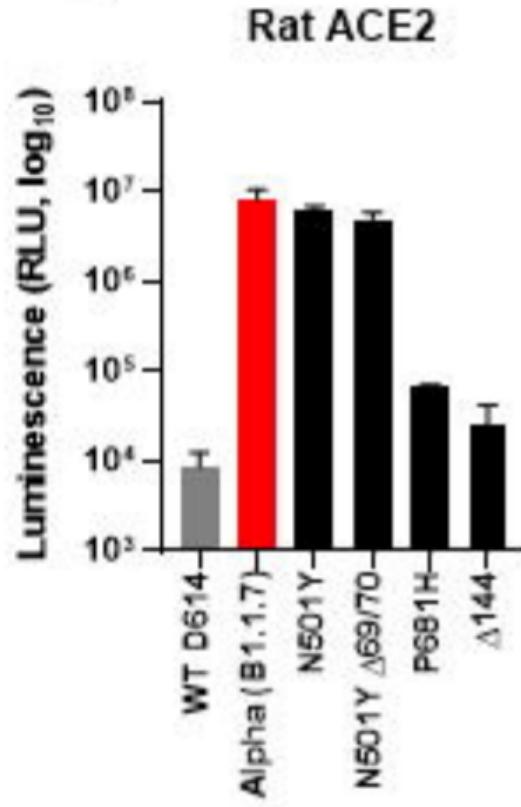
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A.**B.****C.**