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

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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 Δ19 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; Δ69-70, Δ144), however, appeared to have little appreciable effect on entry,
115 with similar results being observed for rat ACE2 (Figure 2B). Of note the Δ69-70 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

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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
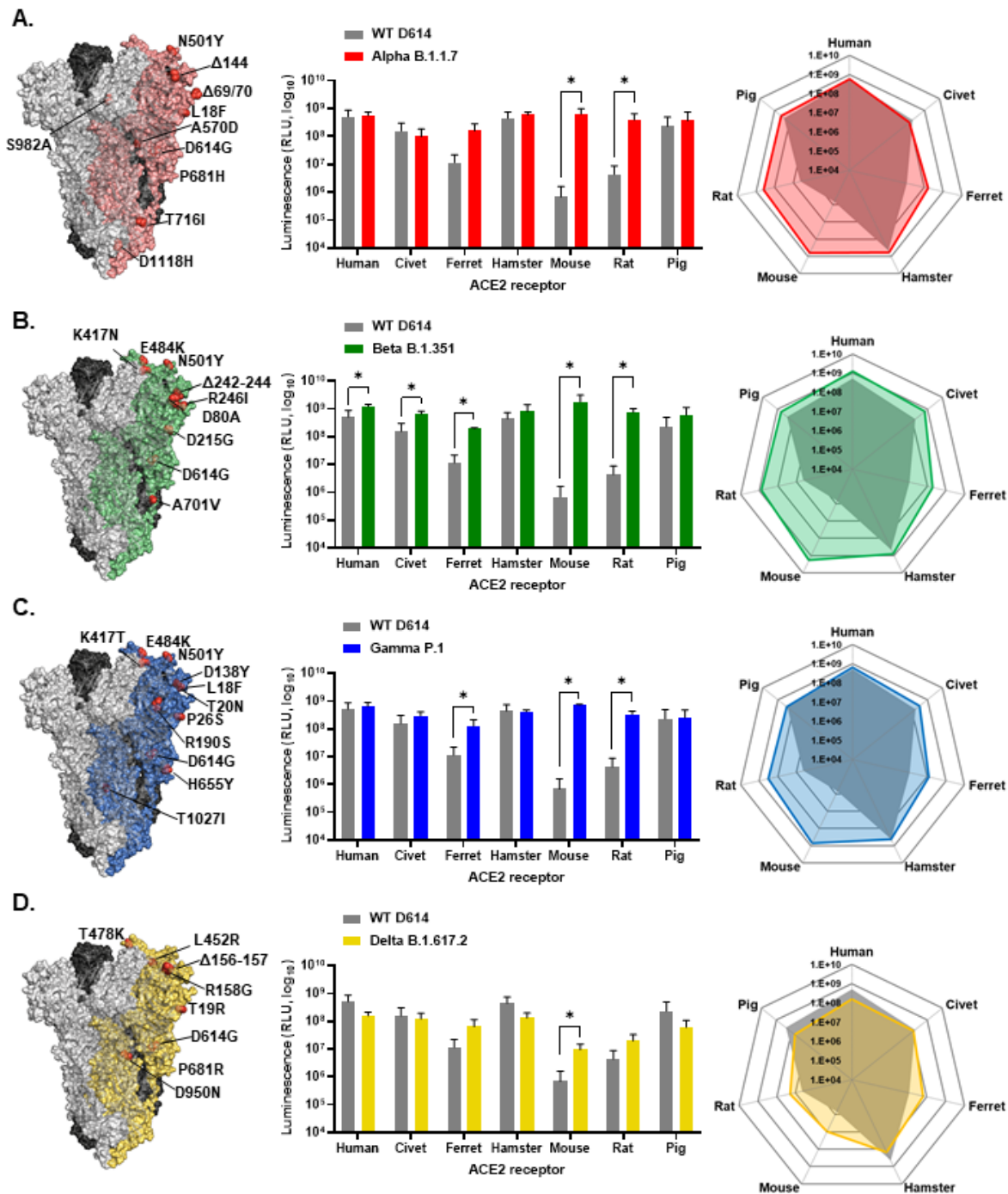
206   determined.

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Conflict of interest:

The authors have no conflicts of interest to declare.

**Figure 1: ACE2 receptor usage screening of SARS-CoV-2 variants of concern (VOC) Spike proteins.** Structural maps and receptor usage analysis are shown for SARS-CoV-2 VOCs (A) Alpha (B.1.1.7), (B) Beta (B.1.351), (C) Gamma (P.1) and (D) Delta (B.1.617.2), compared to WT D614 (Wuhan). **Left panels:** Amino acid differences between the VOC and WT D614 were highlighted on a trimeric surface structure of SARS-CoV-2 Spike using Pymol (PDB: 6XM4). Spike monomers are coloured according to the indicated VOC; red (Alpha),

green (Beta), blue (Gamma), yellow (Delta), with substitutions identified on the coloured structure only. **Middle panels:** Receptor usage was screened using HIV-1 lentiviral pseudotypes bearing the indicated Spike proteins with HEK293 target cells expressing the indicated ACE2 protein. Viral entry was measured by assaying luciferase activity (RLU) using the BrightGlo reagent (Promega). **Right panels:** The same data was replotted on radar plots to illustrate the broadening host-range of the Alpha (red), Beta (green) and Gamma (blue) VOCs, as opposed to Delta (yellow). In each plot the signal for WT D614 is provided in grey (transparent polygon). For entry assays, BHK-21 cells were plated at 5×10^5/well in a 6-well dish and transfected with the indicated ACE2 expression plasmids (human, pig, rat, hamster, ferret, civet, mouse) 24 hours later. The next day, cells were harvested using 2mM EDTA-PBS and diluted to 2×10^5/ml with 100μL being seeded into white-bottomed 96-well plates and incubated for 24 hrs at 37oC, 5% CO2. The media was removed from plated cells and infected with 100μl of the indicated pseudotyped virus and incubated for 48 hours. Firefly luciferase was quantified on a GloMax Multi+ Detection System using Bright-Glo (Promega). Individual experiments were performed in biological triplicates. These experiments were then repeated on three separate occasions (on different days). PERT-standardised RLUs were normalised between experiments based on the average RLU calculated across the whole plate, with the mean RLU for each condition then calculated and plotted. To facilitate interpretation and comparison of the dataset the same WT D614 data has been plotted for each VOC. Statistical significance was measure by students t-test.
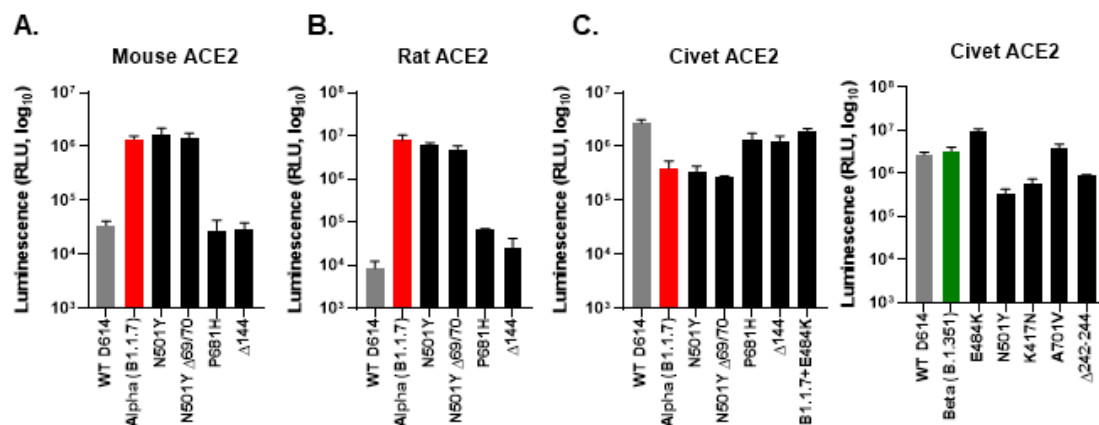
Figure 2: Mutational analysis of the RBD identifies N501Y as a critical amino acid for extending the host-range of SARS-CoV-2 Spike to rodents. The entry efficiency of pseudotypes bearing SARS-CoV-2 Spikes with individual or combinations of mutations found in the Alpha and Beta VOCs was assayed in mouse (A), rat (B) or civet (C) ACE2 expressing cells and compared to Alpha, Beta or WT D614. Viral entry was measured by assaying luciferase activity (RLU) using the BrightGlo reagent (Promega). Individual experiments were

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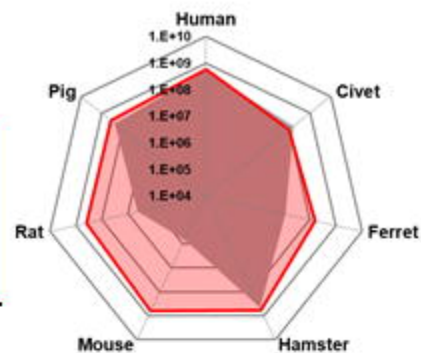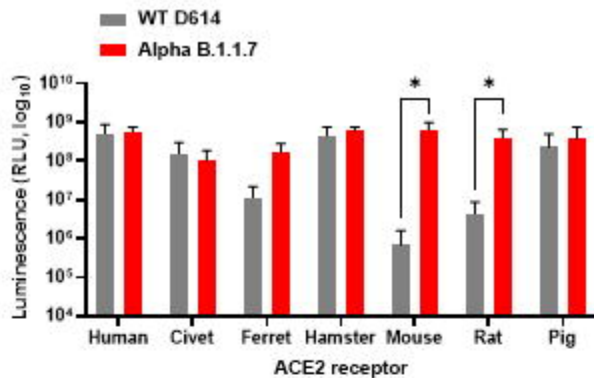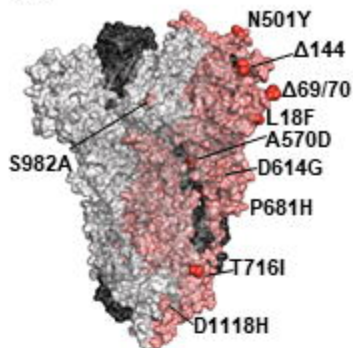
252   plotted.

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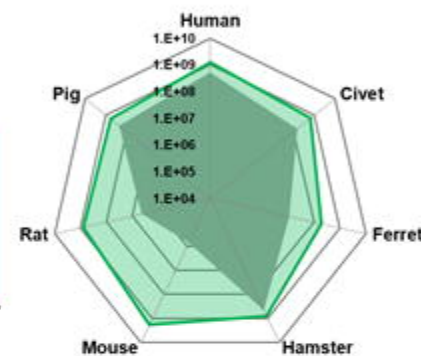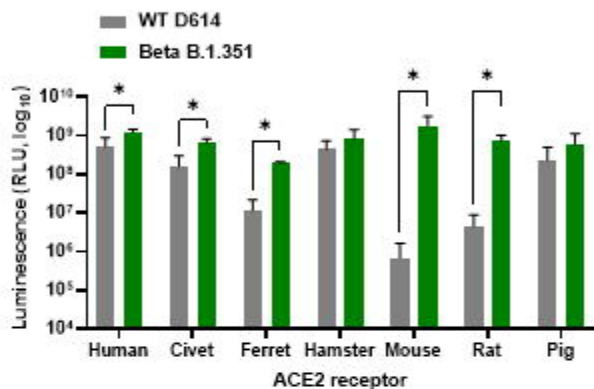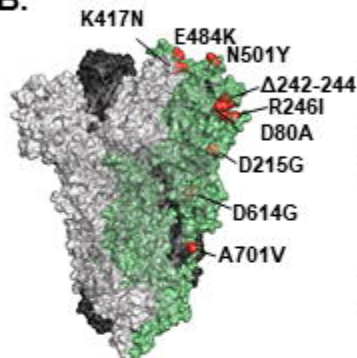298   vaccine efficacy. *Science* 2020;369(6511):1603-1607.

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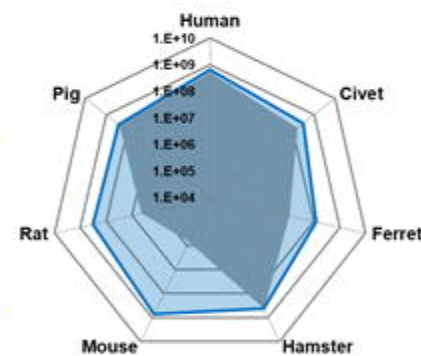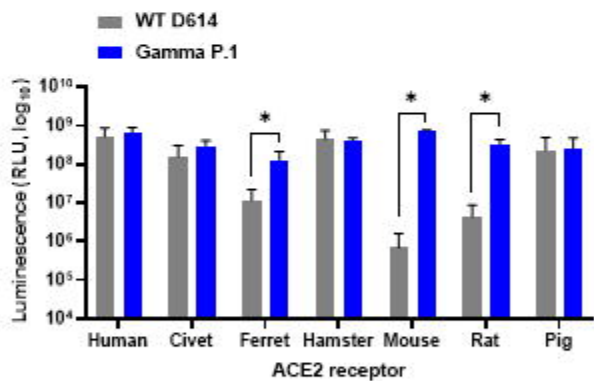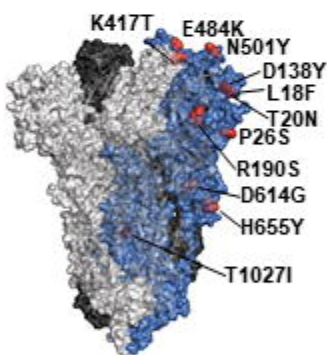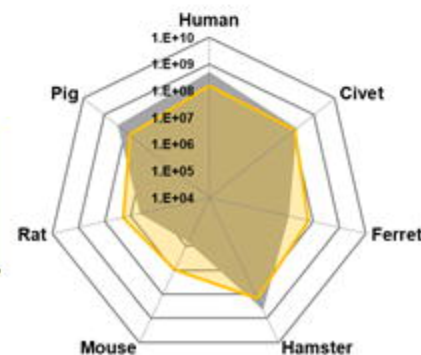
336

A. Alpha B.1.1.7 spike mutations: N501Y, Δ144, Δ69/70, L18F, A570D, D614G, P681H, S982A, T716I, D1118H. WT D614 vs Alpha B.1.1.7 luminescence (RLU, log₁₀) across ACE2 receptors: Human, Civet, Ferret, Hamster, Mouse, Rat, Pig. Radar plot comparing Human, Civet, Ferret, Hamster, Mouse, Rat, Pig.

B. Beta B.1.351 spike mutations: K417N, E484K, N501Y, Δ242-244, R246I, D80A, D215G, D614G, A701V. WT D614 vs Beta B.1.351 luminescence (RLU, log₁₀) across ACE2 receptors.

C. Gamma P.1 spike mutations: K417T, E484K, N501Y, D138Y, L18F, T20N, P26S, R190S, D614G, H655Y, T1027I. WT D614 vs Gamma P.1 luminescence (RLU, log₁₀) across ACE2 receptors.

D. Delta B.1.617.2 spike mutations: T478K, L452R, Δ156-157, R158G, T19R, D614G, P681R, D950N. WT D614 vs Delta B.1.617.2 luminescence (RLU, log₁₀) across ACE2 receptors.

A. Mouse ACE2

B. Rat ACE2

C. Civet ACE2 — Civet ACE2

A. (Mouse ACE2) x-axis: WT D614, Alpha (B1.1.7), N501Y, N501Y Δ69/70, P681H, Δ144

B. (Rat ACE2) x-axis: WT D614, Alpha (B1.1.7), N501Y, N501Y Δ69/70, P681H, Δ144

C. (Civet ACE2) x-axis: WT D614, Alpha (B1.1.7), N501Y, N501Y Δ69/70, P681H, Δ144, B1.1.7+E484K

(Civet ACE2) x-axis: WT D614, Beta (B.1.351), E484K, N501Y, K417N, A701V, Δ242-244

y-axis (all): Luminescence (RLU, $\log_{10}$)