Evaluation of the neutralising antibody response in human and hamster sera against SARS-CoV-2 variants up to and including BA.2.86 using an authentic virus neutralisation assay

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Abstract

New vaccines, therapeutics and immunity elicited by natural infection create evolutionary pressure on SARS-CoV-2 to evolve and adapt to evade vaccine-induced and infection-elicited immunity. Vaccine and therapeutics developers thus find themselves in an “arms race” with the virus. The ongoing assessment of emerging SARS-CoV-2 variants remains essential as the global community transitions from an emergency response to a long-term management plan. Here, we describe how an authentic virus neutralisation assay using low passage clinical virus isolates has been employed to monitor resistance of emerging virus variants to neutralising antibodies from humans and experimentally infected hamsters. Sera and plasma from people who received three doses of a vaccine as well as those who received a bivalent booster were assessed against SARS-CoV-2 variants, up to and including BA.2.86. Contemporary or recent virus variants showed substantial resistance to neutralisation by antibodies from those who had received three vaccines but were still effectively

neutralised by antibodies from individuals who had received a bivalent booster (ancestral/BA.1). Convalescent sera from hamsters that had been experimentally infected with one of seven virus variants (ancestral, BA.1, BA.4, BA.5.2.1, XBB.1.5, XBB.1.16, XBB.2.3) were also tested here. The most contemporary variant, BA.2.86, was effectively neutralised by sera from hamsters infected with XBB.1.5 and XBB.1.16 but it was not neutralised by sera from those infected with BA.5.2.1. These data support the recommendations given by the WHO that a new vaccine was required and should consist of an XBB sub-lineage antigen.

Introduction

Coronavirus 2019 disease (COVID-19) caused by SARS-CoV-2 continues to cause significant disruption to healthcare systems and economies worldwide having caused over 700 million cases and 6.7 million deaths since its emergence in December 2019 [1]. The WHO downgraded the COVID-19 pandemic from a public health emergency of international concern (PHEIC) on 5 May 2023, this marked a transition from emergency response to long-term management [2]. However, the virus continues to evolve in response to changing population immunity leading to the regular identification of new viral variants. Sequences of emerging variants are continuously monitored and assigned as new sub-lineages by the Phylogenetic Assignment of Named Global Outbreak Lineages (PANGOLIN) tool. At the time of writing there are 3,569 designated SARS-CoV-2 lineages with five of these having been classified as variants of concern (VOC) by the WHO Technical Advisory Group on Virus Evolution: Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), Gamma (P.1) and Omicron (B.1.1.529) [3-5].

A global effort to design and manufacture COVID-19 vaccines began as soon as the viral genome was published and the first COVID-19 vaccine was deployed in the UK on 8th December 2020 (Comirnaty) [6]. This was followed by Vaxzevria on 30th December 2020 and Spikevax on 8th January 2021 [7, 8]. These vaccines were the most widely used worldwide for the initial course of three immunisations and all were based on the ancestral (clade A) virus spike glycoprotein. In the first year of vaccines

50 being available it has been predicted that vaccination prevented 14.4 million deaths due to COVID-
51 19 [9]. The emergence of novel SARS-CoV-2 variants brought with it the concern that ancestral-
52 based vaccines would no longer provide sufficient protection to vulnerable people in at-risk groups.
53 Indeed, our data utilising the gold-standard authentic virus neutralisation assay indicated that the
54 latest variants were becoming increasingly resistant to ancestral-based immune responses [10-12].
55 Bivalent vaccines were developed in response to this concern and have been designed to not only
56 target the ancestral strain but also Omicron sub-lineage spike glycoproteins. These were approved
57 for use in the UK (BA.1/ancestral bivalent) for at-risk groups only in the Autumn of 2022, and the US
58 (BA.5/ancestral bivalent) for anyone aged 18 or over in August 2022 [13, 14]. Restricting vaccination
59 to only at-risk groups in the UK and low vaccine uptake in the US, where only 34% adults received a
60 fourth booster, means that most of the population who have received a vaccination are still
61 immunised with an ancestral spike-based vaccine [15]. However, since the relaxing of restrictions
62 worldwide, many people will also have received some degree of natural immunity due to infection
63 with one or more of the circulating variants. The most recent vaccines to be approved elicit
64 immunity against contemporary XBB recombinants, the latest to gain authorisation consists of an
65 XBB.1.5 component [16-18].

66 The recent emergence of the BA.2.86 variant has highlighted the value of sustained sequence
67 monitoring due to the sudden appearance of a virus with vastly different spike sequence compared
68 to other circulating variants or those represented in the latest vaccines. As part of a CEPI-funded
69 "Agility" project, we have been monitoring and performing risk assessments of SARS-CoV-2 variants
70 since November 2020. We previously presented our analysis of 20 different variants using early
71 pandemic human convalescent and triple-vaccinated plasma and serum panels as well as
72 international standards [12]. Here we describe our latest data generated using serum or plasma
73 collected from a panel of individuals who have received up to four doses of a COVID-19 vaccine using
74 an authentic virus neutralisation assay. These assays have been performed using well characterised,
75 low passage SARS-CoV-2 viruses derived from clinical isolates including the BA.2.86 variant. We also

76 present neutralisation data generated using the sera of experimentally infected hamsters which
77 were convalescent from either the ancestral virus (pre-D614G), BA.1, BA.4, BA.5.2.1, XBB.1.5,
78 XBB.1.16 or XBB.2.3 (mono-variant convalescent). We have also compared the antigenic maps of
79 these two data sets.

80 **Methods**

81 Human vaccinee sera and antibody standards

82 SARS-CoV-2 variants were tested against two panels of vaccinee samples collected from staff
83 volunteers. The first panel, consisting of sera from individuals who had received three doses of an
84 ancestral spike-based COVID-19 vaccine, was collected as part of the ESCAPE study as previously
85 described [12, 19]. The second panel was assembled from staff members at MHRA (n=8 plasma
86 samples) and UKHSA (n=14 sera samples) who had received a fourth vaccination consisting of
87 bivalent (BA.1/ancestral) vaccine. Samples were collected at a median of 78 days after the fourth
88 vaccination. When tested for the presence of anti-N antibodies, 19 of the 22 individuals had
89 evidence of convalescence for naturally acquired SARS-CoV-2 infection. Dates of previous infections
90 were provided by 14 individuals which identified an infection range of February 2021 to December
91 2022. Two reference materials were included in this study: a working reagent for anti-SARS-CoV-2
92 immunoglobulin (NIBSC 21/234), which is a pool of several pre-Alpha convalescent plasma, and the
93 International Standard for antibodies to SARS-CoV-2 VOC (NIBSC 21/338), which is a pool of 265
94 individuals who are both vaccinated and convalescent following Alpha, Beta or Delta infection [20,
95 21].

96 Virus bank preparation and quality control checks

97 Unless otherwise stated in Table 1, viruses were isolated from sequence-confirmed clinical swabs at
98 UKHSA. SARS-CoV-2 variants were isolated and propagated on Vero/hSLAM cells (ECACC 04091501)
99 before being subjected to quality control checks as previously described [12]. Briefly, titrations were

100 performed using a focus-forming assay with immunostaining for nucleocapsid to visualise foci [22].
101 Sighting for each variant into the Focus Reduction Neutralisation Test (FRNT) to calculate dilution at
102 which a median of 130 foci could be counted in non-neutralised control wells was performed. Whole
103 genome sequencing (SISPA-Illumina) was performed on final virus assay banks, as described
104 previously, to confirm presence of FCS S1/S2 boundary, check for mixed bases as well as confirm
105 variant identity [12, 23-25].

106 FRNT and statistics

107 Median neutralising antibody titres (ND_{50}) were measured with FRNT and calculated with a Probit
108 regression analysis using R v4.1.2 (R Core Team, Vienna, Austria) as described previously [11, 12, 22].
109 Log_{10}-transformed ND_{50} titres were analysed using a mixed-effects linear model in R, as described
110 previously [12, 26].

111 Antigenic cartography

112 Antigenic maps were generated using the Racmacs (https://acorg.github.io/Racmacs/) package in R
113 as described previously [27, 28]. The number of dimensions was set to 2, number of optimisations to
114 500 and minimum column basis to none. Neutralisation titres from human and hamster sera were
115 used to generate two separate maps.

116 Hamster sera

117 All *in vivo* experimental work was conducted under the authority of a UK Home Office approved
118 project licence that has been subject to local ethical review at either UK Health Security Agency
119 Porton Down or Medicines and Healthcare products Regulatory Agency, South Mimms by their
120 respective Animal Welfare and Ethical Review Body (AWERB) as required by the *Home Office*
121 *Animals (Scientific Procedures) Act 1986*.

122 Sera collected at day 27 post-infection from hamsters exposed to ancestral virus (n=6) and BA.1
123 (n=5) or day 28 post-infection with BA.4 (n=6) and BA.5.2.1 (n=6). These hamster sera were

124 generated as previously described [29, 30]. Sera was collected from hamsters infected with XBB1.16
125 and XBB.2.3 (n=6 per variant) with a target dose of $1x10^4$ FFU/200 µL via intranasal instillation
126 (method previously described [29, 30]). Blood samples were collected during necropsy at 28 days
127 post infection.

128 Sera from XBB.1.5 convalescent hamsters were collected as follows. Four hamsters (approximately
129 10 weeks old) were administered $1x10^5$ FFU/50µL intranasally of XBB.1.5. After infection, oral swabs
130 were taken to confirm infection by lateral flow and RT-qPCR. Hamsters were terminated at 28 days
131 post-infection and clotted whole blood was centrifuged (1,000RPM, 5 mins) to obtain serum.

132 **Results**

133 Through monitoring of the currently circulating SARS-CoV-2 variants, we identified and sourced
134 nasopharyngeal swabs from sequence-confirmed patients through our clinical networks and Pillar 1
135 testing (primarily positive tests conducted in hospital) in England. We then utilised an authentic virus
136 FRNT using low passage SARS-CoV-2 variants of clinical origin to perform risk assessments with
137 regards to cross-reactivity of humoral immunity. SISPA-Illumina sequencing was performed on stocks
138 prior to use in the FRNT to confirm the lineage and check for cell culture-induced mutations. We
139 note that one of the BA.2.86 variants (BA.2.86 – A) described here contains an unexpected amino
140 acid change within the furin cleavage site (FCS) (S:R682W). However, work published by others
141 indicates that changes in this position are unlikely to alter the neutralisation profile, though this
142 mutation makes this stock unsuitable for *in vivo* studies [31-34]. Neutralisation assays were
143 therefore performed with this virus whilst efforts continued to generate a bank of BA.2.86 with a
144 wild-type FCS (BA.2.86 – B). The variants used in this study, and their sources, are detailed in Table 1.

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Table 1 – SARS-CoV-2 variants used in this study

WHO Designation	Pangolin Lineage	GISAID Clade/Lineage	Nextstrain Clade	Date Assigned	GISAID ID of Isolation Swab (Where Known) and/or Source
Ancestral virus	B	O	19A	Jan 2020	EPI_ISL_406844
Alpha	B.1.1.7	GRY	20I (V1)	Dec 2020	EPI_ISL_683466
Beta	B.1.351	GH/501Y.V2	20H (V2)	Dec 2020	EPI_ISL_770441
Delta	B.1.617.2	G/452R.V3	21A	May 2021	EPI_ISL_2742236
Zeta–FioCruz *	P.2	GR/484K.V2	20B/S.484K	Mar 2021	FioCruz, Brazil
Zeta–BEI *	P.2	GR/484K.V2	20B/S.484K	Mar 2021	BEI (NR-55439)
XE	XE	GRA	Recombinant	Mar 2022	EPI_ISL_11586931
XF	XF	GRA	Recombinant	Mar 2022	EPI_ISL_10458256
Omicron	BA.1	GRA	21K	Dec 2021	EPI_ISL_7400555
Omicron	BA.1.1	GRA	21K	Jan 2022	EPI_ISL_8165999
Omicron	BA.2	GRA	21L	Dec 2021	Not available
Omicron	BA.2.12.1	GRA	22C	Apr 2022	Gavin Screaton (University of Oxford)
Omicron	BA.2.75.3	GRA	22D	Jun 2022	EPI_ISL_13882158
Omicron	BA.2.75.2	GRA	22D	Aug 2022	Not available
Omicron	BA.4	GRA	22A	Apr 2022	EPI_ISL_13157810
Omicron	BA.4.6	GRA	22A	Jul 2022	Not available
Omicron	BA.5.2.1	GRA	22B	Apr 2022	EPI_ISL_12810908
Omicron	BQ.1.22	GRA	22E	Sep 2022	EPI_ISL_15581064
XBB.1.1	XBB.1.1	GRA	22F	Oct 2022	EPI_ISL_15682231
Omicron	CH.1.1	GRA	23C	Oct 2022	EPI-ISL-16615644
XBB.1.5	XBB.1.5	GRA	23A	Jan 2023	EPI-ISL-16587654
XBB.1.16	XBB.1.16	GRA	23B	Mar 2023	EPI_ISL_17666853
XBB.2.3	XBB.2.3	GRA	23E	Jan 2023	Not available
EG.5.1.1	EG.5.1.1	GRA	23F	Jun 2023	Not available
Omicron*	BA.2.86 (A)	GRA	21L	Aug 2023	EPI_ISL_18111770
Omicron*	BA.2.86 (B)	GRA	21L	Aug 2023	Not available

* For Zeta and BA.2.86, assays were conducted with stocks derived from viruses of different sources.

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149 Our previously published data indicated that immunity derived from neutralising antibodies was
150 becoming less effective for those who had received three doses of a first-generation COVID-19
151 vaccine against the currently circulating variants [12]. Whilst a newer panel of sera was acquired, we
152 continued our assessment of triple vaccinated individuals who had received an original ancestral
153 based vaccine. This panel had been tested against ten SARS-CoV-2 variants (including ancestral) at
154 the point of publishing our previous paper. We have subsequently tested this panel against a further
155 six variants (Zeta – FC, Zeta – BEI, BA.4.6, BA.2.75.2, BQ.1.22 and XBB.1.1), as shown in Figure 1.

156 Our previous report demonstrated a significant difference in ND_{50} titres of our pre-Alpha
157 convalescent panel when tested against two different Zeta isolates. This was notable due to most
158 other published data for this variant demonstrating comparable neutralisation titres to those against
159 Delta. However, another group demonstrated a similarly large resistance to neutralisation of Zeta
160 with a panel of early convalescent sera but not a panel of vaccinee sera [35]. We therefore sought to
161 investigate whether the same pattern would be observed with our triple vaccinee panel (Figure 1).
162 Geometric mean (GM) neutralisation titres of this panel against Zeta – FC and Zeta – BEI were 199
163 and 671 corresponding to a 26.2-fold and 7.8-fold reduction relative to ancestral, respectively. The
164 difference between the titres measured against the two Zetas was significant (fold-change = 3.4; ME-
165 ANOVA; $p < 0.05$). Of the four remaining additional variants that were tested against this panel since
166 our previous paper, BA.4.6 and BA.2.75.2 resulted in GM titres above the lower limit of detection of
167 the assay (LLOD), thus permitting more accurate estimates of fold-reduction relative to ancestral
168 virus. The fold-reduction for BA.4.6 relative to ancestral was statistically significant at 105. When
169 compared to its close parent lineage, BA.4, there was also a significant fold-reduction of 2.1. Against
170 BA.2.75.2 the triple-vaccinated panel gave a GM titre of 46.1 which equated to a 113-fold reduction
171 relative to ancestral and 3.7-fold reduction relative to a closely related variant, BA.2.75.3. Two of the
172 most recent variants (BQ.1.22 and XBB.1.1) were substantially resistant to neutralisation to sera
173 from triple-vaccinated individuals with most titres falling below the LLOD. Sera which fall below the
174 LLOD are assigned the value of the lowest dilution used in the assay (1/20), thus fold-reduction

175 calculations do not provide accurate estimates. The lower limit of quantification (LLOQ) for the
176 ancestral virus assay (previously determined as 58) is also displayed [11]. Although the LLOQ was not
177 determined for subsequent variants, the precision of the assay is likely to be lower for titres falling
178 below this value. However, the estimated fold-reductions relative to ancestral virus for BQ.1.22 and
179 XBB.1.1 were estimated at 197 and 226-fold.

180 In addition, to assessing the sera from individuals of the panel, the neutralisation titres of the NIBSC
181 working reagents 21/234 and/or International Standard 21/338 were also assessed in every assay.
182 These are displayed in Figure 1 as green squares or blue circles, respectively. As described
183 previously, these are pools of pre-Alpha convalescent plasma (21/234) or individuals who were both
184 vaccinated and convalescent following an Alpha, Beta or Delta infection (21/338). These standards
185 are typically more potent at neutralising the virus compared to the panel. However, it was noted
186 that for BA.2.75.2, BA.4, BA.4.6 and BA.5.2.1, the 21/234 standard was yielding ND_{50} values
187 comparable to those from the lowest responders of our panel and was completely unable to
188 neutralise BQ.1.22. The newest 21/338 standard retains good reactivity and ability to neutralise all
189 variants assessed herein.

190 To continue to assess humoral immunity to currently circulating SARS-CoV-2 variants, a selection of
191 participants who had received a fourth (booster) vaccine were sought. A panel of 22 individuals was
192 assembled from donations given by eight MHRA staff members and 14 UKHSA staff members who
193 had received three doses of a first-generation COVID-19 vaccine followed by a booster with a
194 bivalent (BA.1/ancestral) vaccine. Samples were collected a median of 78 days after the most recent
195 vaccination. Among the 22 individuals, 19 had a known previous SARS-CoV-2 infection between
196 February 2021 and December 2022.

197 This four-dose panel was first assessed against the ancestral virus which resulted in the highest
198 neutralisation titres with a GM of 12,400 (Figure 2). Next, we assessed the titres of this panel against
199 one of the earlier Omicron sub lineages, BA.4. The GM neutralisation titres of the panel against BA.4

200 was 1,060 which equated to a 11.7-fold reduction relative to ancestral virus. To date this panel has
201 been tested against a further eight variants with the largest resistance to neutralisation seen for the
202 Omicron sub lineage, CH.1.1, with a GM titre for the panel of 62.9 and fold-reduction of 180.0.
203 Another Omicron sub lineage, BQ.1.22, also displayed notable resistance to neutralisation by the
204 four-dose panel which generated a GM titre of 92.9 corresponding to a 121.9-fold-reduction relative
205 to ancestral virus. The recombinants, XBB.1.1, XBB.1.5, XBB.1.16, XBB.2.3 and EG.5.1.1 all gave
206 comparable GM titres between 102 and 146. Similarly, the most recently emerged Omicron sub-
207 lineage at the time of writing, BA.2.86, was neutralised equally well by those who have had received
208 four doses of a COVID-19 vaccine. Here, we tested two BA.2.86 isolates with or without an FCS
209 substitution (S:R682W), BA.2.86 – A and BA.2.86 – B, respectively. The GM titres of the panel against
210 each virus was 99.3 and 143, equating to a 124.7 and 89.4-fold reduction relative to ancestral,
211 respectively. The difference between BA.2.86 – A and BA.2.86 – B was a 1.4-fold increase and was
212 not-significant (p=0.32). The numerical breakdown of these data and the model estimates of
213 geometric means are presented in Table S2.

214 Antigenic cartography is a powerful method for visualising the evolution of a pathogen by plotting
215 the neutralisation titres of different panels of sera against successive variants or strains. It was
216 originally developed as a means of monitoring changes in seasonal influenza virus and has since
217 been utilised by numerous groups to compare SARS-CoV-2 variants [28, 36, 37]. Here, we utilise this
218 technique to visualise the antigenic relationships of the SARS-CoV-2 variants tested against the
219 panels of human sera or plasma described in this and our previous publication (Figure 3). The data
220 plotted here include all titres from a panel of pre-Alpha convalescent plasma, triple-vaccinated
221 individuals, boosted (BA.1/ancestral bivalent) individuals and the three standards (20/136, 21/334,
222 21/338). Each square of the map represents one antigenic unit which equates to a two-fold change
223 in neutralisation titres. The ancestral virus is presented as a red circle and sits in the centre of the
224 serum panels. Pre-Omicron variants were coloured as dark green circles and are situated within an
225 inner circle around the serum panels. In the influenza field, vaccines are updated when circulating

226 strains are at least two units away from the vaccine strain, thus marking it as genetically different
227 [36]. Of the pre-Omicron variants, Beta and Zeta-FC are the furthest from ancestral virus with a gap
228 of over 4 antigenic units, though in perpendicular directions from ancestral. Substantial diversity can
229 be seen in post-Omicron variants which are grouped and coloured as Omicron-BA.1x (light green),
230 Omicron-BA.2.x (sky blue), Omicron-BA.4/5.x (yellow), late Omicron (lilac), XE/XF recombinants
231 (pink) and XBB recombinants (neon blue). However, some patterns can be seen, for example the
232 recombinants XF and XE cluster with the virus from which their spike was derived. The recombinant
233 XF is located with BA.1/BA.1.1 at approximately 5.5 units from ancestral and XE with BA.2/BA.2.12.1
234 at 2.5 units from ancestral. Also, the BA.4 and BA.5.2.1 cluster closely at approximately 5 units away
235 from ancestral and, while the BA.4.6 sub lineage is a similar distance from ancestral, it is also
236 approximately 4.5 units away from its parent, BA.4. The XBB family of variants cluster together at
237 approximately six units from ancestral. However, within this cluster there are three antigenic units
238 between the two most distant variants, XBB.1.16 and EG.5.1.1. The late Omicron variants (BQ.1.22,
239 CH.1.1 and BA.2.86) form another separate cluster approximately seven antigenic units from
240 ancestral. Interestingly, BA.2.75.2 and BA.2.75.3 appear on the opposite side of the map to BA.2
241 with BA.2.75.2 located closer to the late Omicrons than to BA.2.75.3.

242 One of the known limitations of generating antigenic maps with human neutralisation data is the
243 diversity of pre-existing immunity that is associated with multi-exposure sera (vaccine and naturally
244 acquired). We therefore utilised single-exposure convalescent sera collected during our *in vivo*
245 hamster infection studies to generate more antigenically defined maps. In Figure 4, we have plotted
246 neutralisation titres of sera from hamsters collected on day 27 (ancestral and BA.1) or day 28 (BA.4,
247 BA.5.2.1, XBB.1.5, XBB.1.16, XBB.2.3) after a single virus infection by the intranasal route. Each panel
248 of sera was tested against the variant used as the challenge virus. Fold-changes against subsequent
249 variants tested are presented as relative to the virus used for infection (Figure 4). Data for hamsters
250 infected with ancestral, BA.1, BA.4 and BA.5.2.1 are described previously but also presented here
251 [29, 30]. As our results from the human data indicated that the FCS substitution in the BA.2.86 – A

252 virus did not affect neutralisation titres, repeat assays with these volume-limited sera were not
253 performed with the second virus.

254 Briefly, sera taken from hamsters infected with ancestral virus gave significantly lower GM titres
255 with a 3.3- and 3.5-fold reduction for Alpha and Delta relative to ancestral, respectively. Sera taken
256 from BA.1 convalescent hamsters showed a similar ability to neutralise both BA.1 and ancestral but
257 were unable to neutralise BQ.1.22 and XBB.1.1 at the lowest dilution used here (1/30). For BA.4
258 convalescent hamster sera, BQ.1.22 and XBB.1.5 were the only variants which were significantly
259 more resistant to neutralisation by these sera, with the latter resulting in GM titres all below the
260 LLOD. In hamsters that were convalescent for BA.5.2.1, GM titres against ancestral virus relative to
261 BA.5.2.1 were not significantly different. Titres against all other variants tested against BA.5.2.1
262 convalescent sera were significantly lower relative to BA.5.2.1. Against BQ.1.22, XBB.1.1, XBB.1.5
263 and EG.5.1.1 titres were above the LLOD meaning fold changes could be determined accurately with
264 7.5-, 94.4-, 80.1-, 73.8-fold reductions, respectively. Notably, when tested against BA.2.86 – A, none
265 of the sera from BA.5.2.1 infected hamsters were able to neutralise the virus with all titres falling
266 below the LLOD. GM titres of convalescent sera from hamsters infected with XBB.1.5 tested against
267 EG.5.1.1 were significantly reduced relative to XBB.1.5 with a 1.8-fold reduction. When these sera
268 were tested against BA.2.86 there was no significant difference in GM neutralising titres.
269 Convalescent sera from hamsters experimentally infected with XBB.1.16 were tested against
270 ancestral virus, Beta, BA.1, BA.5.2.1, XBB.1.5, EG.5.1.1, XBB.2.3 and BA.2.86 – A. GM titres for this
271 panel against all variants were significantly reduced relative to XBB.1.16 with fold-reductions of 6.0,
272 77.5, 55.0, 17.1, 6.8, 10.4, 12.2 and 33.1, respectively. Finally, convalescent sera from hamsters
273 experimentally infected with XBB.2.3 were able to neutralise ancestral, XBB.1.16 and EG.5.1.1
274 equally well to XBB.2.3. Against all other variants (Beta, BA.1, BA.5.2.1, XBB.1.5, BA.2.86 – A), the
275 neutralisation titres for these sera were significantly lower with Beta displaying the most resistance
276 to neutralisation by this panel. The numerical breakdown of these data and the model estimates of
277 geometric means are presented in Tables S3-S9.

278 When these data were used to generate an antigenic map (Figure 5) the points corresponding to
279 individual sera were more widely distributed compared to the human map. As expected, each
280 antigenic variant clusters with sera from animals infected with homologous virus. Of the pre-
281 Omicron variants, Beta is again the most antigenically distant at approximately three units from
282 ancestral. BQ.1.22 is located in the same area of the map as Beta but another antigenic unit further
283 from ancestral. The most antigenically distinct variant with the hamster map was XBB.1.1 at 5.5 units
284 from ancestral and three units away from the nearest variant on the map, BA.1. The most
285 contemporary variant tested here, BA.2.86 – A, is approximately five units from ancestral but
286 clusters closely to XBB.1.5 with just over one antigenic units' distance.

287 **Discussion**

288 SARS-CoV-2 continues to cause worldwide disruption, despite the downgrading of the COVID-19
289 pandemic marking the end of the public health emergency. Since its emergence, SARS-CoV-2 has
290 been acquiring fitness-enhancing mutations in response to changing population immunity driven by
291 both infection and vaccination [38]. It is thought that additional pressures on the virus such as the
292 clinical use of antiviral drugs, long-term infections in immunocompromised individuals and spillback
293 events from zoonotic infections are also likely to contribute to the increased diversity in viral
294 sequences [39, 40]. Here, we present our updated assessment of emerging SARS-CoV-2 variants in
295 an authentic virus neutralisation assay against panels of sera or plasma from individuals who have
296 received three doses of a COVID-19 vaccine as well as those who received a bivalent (BA.1/ancestral)
297 booster. We also assessed neutralisation titres from experimentally infected hamsters that were
298 convalescent for seven SARS-CoV-2 variants (ancestral, BA.1, BA.4, BA.5.2.1, XBB.1.5, XBB.1.16,
299 XBB.2.3) and map both these and the human data antigenically.

300 In our previous publication we described a distinct difference in neutralisation titres against two
301 different isolates of the Zeta variant when tested against our pre-Alpha convalescent panel. Another
302 group from the University of Geneva showed a similar large reduction in neutralisation titres with an

303 early pandemic convalescent panel of sera against Zeta that was not seen against triple vaccinated
304 individuals [41]. We therefore tested our two Zeta isolates against our three-dose vaccinee panel,
305 however these viruses continued to show substantial resistance to neutralisation by this panel with a
306 significant difference between the two isolates. A sequence comparison with data kindly supplied by
307 the University of Geneva revealed identical spike sequences for all three viruses but with a three
308 amino acid deletion in the N gene of the virus discussed in the Bekliz paper (208-210). Zeta – BEI and
309 the University of Geneva virus also have three additional non-synonymous mutations in Orf1a
310 leading to amino acid changes: V1071A, P1810L and S3149F. With no differences in the main viral
311 proteins exposed to neutralising antibodies (spike, membrane, envelope), it appears that the
312 sequence is not responsible for the variation in titres seen here. Other potential explanations were
313 described in our previous paper and include the potential impact of secretion of free spike protein,
314 defective interfering particles and differences in spike glycosylation [12, 42-46].

315 Our data from individuals who had received three doses of an ancestral spike-based vaccine
316 indicated that antibodies against the two variants, BA.2.75.2 and BA.4.6, were less able to neutralise
317 these viruses with titres nearing the LLOD. BA.2.75.2 has three additional amino acid changes
318 relative to BA.2.75.3 (identical spike to BA.2.75) which are thought to increase immune evasion
319 (S:R346T, S:F486S, S:D1199N) [47, 48]. Similarly, BA.4.6 also contains S:R346T relative to its parent,
320 BA.4. Our neutralisation data supports this prediction with a higher immune escape of BA.2.75.2
321 compared to BA.4.6 and agrees with others who show similar trends [49, 50].

322 Further testing of this population demonstrated that the sera in this panel had no detectable
323 neutralising activity against the later variants, BQ.1.22 and XBB.1.1. The BQ.1.22 variant used in this
324 study has the same spike sequence as the BQ.1.1 variant, which was dominant in December 2022,
325 with three additional changes relative to its parent BA.5 (S:R346T, S:K444T, S:N460K). Similarly, the
326 recombinant XBB.1.1, circulating around the same time, had changes in several of the same
327 locations including S:R346T, S:L368I, S:N460K, S:F486S, S:R493Q and S:F490S. These two variants

328 were also resistant to neutralisation by panels of triple vaccinated volunteers in other studies as well
329 as in those with breakthrough infections with BA.1 and BA.2 [51, 52].

330 In the UK, as boosters containing bivalent antigens were deployed in the Autumn of 2022, we
331 continued our assessment of emerging variants with samples from individuals who had received a
332 BA.1/ancestral booster. The majority also had at least one breakthrough infection between February
333 2021 and December 2022 (Alpha to XBB.1.5). Results from this panel showed that neutralisation
334 titres for the two variants which were no longer neutralised by the three-dose panel (BQ.1.22 and
335 XBB.1.1) were effectively neutralised by this four-dose panel. Indeed, all the subsequent
336 recombinants and Omicron sub-lineages tested here showed similar neutralisation titres for all
337 variants between CH.1.1 to BA.2.86. This includes the two BA.2.86 isolates tested here, with and
338 without the presence of an amino acid change in the FCS (S:R682W), with no significant difference
339 seen for sera tested against the two viruses. These data are in alignment with others who have
340 demonstrated that changes in the FCS are not important in relation to virus neutralisation *in vitro*
341 but alter how the virus behaves *in vivo* [31-34]. All variants tested (CH.1.1, BQ.1.22, XBB.1.1, XBB.1.5,
342 XBB.1.16, XBB.2.3, EG.5.1.1, BA.2.86) displayed increased resistance to neutralisation to this four-
343 dose panel relative to both ancestral and BA.4, with similar GM titres for all. This aligns with results
344 presented by other groups using both pseudovirus (PSV) and authentic virus assays against panels of
345 vaccinated (three- and four-dose) with additional break-through infection in a globally diverse
346 population [53-60]. It is also of interest that the GM titres are comparable despite the genetic
347 diversity in the variants tested against our four-dose panels. This is likely due to the range of variants
348 to which these individuals have acquired natural immunity via break-through infections. We note
349 however that, due to vaccination campaigns targeting at-risk subgroups within the population, the
350 sera or plasma from individuals in our panel of boosted vaccinees are part of a limited subset of the
351 population considered to be at risk. Whilst this is informative in terms of the effects of vaccines on
352 immunity it may not be representative of levels of vaccine-induced immunity in the general
353 population.

354 Antigenic mapping of the human data shows co-localisation of the pre-Omicron variants in the
355 centre of the map with a larger antigenic spread following the emergence of Omicron and
356 recombinants. This is comparable to maps presented by others using human data, though direct
357 comparisons are challenging given the diversity in different panels of human sera used between
358 studies [28, 61-64]. For example, many will have had different vaccination schedules/ formulations
359 and exposure to unidentified viral variants in addition to human genetic diversity leading to a range
360 of humoral immune responses. However, while distances between variants in different maps may
361 vary, the general pattern is the same with pre-Omicron variants localised to the centre and clusters
362 of related variants moving out from the centre in a chronological manner. As others have shown, the
363 XBB family of variants cluster further away from ancestral than BA.4/5 and late Omicron variants
364 such as BQ.1.22, CH.1.1 and BA.2.86 are more distant again [60, 65].

365 One limitation of ours and others human antigenic maps is the clustering of sera in one location on
366 the map, corresponding to the ancestral spike that drove the pandemic and was used as the basis of
367 the first-generation vaccines. This effect is less prominent when using the neutralisation titres from
368 sera taken from hamsters 27- or 28-days post-infection with a single, known SARS-CoV-2 variant at a
369 standard infectious dose. Antigenic mapping of these data highlighted two antigenically distinct
370 viruses: BQ.1.22 and XBB.1.1. Although the most contemporary variant to date, BA.2.86, is one of
371 the furthest antigenically from ancestral, it clustered closely with XBB.1.5. This suggests that
372 antibodies raised against the XBB.1.5 spike antigen have good cross-reactivity against BA.2.86, thus
373 supporting the transition to XBB.1.5 based vaccines. Others have also shown XBB.1.1, BQ.1.1 and
374 BA.2.86 to be distinct in antigenic maps generated with hamster or mouse data [60, 65]. Using single
375 exposure sera from hamsters has the added benefits of being generated by a known priming
376 antigen, given at the same dose with the duration since infection being known and fixed. These are
377 therefore more predictive of antigenic relationships when mapped. Our hamster data also indicate
378 that a BA.5 spike alone does not generate a detectable neutralising antibody response to one of the
379 most recent variants, BA.2.86. Taken together, these data suggested a turning point in the virus'

380 evolution and signalled to vaccine developers that an updated vaccine was required. On 18 May
381 2023 the WHO Technical Advisory Group on COVID-19 Vaccine Composition (TAG-CO-VAC) published
382 their recommendations to develop vaccines to target contemporary XBB-descendent lineages [66].

383 Numbers of sequenced SARS-CoV-2 genomes are decreasing due to reduced global surveillance
384 activity since the end of the pandemic. As a result, identifying variants of concern in a timely manner
385 is increasingly challenging. This was highlighted by the apparent sudden emergence of BA.2.86 in
386 August 2023 despite some models retrospectively predicting it had been circulating since May 2023
387 [56, 67]. Continued monitoring of emerging variants and their assessment in programmes such as
388 Agility is essential to build on existing knowledge of immune evasion of contemporary and newly
389 emerging variants and to better advise policymakers and vaccine manufacturers on future strategies.

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398 **Ethics**

399 The ESCAPE study was conducted in accordance with the Declaration of Helsinki and the Governance
400 Arrangements for Research Ethics Committees (GAfREC). It was approved by the PHE Research Ethics
401 and Governance Group (R&D REGG Ref NR0190, March 2020). Informed consent was obtained from
402 all subjects involved. Donations from MHRA staff members were collected with written informed

403 consent from donors, study was approved by the MHRA local ethics committee (HuMAC ref:
404 20/05/SK).

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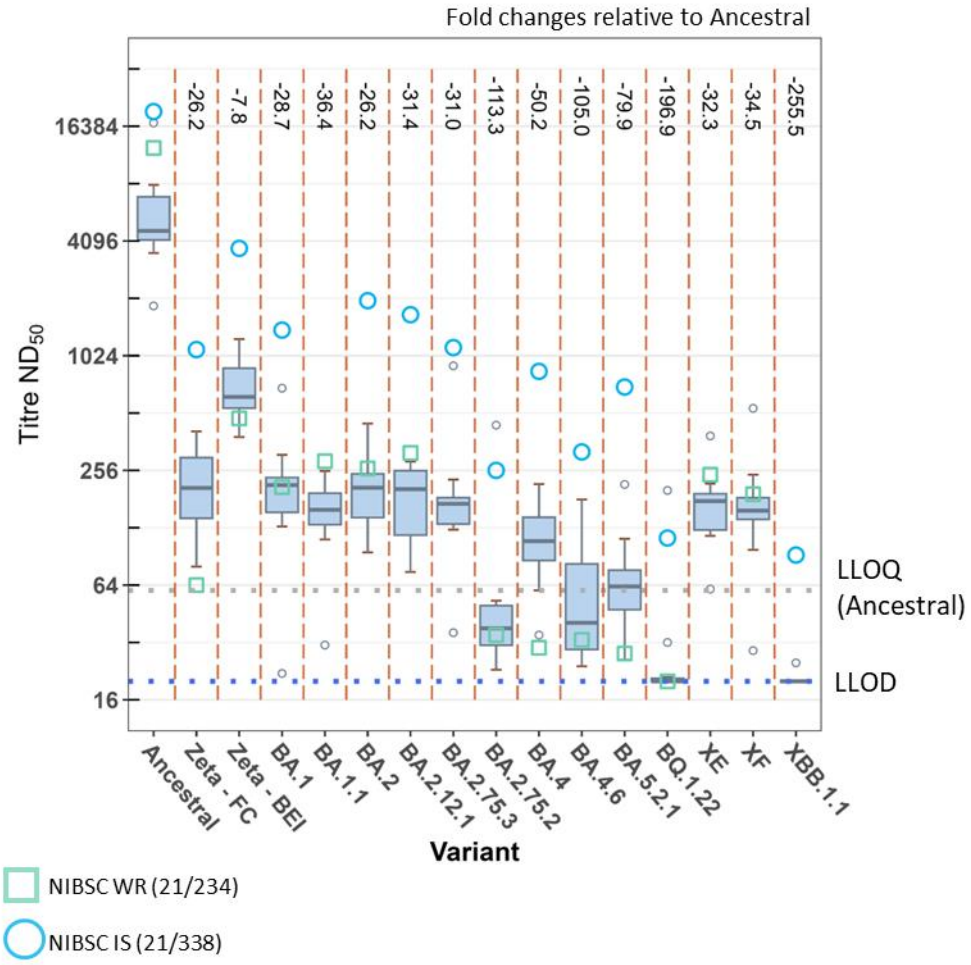

Figure 1. Neutralising antibody titres of a panel of triple vaccinated human participant sera determined by Focus Reduction Neutralisation Test (FRNT) against authentic SARS-CoV-2 Variants.

A panel of ten sera from triple vaccinated human volunteers were assessed by FRNT with 16 authentic SARS-CoV-2 variants. Data are presented as median and interquartile ranges of titre ND_{50}, titres of panel members falling outside of these ranges are plotted as open small circles. Fold-changes relative to ancestral virus were calculated by regression analysis using a mixed-effect model and are presented where the difference is statistically significant ($p<0.05$, two-way ANOVA with Tukey's *post hoc* test). The Lower Limit of Detection (LLOD) of 1/20 is indicated by the blue dotted line. The Lower Limit of Quantification (LLOQ) of 1/58 as determined for the ancestral assay, is indicated by the grey dotted line. Neutralisation titres of the standards; NIBSC WR 21/234 and 21/338, are presented as a green box and blue circle, respectively.

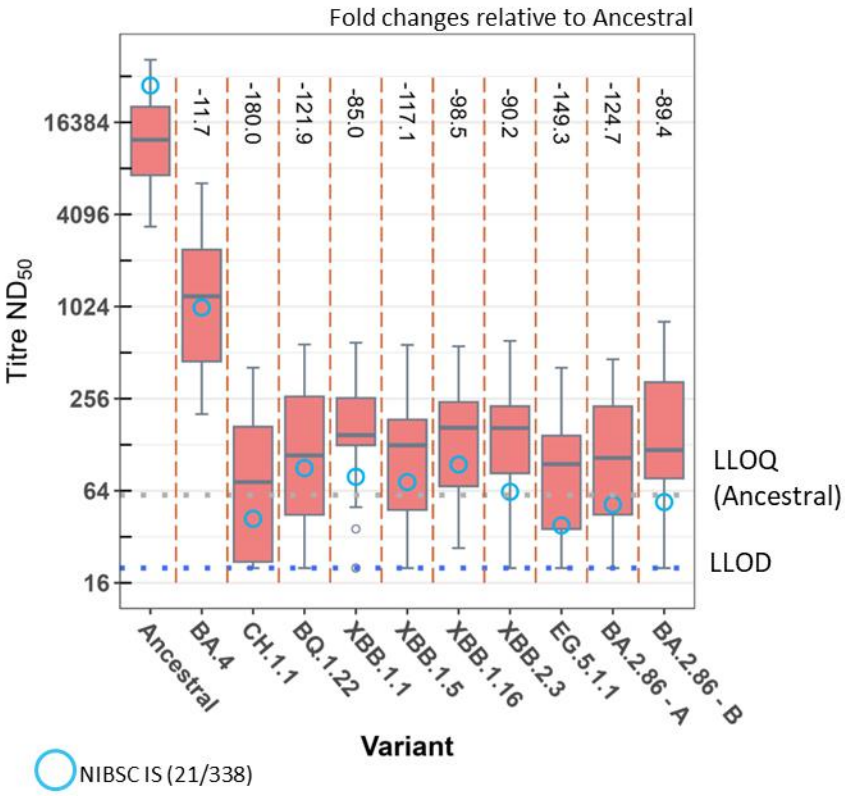

Figure 2. Neutralising antibody titres of a panel of sera/plasma from human volunteers who received three doses of an ancestral based vaccine as well as a BA.1 bivalent booster as determined by Focus Reduction Neutralisation Test (FRNT) against authentic SARS-CoV-2 Variants.

Sera/plasma from participants who received four doses of a vaccine were assessed by FRNT with 11 authentic SARS-CoV-2 variants. The panel consists of sera (n=14) and plasma (n=8) samples from human participants who received three doses of an ancestral based vaccine as well as a BA.1 bivalent booster. Of these, 19 out of 22 had at least one confirmed breakthrough infection with SARS-CoV-2. Data are presented as median and interquartile ranges of titre (ND_{50}). Fold-changes relative to ancestral virus were calculated by regression analysis using a linear mixed effect model and are presented where the difference is statistically significant ($p<0.05$, two-way ANOVA with Tukey's *post hoc* test). The Lower Limit of Detection (LLOD) of 1/20 is indicated by the blue dotted line. The Lower Limit of Quantification (LLOQ) of 1/58 as determined for the ancestral assay, is indicated by the grey dotted line. Neutralisation titres of the NIBSC WR 21/338 is presented as a blue circle.

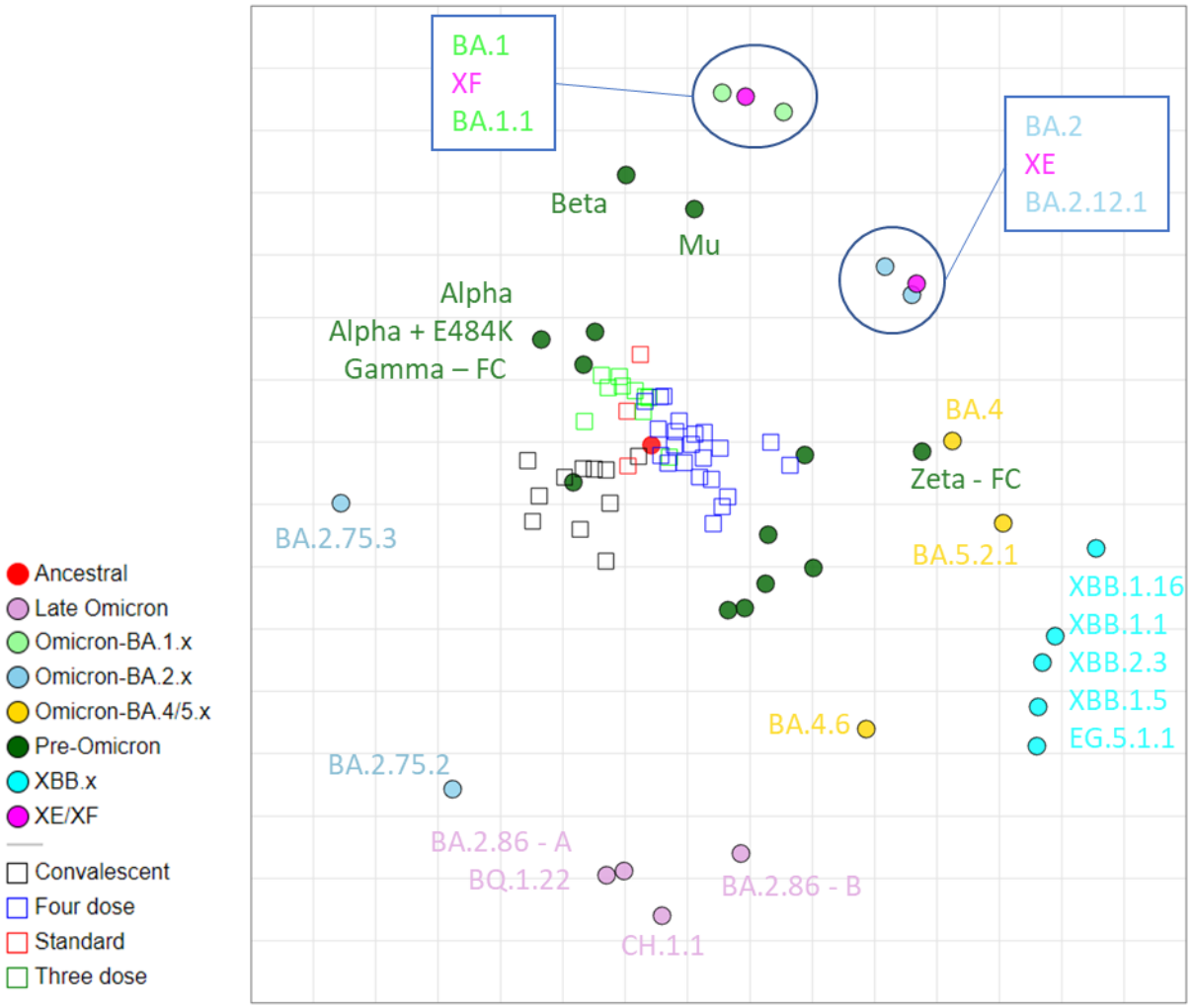

Figure 3. Antigenic map of SARS-CoV-2 variants assessed against human pre-Alpha convalescent, vaccinee sera or plasma and antibody standards

Median neutralisation titres (ND_{50}) for human sera/plasma were generated against 34 authentic SARS-CoV-2 variants and plotted as antigenic maps. Serum/Plasma are plotted as open-coloured boxes and virus variants (antigens) as filled circles. Convalescent plasma (n=11) collected early in the pandemic (pre-Alpha), sera (n=10) from triple-vaccinated individuals and sera/plasma (n=22) from those who had received a BA.1 bivalent booster are shown as black, green, and blue boxes, respectively. The standards, WHO IS 20/136, NIBSC WR 21/334 and IS 21/338 are also plotted as red boxes. Virus variants are shown as coloured circles.

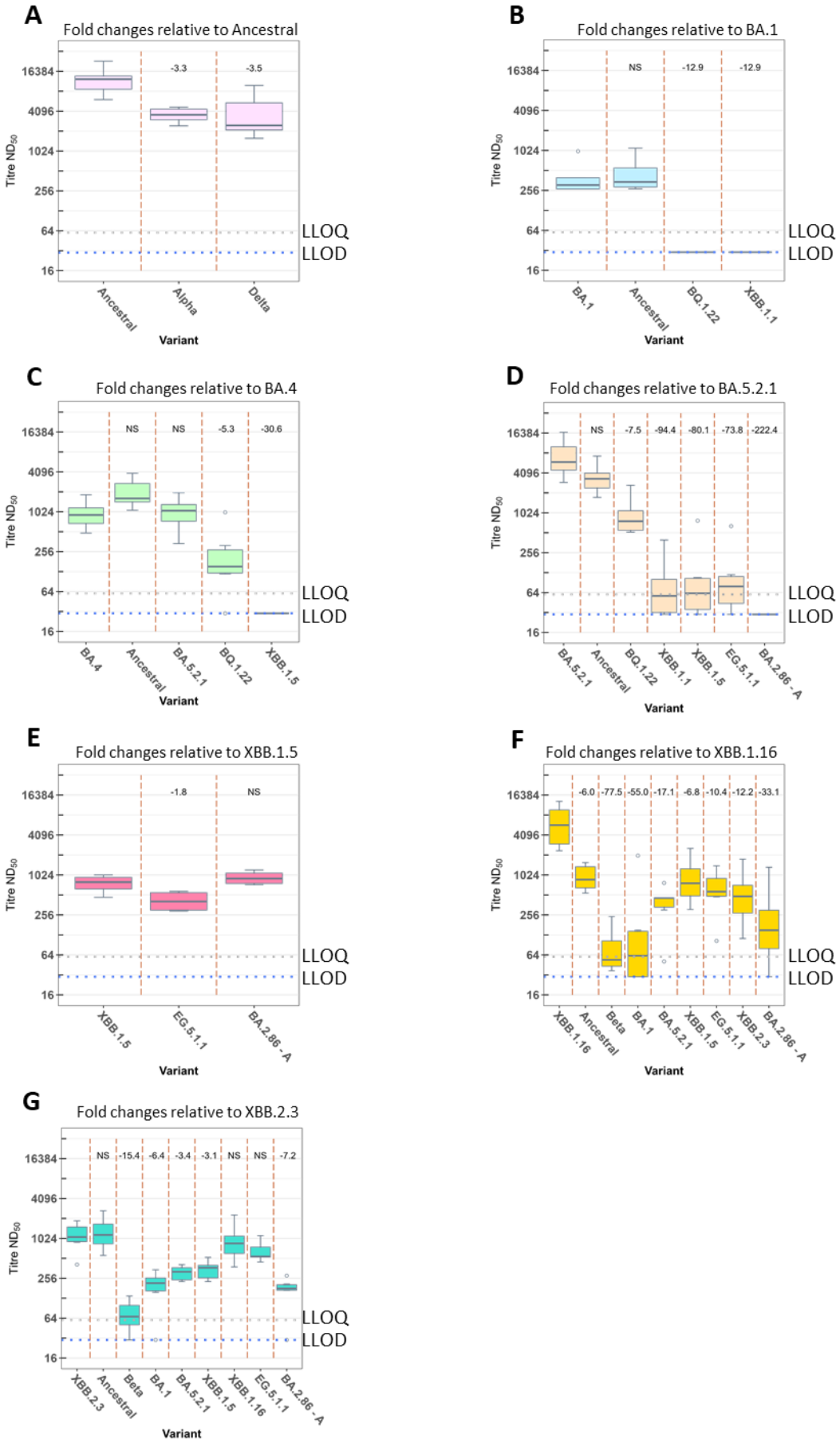
A
Fold changes relative to Ancestral
-3.3
-3.5
Titre ND50
16384
4096
1024
256
64
16
LLOQ
LLOD
Ancestral
Alpha
Delta
Variant
B
Fold changes relative to BA.1
NS
-12.9
-12.9
BA.1
Ancestral
BQ.1.22
XBB.1.1
C
Fold changes relative to BA.4
NS
NS
-5.3
-30.6
BA.4
Ancestral
BA.5.2.1
BQ.1.22
XBB.1.5
D
Fold changes relative to BA.5.2.1
NS
-7.5
-94.4
-80.1
-73.8
-222.4
BA.5.2.1
Ancestral
BQ.1.22
XBB.1.1
XBB.1.5
EG.5.1.1
BA.2.86 - A
E
Fold changes relative to XBB.1.5
-1.8
NS
XBB.1.5
EG.5.1.1
BA.2.86 - A
F
Fold changes relative to XBB.1.16
-6.0
-77.5
-55.0
-17.1
-6.8
-10.4
-12.2
-33.1
XBB.1.16
Ancestral
Beta
BA.1
BA.5.2.1
XBB.1.5
EG.5.1.1
XBB.2.3
BA.2.86 - A
G
Fold changes relative to XBB.2.3
NS
-15.4
-6.4
-3.4
-3.1
NS
NS
-7.2
XBB.2.3
Ancestral
Beta
BA.1
BA.5.2.1
XBB.1.5
XBB.1.16
EG.5.1.1
BA.2.86 - A

Figure 4. Neutralising antibody titres of panels of convalescent sera from hamsters experimentally infected with SARS-CoV-2 variants determined using an authentic virus Focus Reduction Neutralisation Test (FRNT)

Sera was collected from Golden Syrian hamsters at day 27 or day 28 post-infection with ancestral (A), BA.1 (B), BA.4 (C), BA.5.2.1 (D), XBB.1.5 (E), XBB.1.16 (F) or XBB.2.3 (G) SARS-CoV-2 variants. Median neutralisation titres (ND_{50}) were generated by FRNT using 14 authentic SARS-CoV-2 viruses. Data are presented as median and interquartile ranges of titre (ND_{50}). Fold-changes relative to the homologous virus were calculated by regression analysis using a mixed effect model and are presented where the difference is statistically significant ($p<0.05$, two-way ANOVA with Tukey's *post hoc* test). The Lower Limit of Detection (LLOD) of 1/30 is indicated by the blue dotted line. The Lower Limit of Quantification (LLOQ) of 1/58 as determined for the ancestral assay, is indicated by the grey dotted line. NS = not significant at the $p<0.05$ level.

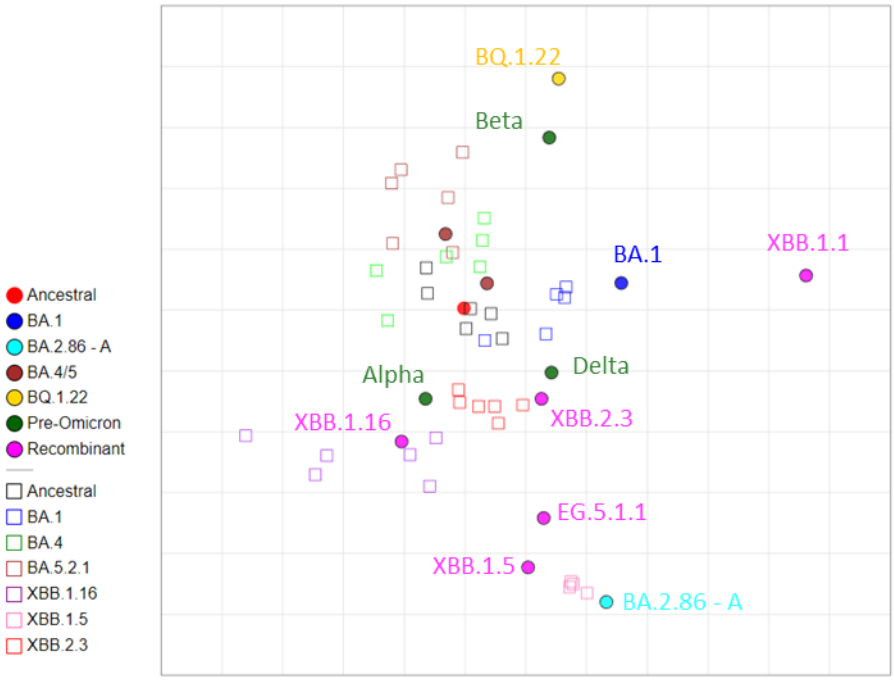

Figure 5. Antigenic map of SARS-CoV-2 variants assessed against convalescent sera collected from experimentally infected hamsters

Sera was collected from Golden Syrian hamsters at day 27 or day 28 post-infection with ancestral, BA.1, BA.4, BA.5.2.1, XBB.1.5, XBB.1.16 or XBB.2.3 SARS-CoV-2 variants. Median neutralisation titres (ND_{50}) were generated by Focus Reduction Neutralisation Test (FRNT) using 14 authentic SARS-CoV-2 viruses and plotted using antigenic cartography. Serum/Plasma are plotted as open-coloured boxes and virus variants (antigens) as filled circles.