Vaccine-induced and hybrid immunity against SARS-CoV-2 variants of concern in two cohorts in Queensland, Australia (2021-2022).

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Abstract

The spike glycoprotein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the main target for vaccine development, as antibodies generated against the spike protein are the most immunodominant and neutralizing against the virus. However, variants of concern (VOC), often containing multiple mutations within neutralizing epitopes, confer immune evasion of the response generated by current SARS-CoV-2 vaccines. To assess the immunogenicity and virus-neutralisation ability of antibodies induced by individual or combination of Vaxzevria (AstraZeneca: AZ) and Comirnaty (Pfizer: PZ) vaccines and of a hybrid immunity induced in people who were also infected with SARS-CoV-2, longitudinal sera samples of participants recruited through the David Serisier Research biobank (Mater Research Hospital) or at the University of Queensland were collected. ELISA with a panel of purified Spike proteins from ancestral, alpha, delta and omicron BA.1 and BA.2 VOCs showed significantly (by ~2 to 6-fold) reduced IgG antibody titres against Spike proteins from Omicron BA.1 and BA.2 compared to ancestral strain regardless of the number of vaccinations or presence of infection. Neutralisation assays showed reduced activity against delta and omicron BA.1, BA.5 and BA.5 VOCs. However, the differences were in general less pronounced than in the ELISA assay and some were not statistically significant, particularly after four (two AZ and two PZ) vaccinations. We also generated by circular polymerase extension reaction an attenuated SARS-CoV-2 strain with deletion of all accessory genes, ORF 3, 6, 7 and 8, based on the ancestral (QLD02) virus backbone (QLD02$_{\Delta 3678}$) and validated it in virus-neutralization assays with our panel of sera samples. We showed the attenuation of the QLD02$_{\Delta 3678}$ virus in Vero E6 and human Caco2 cells.

We demonstrated that neutralization assays with the wild-type QLD02 virus and QLD02$_{\Delta 3678}$ virus were concordant, providing a safe platform for neutralisation assays in BSL2/PC2 settings.

Introduction

The COVID-19 pandemic, caused by the SARS-CoV-2 virus, has highlighted the importance of monitoring adaptive immunity elicited from vaccination and natural infection to assess the duration of protection against SARS-CoV-2. Longitudinal monitoring of individuals allows to assess the duration of protection against SARS-CoV-2, compare the durability of different vaccines, track the dynamics of waning antibody titers, and identify the emergence of neutralization-resistant variants of concern.

Following SARS-CoV-2 infection or vaccination, the immune system mounts a robust antibody response, generating IgG, IgA, and IgM immunoglobulins. Seroconversion typically occurs within 15 days post-infection, with IgM and IgG peaking around 18 and 20 days, respectively[1,2]. Spike-specific antibodies typically neutralize the virus by binding to the receptor-binding domain (RBD), and block its interaction with cell surface receptor proteins, as well as mediating antibody-dependent cellular cytotoxicity and phagocytosis through Fc receptors on immune cells.

In Australia, Pfizer/BioNTech's BNT162b2 mRNA vaccine (PZ) formulated as RNA–lipid nanoparticles of nucleoside-modified mRNA[3] and the Oxford/AstraZeneca's ChAdOx1-S non-replicating adenovirus-vectored vaccine (AZ)[4] were the first approved COVID-19 vaccines by the Therapeutics Goods Administration (TGA). Both vaccines express the SARS-CoV-2 spike protein, and primary administration involves two doses of AZ vaccine (AZ_1/AZ_2) with an 8-12-week interval, and in some individuals followed by two doses of PZ vaccine (PZ_1/PZ_2) administered 4-8 weeks apart. Peak antibody titres are observed within 2-4 weeks, followed by a log-linear decline over 4 to 16 weeks[5-7]. The second dose significantly boosts antibody responses, leading to higher overall antibody titers[8]. Factors such as, age, sex, infection severity and hospitalisation status influence the peak magnitude of the IgG response[5,9-11].

While the primary regimen of AZ and PZ vaccines confer strong immunogenicity and higher short-term efficacy, their effectiveness wanes considerably 5-8 months post-vaccination. Despite this, they maintain effective real-world protection against hospitalisation and death after 6 months with 79% and 90% efficacy, respectively[12]. Although absolute IgG titres against the ancestral spike protein decline over time, durable, long-lived neutralising antibodies, particularly those targeting the RBD, exhibit a sustained plateau phase[2,13].

Variants of concern (VOC) are strains of SARS-CoV-2 that have undergone multiple mutations, leading to changes in transmissibility, disease severity, or immune escape potential[14]. The first VOC,

Alpha (B.1.1.7), identified in England, was estimated to have a 40–80% increased transmissibility than ancestral SARS-CoV-2[15]. Key defining mutations in the spike protein (D614G and ΔH69/V70) contribute to this phenotype[16,17]. The Delta VOC (B.1.617.2) demonstrated significant immune evasion with several studies reporting between 3.2-to 5-fold reduction in neutralisation with a primary AZ or PZ regimen[18-20]. The Omicron variant (B.1.1.529) includes several sublineages (BA.1, BA.2, BA.5) that are the most immunoevasive, with more than 15 mutations on average in the RBD. The neutralizing activity of sera from convalescent and double-vaccinated individuals was undetectable or very low against Omicron variants compared with the ancestral virus[21,22], whereas neutralizing activity of sera from individuals who had been exposed to spike three or four times through infection and/or vaccination was maintained, although at significantly reduced levels[22]. It was later shown that Omicron BA.1 escapes the majority of existing SARS-CoV-2 neutralizing antibodies[21].

The overall reduction in circulating IgG against the Spike protein and the emergence of immunoevasive VOCs suggested that the initial primary two-dose regimen was becoming less effective overall against VOC with less than 20% effectiveness against laboratory-confirmed Omicron infection at 6 months[23]. To counteract waning immunity and reduced neutralisation of VOCs, booster doses have been introduced[24]. These booster doses, typically administered six months after the primary vaccination series, have been shown to significantly enhance antibody levels and improve protection against emerging VOCs, although 9 months after booster administration vaccine effectiveness was less than 30% against laboratory-confirmed infection and symptomatic disease[23]. In Australia, the TGA approved the Pfizer COVID-19 vaccine on October 27, 2021 after interim results showed up to 95.3% vaccine efficacy[25].

To evaluate the immunogenicity and virus-neutralizing efficacy of responses induced by vaccinations we sampled two cohorts in Brisbane, Australia, one containing health workers recruited through David Serisier Research biobank (Mater Research Hospital) and another one containing participants from the University of Queensland. Samples were collected throughout the study from the same participants following 2, 3 and 4 vaccinations with combinations of Astra Zeneca (AZ) and Pfizer (PZ) vaccines. A separate set of samples was collected from participants who were vaccinated and infected with SARS-CoV-2 to assess hybrid immunity. The IgG antibody titres were assessed by ELISA against purified Spike proteins from different VOCs and virus-neutralising antibody titers were assessed against different VOCs in high throughput microneutralizations assay. Towards the development of a safer virus neutralization assay that can be performed in BSL2 facilities, we generated an attenuated SARS-CoV-2 with deletion of all accessory genes and compared its efficacy in neutralization assay with that of full-length infectious virus.

Materials and Methods

Human ethics, sera and nasal swab collection and processing

The collection of human sera was carried out following the approval of the Mater Misericordiae Ltd Human Research Ethics Committee (Reference: HREC/MML/68320) or the University of Queensland Human Research Ethics Committee (References: 2021/HE000362, 2021/HE000139). Blood samples, approximately 5mL in volume, were collected in serum-separating tubes and processed by centrifugation at 1000g for 10 minutes at 4ºC. After centrifugation, the sera were aliquoted into individual, single-use tubes and stored at -80°C. Nasopharyngeal aspirates of infected individuals were collected from consenting COVID-19-positive patients in Brisbane, Australia and collected for the recovery of SARS-CoV-2 isolates as previously described[26].

Cell lines

Vero E6 cells, derived from African green monkey kidney epithelia and Vero E6 stably expressing human TMPRSS2 (VE6-hTMPRSS2 Clone #1), as previously described[27], were cultured at 37 °C and 5% CO_2 in Dulbecco modified Eagle medium (DMEM; Gibco, Thermo Fisher, USA) supplemented with 10% fetal calf serum (FCS; Bovogen; USA), 1% GlutaMAX™ (Cat: 35050079, Thermo Fisher, USA), and 100 U/mL penicillin-streptomycin (Gibco, Thermo Fisher, USA), VE6-hTMPRSS2 cells were grown in the same media as Vero E6 cells with addition of 30µg/mL of puromycin dihydrochloride (A1113803, Thermo Fisher, USA). Caco2 (human colorectal adenocarcinoma cells) were cultured in RPMI Medium 1640 (Gibco, Thermo Fisher, USA) supplemented with 10% FCS and 100 U/mL penicillin-streptomycin. Human embryo kidney tissue cells stably expressing ACE2 and TMPRSS2 (HEK293T-ACE2-TMPRSS2) cells were generated by transduction of HEK293T-ACE2 cells, provided by Jesse Bloom (Fred Hutchinson Cancer Research Centre, Washington, USA) with a lentivirus containing puromycin-selectable codon-optimized human TMPRSS2 gene[28] and validated with anti-TMPRSS2 antibody (ab109131, Abcam, UK). These cells were selected and propagated in the media supplemented with 2µg/mL puromycin dihydrochloride.

SARS-CoV-2 viruses

This study used five low-passage SARS-CoV-2 viruses recovered from nasopharyngeal aspirates of infected individuals provided by the Queensland Health Forensic and Scientific Services, Queensland Department of Health, and the Queensland Institute of Medical Research. These strains included: I) an early ancestral (PANGO v.4.2 Lineage A) Australian isolate hCoV-19/Australia/QLD02/2020 (QLD02) sampled on 30/01/2020 (GISAID Accession ID; EPI_ISL_407896); II) the Delta variant (B.1.617.2), specifically hCoV-19/Australia/QLD1893C/2021 collected on 05/04/2021 (GISAID accession ID EPI_ISL_2433928); III) the Omicron BA.1, named hCoV-19/Australia/QLD2639C/2021 collected on 14/12/2021 (GISAID accession ID

141 EPI_ISL_8186598); IV) the Omicron BA.2 variant named as hCoV-19/Australia/QLD-QIMR02/2022,
142 collected on 12/05/2022 (GISAID accession ID: EPI_ISL_15671877.2); and V) an Omicron BA.5
143 sub lineage BE.1 variant named hCoV-19/Australia/QLD-QIMR03/2022 (GISAID accession ID:
144 EPI_ISL_15671874.2) collected 19/07/2022. All working virus stocks were propagated and titred on
145 VE6-hTMPRSS2 cells using an immuno-plaque assay[29].

146 **SARS CoV-2 HexaPro Spike protein expression**

147 SARS-CoV-2 HexaPro S proteins for the Ancestral (QLD02), Delta, BA.1 and BA.2 variants were
148 produced with the six proline substitutions and GSAS substitution of the furin cleavage site at
149 residues 682–685[30]. Codon-optimised gBlocks™ (IDT, Singapore) encoding the mutated HexaPro
150 S protein sequences were subcloned into the mammalian expression vectors pNBF-Hv or pNBF-Lv
151 in-frame with an IgK signal peptide and an affinity C-tag (EPEA) for purification. Expression of these
152 proteins was carried out in stable Chinese Hamster Ovary (CHO) bioreactors following previously
153 described protocols[31]. Specific mutations incorporated into the HexaPro S proteins for each variant
154 of concern were as follows: for the Alpha variant (B.1.1.7; ΔH69, ΔV70, ΔY144, N501Y, A570D,
155 D614G, P681H, T716I, S982A, and D1118H), Delta variant (B.1.617.2; T19R, G142D, Δ156E,
156 Δ157F, R158G, L452R, T478K, D614G, P681R, and D950N), Omicron BA.1 (A67V, D614G, D796Y,
157 E484A, G142D, G339D, G446S, G496S, ΔH69, H655Y, ins214EPE, K417N, L212I, L981F, ΔN211,
158 N440K, N501Y, N679K, N764K, N856K, N969K, P681H, Q493R, Q498R, Q954H, S371L, S373P,
159 S375F, S477N, T95I, T478K, T547K, ΔV70, ΔV143, ΔY144, ΔY145, Y505H) and Omicron BA.2
160 variant (A27S, D405N, D614G, D796Y, E484A, G142D, G339D, H655Y, K417N, ΔL24, N440K,
161 N501Y, N679K, N764K, N969K, ΔP25, ΔP26, P681H, Q493R, Q498R, Q954H, R408S, S371F,
162 S373P, S375F, S477N, T19I, T376A, T478K, V213G, Y505H).

163 **Serum anti-Spike IgG ELISA**

164 Antigen-specific IgG titres were quantified using the indirect ELISA method as previously
165 described[32,33]. Briefly, high-bind protein plates (Cat: 655061, Greiner, Bio-One, Austria) were coated
166 with 1 µg/mL of the HexaPro S antigen in PBS overnight at 4°C. The following day, plates were
167 blocked with 5% KPL Milk Diluent/Blocking Solution (Cat: 5140-0011; SeraCare, USA) for 30 min at
168 37ºC. Sera samples were diluted in blocking buffer starting at a dilution of 1:10 and subsequent
169 serial dilutions at fivefold increments. A negative control of pooled pre vaccination baseline was
170 included with blocking buffer, while a human anti-Spike monoclonal (CR3022, 50 µg/mL)[34]. After
171 blocking 40 µL of each serial dilution was added to the ELISA plates and incubated at 37ºC for 1
172 hour. Plates were washed three times in PBS-Tween 20 (PBS-T, 0.1%). After primary incubation
173 and washing, 50 µL of goat anti-Human IgG Fc HRP antibody (0.5 µg/mL, Cat: A18829, Thermo
174 Fisher Scientific, USA) with blocking buffer was added to each well. The plates were incubated for
175 1 hour at 37°C. After five washes with 0.1% PBST, 50µL of 3,3',5,5'-tetramethylbenzidine (Cat:

176 002023, Life Technologies, USA) substrate was added. The plates were incubated at room
177 temperature for 5 minutes and the reaction was stopped by adding 1M sulfuric acid. Absorbance
178 was measured at 450 nm using a Varioskan LUX Microplate reader (Thermo Fisher Scientific, USA)
179 using the SkanIt™ Software v6.0.1 (Thermo Fisher Scientific, Waltham, MA, USA).

180 **SARS-CoV-2 virus microneutralization assay**

181 For the virus neutralization assays, $3x10^5$ VE6-hTMPRSS2 cells were seeded in 96-well plates in
182 complete puromycin-containing medium as previously described and incubated overnight at 37°C
183 with 5% CO_2. On the day of assay, serum samples were diluted in Gibco™ minimum essential
184 medium (MEM) supplemented with 2% (FCS) in sterile round-bottom 96-well plates. The initial
185 dilution was 1:10, and serial 1:2 dilutions were performed. The media control wells contained
186 MEM+2% FCS medium only. Each contained a media-only control and a pooled baseline sample
187 from non-infected and non-vaccinated individuals from the Mater cohort. SARS-CoV-2 viruses were
188 diluted in MEM+2% FCS and added to each well of the 96-well plates containing sera except control
189 (virus-negative) wells. The media control wells contained MEM+2% FCS only. The plates were then
190 incubated at room temperature for 1 hour. After incubation, 120µL of virus plus sera was transferred
191 to VE6-TMPRSS2 cells and incubated at 37°C for 1 hour. Following the incubation period, the
192 medium was aspirated, and 200 µL of MEM+2% FCS was added to each well. The plates were then
193 incubated for 48 hours at 37°C. After 48 hours, the medium was aspirated, and the plates were fixed
194 in ice-cold 80% acetone in PBS and placed at -20°C for 30 minutes before removal from the facility
195 and processing in a PC2 laboratory. After acetone removal from the plates, the plates are dried, and
196 PBS is added to flush out the remaining acetone. The plates were then blocked in 5% KPL solution
197 diluted in PBS-T for 1 hour at 37°C. For primary incubation, the human Fc-linked anti-SARS-CoV-2
198 nucleocapsid nanobody C2[35] was added at 1 µg/mL and incubated for 1 hour at 37°C. Plates were
199 then washed three times with PBS-T. Subsequently, 0.2 µg/mL anti-human IgG Fc Highly Cross-
200 Adsorbed Secondary Antibody (Cat: A18829, Thermo Fisher Scientific, USA) for 1 hour at 37°C. The
201 plates were incubated with TMB as previously described, and the reaction was stopped by adding
202 1M sulfuric acid. Absorbance was measured at 450 nm using a Varioskan LUX Microplate reader
203 (Thermo Fisher Scientific, USA).

204 **Generation of SARS-CoV-2 QLD02$_{\Delta ORF3678}$ with Circular Polymerase Extension Reaction**

205 Design of the attenuated recombinant SARS-CoV-2 QLD02$_{\Delta ORF3678}$ virus followed that of a live-
206 attenuated SARS-CoV-2 vaccine candidate with accessory protein deletions[36] with some minor
207 modifications. The virus was generated using the Circular Polymerase Extension Reaction (CPER)
208 as per our previously described method[27] with the following modifications (see Figure 4A). The
209 CPER amplicon design was refined to enable seamless incorporation (and exchange) of the
210 complete spike gene into fragment 5, involving updated primer sets (ext2), allowing for longer

sequence overlaps in amplicons used for CPER assembly. Fragment 1 containing part of 5'UTR sequence and NSP1-NSP3 genes (5025nt of viral genome) was amplified using primers F1-F 5'-ATTAAAGGTTTATACCTTCCCAGGTAACAAACCAACCAACTTTCG-3' and F1-R 5' -GACATGTCCACAACTTGCGTATGTAGGTTAATGTTGTCTACTG – 3'. Fragment 2 containing NSP3-NSP5 genes and the start of NSP6 gene (6014nt of viral genome) was amplified using primers F2-F 5'-AACAGTAGACAACATTAACCTACATACGCAAGTTGTGGACATGTC-3' and F2-R 5'-TGATTGTTCTTTTCACTGCACTTTGGAAAGTAACACCTGAGCATT-3'. Fragment 3 containing NSP6-NSP13 genes (6431nt of viral genome) was amplified using primers F3-F 5'-AATGCTCAGGTGTTACTTTCCAAAGTGCAGTGAAAAGAACAATCA-3' and F3-R 5'-AATTTGTGGCCATTGAAATTTCATCAAAGACAACTATATCTGCTG-3'. Fragment 4 containing NSP13-NSP16 genes (4189nt of viral genome) was amplified using primers 4-F 5'-CAGCAGATATAGTTGTCTTTGATGAAATTTCAATGGCCACAAATT-3' and 4-R 5'-ACAACTCTGTTGTTTTCTCTAATTATAAGTCTACCTTTACTAAGAAGAGAT-3'. Fragment 5 containing the c-terminal part of the NSP16 gene and S gene (3911nt of viral genome) was amplified using primers F5-F 5'-ATCTCTTCTTAGTAAAGGTAGACTTATAATTAGAGAAAACAACAGAGTTGT-3' and F5-R 5'-TTATGTGTAATGTAATTTGACTCCTTTGAGCACTGGCTCAGAGTC-3'. Fragment 6A (1081nt in total) containing the ORF3 deletion amplified using primers 6A-F 5'-GACTCTGAGCCAGTGCTCAAAGGAGTCAAATTACATTACACATAAGCACAAGCTGATGAGTAC GAACTTATGTACT-3' and 6A-R 5'-TTACTGTACAAGCAAAGCAATATTGTCACTGCTACTGG-3'. Fragment 6B (1675nt in total) containing deletion of ORF 6,7 and 8 genes was amplified with primers 6B-F 5'-CCAGTAGCAGTGACAATATTGCTTTGCTTGTACAGTAATTCGTGTTGTTTTAGATTTCATCTAA-3' and 6B-R 5'-GTCATTCTCCTAAGAAGCTATTAAAATCACATGGGGATAGCAC-3'. The linker fragment containing the last 43 nucleotides of the viral genome, 30A nucleotides, HDV ribozyme, spacer region, CMV promoter and the first 45 nt of the viral genome was generated using primers Linker-ext2-F 5'-GTGCTATCCCCATGTGATTTTAATAGCTTCTTAGGAGAATGAC-3' and Linker-ext2-R 5'-CGAAAGTTGGTTGGTTTGTTACCTGGGAA-5'. Each purified SARS-CoV-2 and linker fragments were pooled in equimolar amounts (0.1 pmol) in a 50µL CPER reaction with 2 µL of PrimeSTAR GXL DNA Polymerase (Takara Bio, Japan). The cycling conditions included: initial denaturation at 98°C for 1 minute, followed by 12 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 20 seconds, and extension at 68°C for 10 minutes, followed by a final extension at 68°C for 10 minutes. Post-CPER assembly, the 50µL reaction mixture was transfected into HEK293T-ACE2-TMPRSS2 cells with Lipofectamine LTX Plus reagent (Invitrogen, USA) as per manufacturer's protocol. Twelve hours post-transfection, cells were trypsinised and overlayed onto VE6-hTMPRSS2 cells. The cells were monitored daily for cytopathic effect (CPE), and culture

247 supernatant was tested using Rapid Antigen Tests (Medomics, China) until a positive result was
248 obtained. The recovered virus was titred on VE6-hTMPRSS2 cells using an immuno-plaque assay[29].

249 **Statistical analysis**

250 Statistical analysis and visualisation were conducted with GraphPad Prism 10.0.2 (GraphPad
251 Software Inc., San Diego, CA, USA). The IC_{50} value of sera was calculated by regression analysis
252 (Sigmoidal, 4PL). The IC_{50} was used to indicate the IgG antibody titer. The neutralization index was
253 calculated by the following equation: neutralization level = 1- $[(|OD_s-OD_b|-OD_m)/OD_v-OD_m]$%. ($OD_s$:
254 absorbance of sample sera, OD_b: absorbance of baseline sera, OD_m: absorbance of media control,
255 OD_v: absorbance of virus control). Nonlinear regression ($log_{inhibitor}$ vs. normalized response) was
256 used to calculate the IC_{50} for neutralization assay. A linear regression analysis was undertaken to
257 compare recombinant QLD02ΔORF3678 and QLD02 WT virus for viral neutralisation assays. For
258 comparisons between VOCs in immunogenicity and neutralisation data, groups were first tested for
259 normal distribution using the Kolmogorov-Smirnov test.

260 ## Results

261 **Sample collection and demographic characteristics of Mater Hospital and University of**
262 **Queensland cohorts.**

263 To assess the immune response elicited by vaccines or a hybrid immunity, blood samples were
264 collected approximately six weeks after the double primary vaccinations AZ_1AZ_2 or PZ_1PZ_2 and
265 heterologous booster vaccinations with PZ; $AZ_1AZ_2PZ_3$ and $AZ_1AZ_2PZ_3PZ_4$ (Figure 1A). We
266 conducted longitudinal sampling for sera of individuals at two locations: Mater Hospital, Brisbane,
267 Queensland, Australia (Mater) and the University of Queensland (UQ), Brisbane, Australia, from
268 March 2021 to December 2022 (Figure 1A). Primary vaccinations of AZ/PZ coincided with the
269 emergence and dominance of the Delta VOC (June 2021 – December 2021) (Figure 1B).
270 Heterologous and homologous PZ for booster vaccination, coinciding with the dominance of the
271 Omicron BA.1 lineage (January – February 2022). Subsequent Omicron sub-lineages BA.2 and
272 BA.5 coincided with the double booster $AZ_1AZ_2PZ_1PZ_2$ cohort.

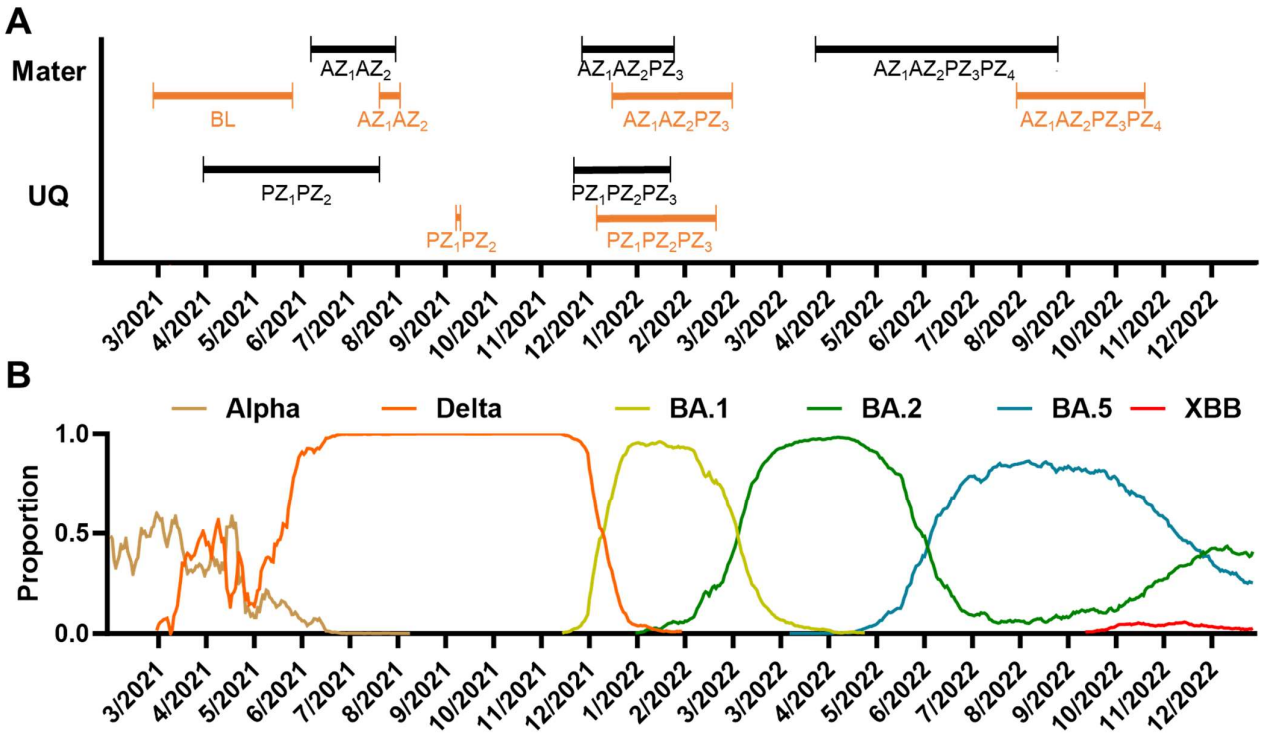

Figure 1: **Timescale of vaccination and sera collection for study cohorts and genetic epidemiology of SARS-CoV-2 VOC lineages in Australia.** A) The vaccination (black) and sera collection (orange) for AZ and PZ doses from the Mater and UQ cohorts with baseline (BL) collection indicated B) Genetic epidemiology of Australian SARS-CoV-2 variants of concern from March 2021-December 2022 generated using CoV-Spectrum[37]

At Mater, 286 healthcare workers were initially enrolled in the study, and baseline pre-vaccination sera were sampled for 191 participants. Out of these participants, sera samples were collected from 62 individuals who received double doses of the AZ vaccine (AZ_1AZ_2 group). Out of this group, 43 individuals received a heterologous booster of the PZ vaccine ($AZ_1AZ_2PZ_3$), and within this subgroup, 34 individuals received a second PZ booster ($AZ_1AZ_2PZ_3PZ_4$). Longitudinal samples from these 34 participants were used to assess the IgG and virus-neutralising antibody responses. Additionally, 11 subjects from the $AZ_1AZ_2PZ_3$ booster group were diagnosed with the SARS-CoV-2 virus, with sera sampled at the point of care; this group is considered a hybrid immunity group and was sampled between January to August 2022, during the Omicron BA.1 to BA.2 period, which are implied as the likely infection.

The overall Mater cohort was predominantly female (79.6%), with a mean age of 46.6 years (SD= 12.4, range 22-74 years). There were minimal differences in the average ages between the vaccination groups, with the AZ_1AZ_2 group being, on average, the youngest (43.9 ± 12.0 years), followed by the $AZ_1AZ_2PZ_3$ group (47.4 ± 11.0 years) and the four-dose group (49.8 ± 11.0 years). The hybrid immunity group was 40.8 ±12.6 years.

294 For the PZ cohort, sera were also collected from 15 researchers at the University of Queensland
295 (UQ). Blood draws occurred seven to nine weeks after the initial two-dose vaccination (PZ_1PZ_2
296 group) for these 15 individuals. Samples from a three-dose group that received a homologous PZ
297 booster ($PZ_1PZ_2PZ_3$ group, n=10 were collected two to three weeks after the third vaccine dose.
298 Within the UQ cohort, 5 individuals (22.7%) were diagnosed with COVID-19 through positive rapid
299 antigen or PCR tests. Of the 5 participants in the COVID+ group, two contracted SARS-CoV-2 after
300 receiving two PZ doses, and three after receiving three PZ doses - all of whom have been allocated
301 into the hybrid immunity group, which was collected between January – Feb 2022 and are implied
302 as being infected with BA.1 (Figure 1B). These were pooled with COVID+ samples from the Mater
303 cohort into a hybrid immunity group (n=16), none of these individuals had COVID prior to
304 vaccination.

305 Table 1: Demographic information of the Mater and UQ cohorts.

Cohort	Group	n	Age ($\bar{x}$ ± SD)	Male	Female
Mater	AZ_1AZ_2	62	43.9±12.0	12 (16.7%)	50 (83.3%)
	$AZ_1AZ_2PZ_3$	44	47.4±11.0	9 (20.5%)	35 (79.5%)
	$AZ_1AZ_2PZ_3PZ_4$	34	49.8±12.5	7 (20.6%)	27 (79.4%)
	COVID+	11	40.8±12.6	4 (36.3%)	7 (63.6%)
UQ	PZ_1PZ_2	15	31.3±9.9	8 (53.3%)	7 (46.7%)
	$PZ_1PZ_2PZ_3$	10	36.2±11.2	6 (60.0%)	4 (40.0%)
	COVID+	5	24.1±3.2	4 (80.0%)	2 (20.0%)

306

307 The mean age of the total UQ cohort was 30.5 ± 9.3 years, with 54.5% of participants being male
308 (Table 1). The oldest average age was in the $PZ_1PZ_2PZ_3$ group (36.2 ± 11.2 years), while the
309 COVID+ group had the youngest mean age (24.1 ± 3.2 years) (Table 1).

310 **IgG antibody responses after vaccinations and infection.**

311 We first assessed the sera samples from individuals vaccinated with AZ and PZ vaccines and those
312 with hybrid immunity against the purified Spike proteins of various SARS-CoV-2 variants of concern
313 (VOCs). Serum IgG antibody titres against the HexaPro S protein derived from the ancestral QLD02
314 strain and Alpha, Delta, Omicron BA.1 and BA.2 variants of concern were measured using indirect
315 ELISA. Antibody titres were represented by quantifying the half-maximal inhibitory concentration
316 (IC_{50}) values, with decreases in fold change relative to the ancestral QLD02 strain (Figure 2).

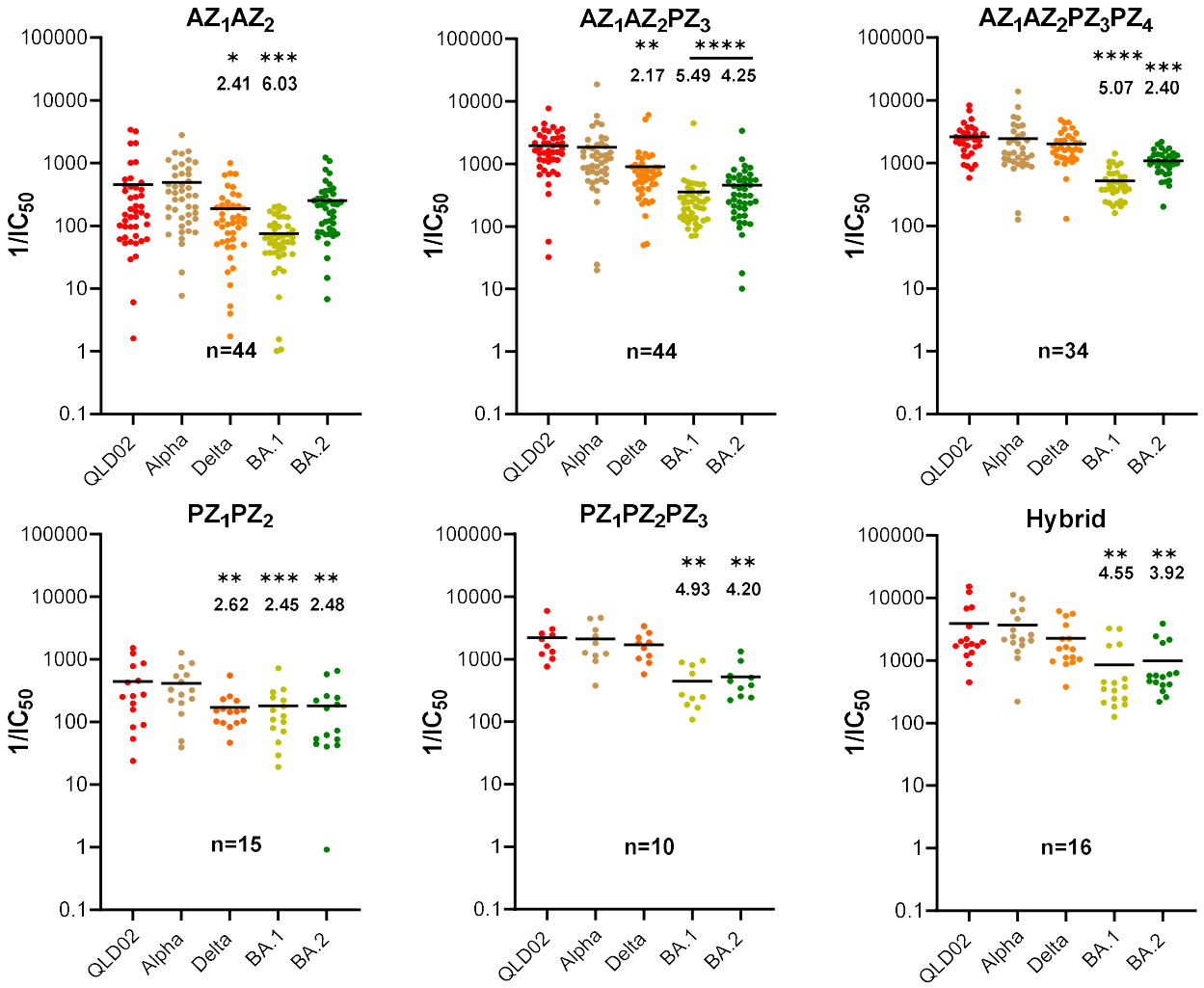

Figure 2: IgG antibody titres against purified Spike proteins from different SARS-CoV-2 VOCs. Average serum IgG antibody titres against HexaPro S protein derived from QLD02, Delta, and Omicron BA.1 and BA.2 variants measured by ELISA and shown by IC_{50} value. The decreased fold change compared with the ancestral virus QLD02 are shown in the figure. Ordinary one-way ANOVA, with Dunnett's multiple comparisons post hoc test, was used to compare samples from the same subject population against QLD02 and the fold change against QLD02 IC_{50} value is shown. Number of participants in each group is given in Table 1. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$ and non-significant (ns).

Generally, the third vaccination with the PZ vaccine elicited higher IgG antibody titres than the first two vaccinations, regardless of whether the primary regimen was AZ or PZ (Figure 2). The fourth vaccination in the cohort that received two AZ shots and one PZ shot before the fourth PZ booster did not elicit a significant increase in IgG antibody titres against the Spike proteins from ancestral virus and alpha variant while slightly improving IgG antibody titres against later VOCs (Figure 2).

Sera from vaccinated individuals, irrespective of the vaccine received, showed diminished IgG titres against Spike from Delta and Omicron VOCs, particularly against the Omicron BA.1 and BA.2 variants (Figure 2).

334 All vaccine groups demonstrated a consistent order of immunogenicity from highest to lowest
335 against the Spike proteins from ancestral QLD02 to Delta, BA.2, and then BA.1 VOCs, irrespective
336 of vaccine status. In the AZ_1AZ_2 group, IgG titres against Delta and Omicron BA.1 exhibited
337 decreases by 2.41- and 6.03-fold, respectively, compared to QLD02 while decrease against BA.2
338 was not significant. Within the $AZ_1AZ_2PZ_3$ cohort, the observed fold decreases were 2.17 for Delta,
339 5.49 for BA.1, and 4.25 for BA.2. Participants in the $AZ_1AZ_2PZ_3PZ_4$ group showed less of a decrease
340 in titres against VOCs, with a 5.07 for BA.1, and 2.40 for BA.2. The hybrid immunity group exhibited
341 a 4.55-fold decrease for BA.1 and 3.92-fold decrease for BA.2 while no significant decrease was
342 detected for Delta variant.

343 For the PZ-vaccinated cohorts, the PZ_1PZ_2 group demonstrated a 2.62-fold decrease in IgG titres
344 against Delta, 2.45 for BA.1, and 2.48 for BA.2 compared to QLD02. The $PZ_1PZ_2PZ_3$ group had a
345 4.93-fold decrease for BA.1 and 4.20 for BA.2, while no significant difference in antibody titres were
346 detected against the Delta variant.

347 **Neutralising antibody responses against SARS-CoV-2 VOCs**

348 The virus neutralizing capacity of sera was tested against a panel of SARS-CoV-2 strains, including
349 the ancestral strain QLD02 and the variants of concern (VOCs) Delta, and the Omicron lineages
350 BA.1, BA.2, and BA.5, across different vaccination regimens: AZ_1AZ_2, $AZ_1AZ_2PZ_3$, $AZ_1AZ_2PZ_3PZ_4$,
351 PZ_1PZ_2, $PZ_1PZ_2PZ_3$ and the hybrid immunity group (Figure 3). The Alpha VOC was not tested for
352 neutralization as it did not show significant differences to QLD02 in immunogenicity tested by ELISA
353 in these sera samples (Figure 2). BA.5 VOC was added to the testing as the latest Omicron variant
354 circulating at the time of the study. The neutralizing antibody titres were quantified using a
355 microneutralization assay, expressed as $1/IC_{50}$ values, representing the serum dilution required to
356 inhibit 50% of the virus infectivity.

357 In the AZ_1AZ_2 group, the sera showed a slightly reduced but significant neutralization against BA.1
358 and BA.5 with fold changes of 1.48 and 1.50, respectively. In contrast, the $AZ_1AZ_2PZ_3$ group
359 exhibited a significant reduction in neutralization capacity against all VOCs ranging from 2.23 (BA.2)
360 to 3.88 (Delta). Notably, the $AZ_1AZ_2PZ_3PZ_4$ group demonstrated no significant virus neutralization
361 differences against all tested VOCs, including all Omicron variants, clearly demonstrating the benefit
362 from an additional PZ booster.

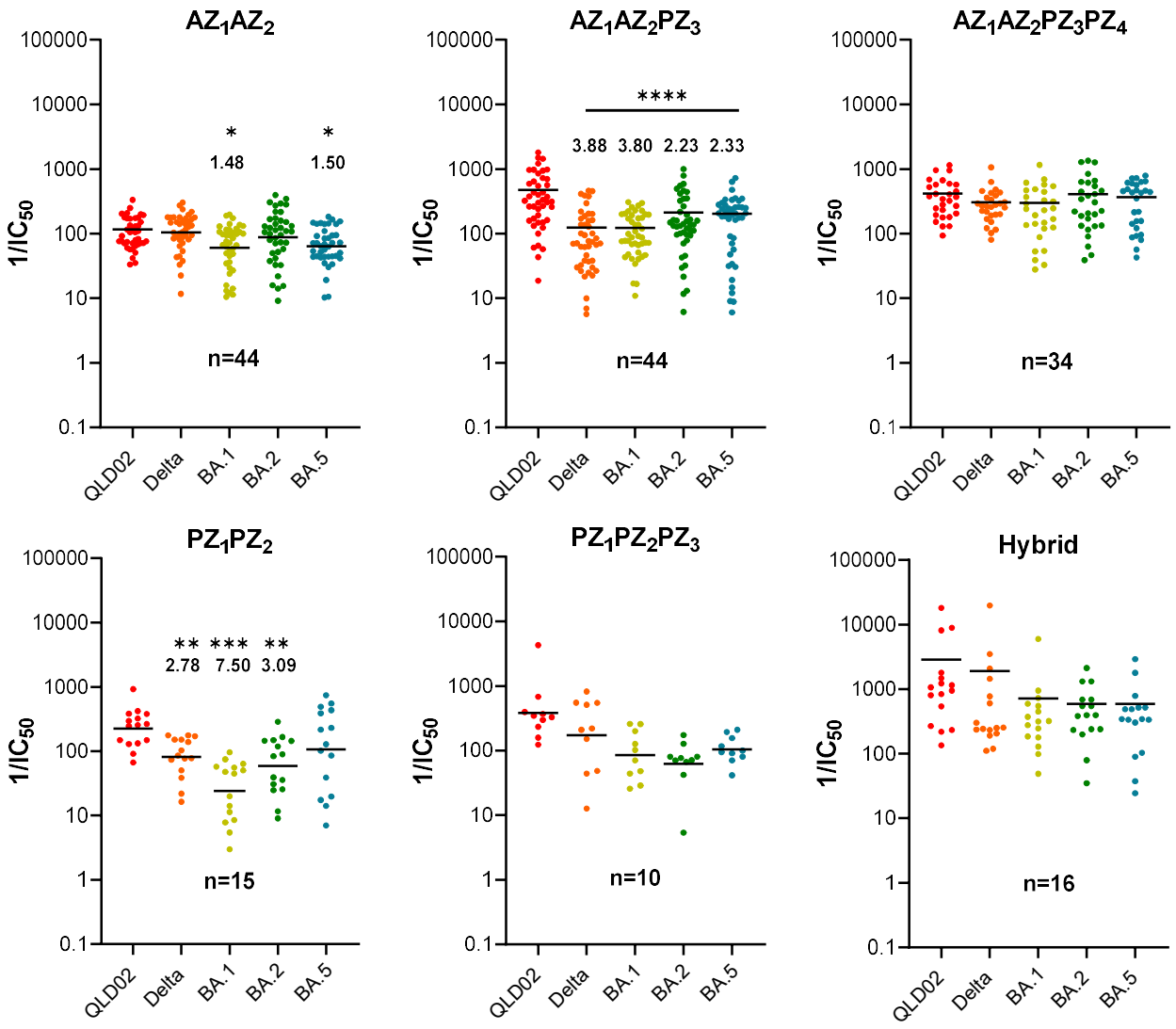

Figure 3: Virus-neutralizing antibody titres against SARS-CoV-2 VOCs. Sera neutralization antibody titres against QLD02, Delta, BA.1, BA.2, and BA.5 variants detected by microneutralization assay are shown by $1/IC_{50}$ values. Fold change reductions against VOCs compared with QLD02 ancestral virus are indicated. The mean and SEM are shown by the black bar. P value was calculated by using the two-way ANOVA with Dunnett's multiple comparisons test against QLD02. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$ and non-significant (ns). Number of participants in each group is given in Table 1.

Participants in the PZ_1PZ_2 group displayed the greatest reduction in neutralizing titres against BA.1 (7.5-fold, $p < 0.001$), followed by BA.2 (3.09-fold, $p < 0.01$) and Delta (2.78-fold, $p < 0.01$), with no significant reduction in neutralization for the BA.5 variant (Figure 3). The $PZ_1PZ_2PZ_3$ cohort showed no significant difference in neutralization for all VOCs, although this could be due to a smaller number of samples (Figure 3).

376 For the hybrid immunity group, no significant differences were also observed for all VOCs despite
377 visual difference, although this could also be due to the smaller cohort size (Figure 3).

378 Overall, the results indicate that while initial vaccinations with AZ or PZ vaccines showed a tendency
379 for decreased neutralization of Delta and Omicron OCs, additional booster(s) with PZ vaccine
380 appear to elicit more effective neutralization responses against theses VOCs.

381 **Generation of an attenuated SARS-CoV-2 virus with deletion of all accessory genes and its**
382 **application for virus-neutralization assays.**

383 Currently, live virus neutralisation assays of sera samples need to be undertaken in facilities with
384 BSL3/PC3 containment. SARS-CoV-2 virus with deletion of all accessory genes, ORF 3, 6, 7 and 8
385 (ΔORF3678), was recently developed and was shown to be severely attenuated in interferon
386 response-competent human cells and in animals while still allowing efficient propagation in
387 interferon-deficient Vero E6 cells[36]. Such attenuated viruses allow neutralization assays to be
388 performed in less stringent BSL2/PC2 containment facilities while retaining the characteristic
389 features of SARS-CoV-2 infection[38].

390 Herein, we employed our previously developed for SARS-CoV-2 circular polymerase extension
391 reaction (CPER)[27] to generate SARS-CoV-2 virus with deletion of ORFs 3, 6, 7 and 8 based on
392 ancestral QLD02 strain $QLD02_{\Delta ORF3678}$. To facilitate rapid adaptation of neutralization analysis with
393 future VOCs we reconfigured our previous CPER amplicon strategy to allow seamless swapping of
394 the complete Spike genes from any future VOCs in fragment 5 (Figure 4A). Using our new CPER
395 design, we recovered the $QLD02_{\Delta ORF3678}$ virus following transfection of CPER mix into HEK293-
396 ACE2-TMPRSS2 cells and propagation in VE6-hTMPRSS2 cells yielding viral titre of 1.34 x 10^6
397 FFU/mL. RT-PCR from isolated viral RNA confirmed ORF3678 deletion (Figure 4B). Immunoplaque
398 assay showed that the virus produced smaller foci compared to the parental QLD02 virus in Vero
399 E6 cells (Figure 4C).

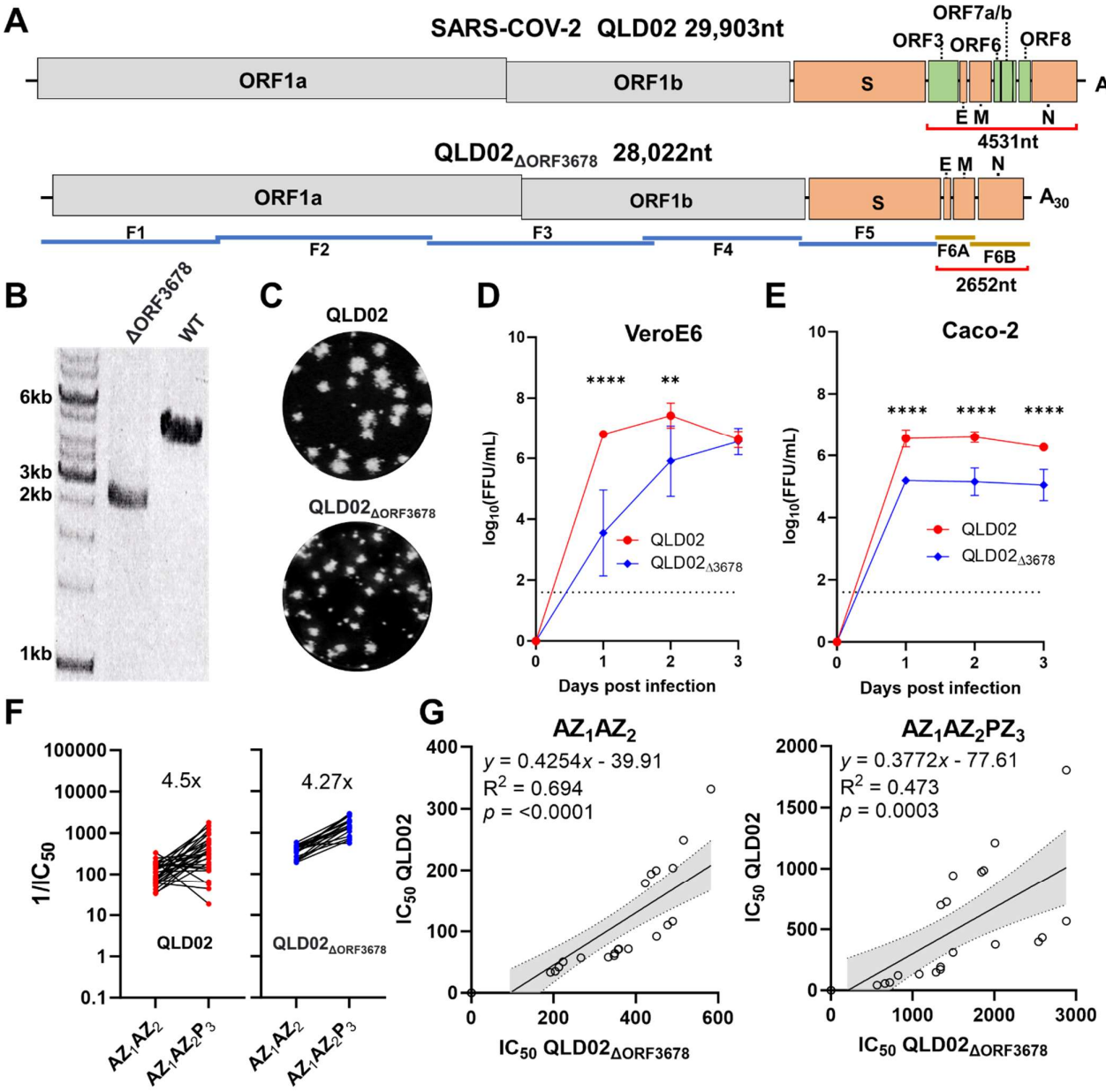

Figure 4: **Generation and characterisation of an attenuated recombinant QLD02$_{\Delta ORF3678}$ virus as a substitute for native SARS-CoV-2 in virus-neutralization assay.** A) Schematics of the SARS-CoV-2 QLD02 and the recombinant QLD02$_{\Delta ORF3678}$ virus genomes and amplicon fragments used for CPER assembly. Red lines with nucleotide sizes show RT-PCR amplicons of fragment 6 for the QLD02 and QLD02$_{\Delta ORF3678}$ viruses shown in B). C) Immuno-plaque assay with anti-SARS-CoV-2 nucleocapsid nanobody at 20 hours post-infection. Growth kinetics of WT QLD02 (red) and QLD02$_{\Delta ORF3678}$ (blue) viruses over a 3-day time course in D) Vero E6 cells and E) Caco2 cells infected at a multiplicity of infection MOI = 0.01, n = 2 independent experiments with three replicates in each, statistical analysis was performed by two-way analysis of variance with Tukey's multiple comparisons test against WT virus. Mean values for each virus at each time point are shown ± SD. F) Sera neutralization antibody titres against QLD02 and QLD02$_{\Delta ORF3678}$ SARS-CoV-2 virus by microneutralization assay are shown by IC_{50} value against 20 matched samples that were in both the AZ_1AZ_2 and $AZ_1AZ_2PZ_3$ groups. F) The linear relationship between matched samples of IC50 values from both AZ_1AZ_2 and $AZ_1AZ_2PZ_3$ samples (n=20) and baseline unvaccinated samples (n=3) from G) The linear regression function, the coefficient of determination (R^2) and the *p*-value for the function are given on both graphs, a 95% confidence band for the line of best fit are shown.

417 We then compared the growth of WT QLD02 and QLD02$_{\Delta ORF3678}$ viruses in interferon-deficient Vero
418 E6 cells and interferon-competent human Caco-2 cells at a MOI 0.01 over a three-day time course.
419 Both viruses showed pronounced growth in Vero E6 cells (Figure 4D), with the QLD02$_{\Delta ORF3678}$
420 exhibiting delayed replication and significant differences between titres of the two viruses in the first
421 two days (Day 1 $p<0.0001$, Day 2 $p=0.0020$, two-way ordinary ANOVA with Tukey's multiple
422 comparisons test) while reaching comparable titres by day 3 ($p=0.9997$) (Figure 4D). In the
423 interferon competent human Caco2 cell line, QLD02$_{\Delta ORF3678}$ grew to significantly lower viral titres
424 than WT QLD02 at all time points (Figure 4E).

425 To examine if QLD02$_{\Delta ORF3678}$ virus could be used as a surrogate for virus-microneutralization assays,
426 sera samples from 20 individuals that matched between AZ_1AZ_2 and $AZ_1AZ_2PZ_3$ groups were
427 examined for virus neutralisation assays with the ΔORF3678 virus and compared with neutralization
428 by WT QLD02 virus. The serum neutralization titres for the ΔORF3678 compared to the WT QLD02
429 virus were on average 3.29-fold higher for the AZ_1AZ_2 samples and 3.11-fold higher for the
430 $AZ_1AZ_2PZ_3$ samples indicating higher assay sensitivity. In addition, the assay with QLD02$_{\Delta ORF3678}$
431 virus showed lower variability between individual samples than WT QLD02 virus while maintaining
432 similarly increased neutralization between pre- and pos-booster immunization groups (Figure 4F).

433 To further examine the harmony between individual samples being assayed for IC_{50} using both
434 viruses we plotted the relationship between IC_{50} values of the WT virus and ΔORF3678 virus for the
435 same samples (Figure 4G). Three pre-immunisation sera samples were added to calibrate the
436 baseline at IC_{50} 0. Generally, there was a strong linear relationship between WT QLD02 IC_{50} values
437 and the ΔORF3678 virus for the AZ_1AZ_2 (R^2 = 0.694) and a slightly weaker relationship for the
438 $AZ_1AZ_2PZ_3$ samples (R^2 = 0.473). There was also a significant linear relationship between IC_{50}
439 values for the WT virus and ΔORF3678 virus for both pre- and post-booster immunization groups
440 as indicated by the slope test (AZ_1AZ_2, p = <0.0001; $AZ_1AZ_2PZ_3$, p = 0.0003). The data therefore
441 show that using the ΔORF3678 virus in virus neutralisation assays recapitulates results generated
442 using WT QLD02 virus, and therefore an attenuated ΔORF3678 virus could be used as a suitable
443 substitute for the WT virus in neutralization assays in BSL-2/PC2 facilities.

444 Discussion

445 Here we examined the total IgG and virus-neutralizing antibodies in the longitudinal sera from
446 participants undergoing SARS-CoV-2 vaccinations with Vaxzevria (AstraZeneca: AZ) and Comirnaty
447 (Pfizer: PZ) vaccines and some of them infected with SARS-CoV-2 in two cohorts in Queensland,
448 Australia. We show that similar to other published findings, Spike-specific IgG and virus-neutralising
449 antibody titres were reduced for VOCs Delta and Omicron sublineages. Neutralization assays with
450 natural isolates of SARS-CoV-2 revealed reductions in neutralizing antibody titers against Omicron
451 sublineages: in the AZ_1AZ_2 group with a follow up single PZ ($AZ_1AZ_2PZ_3$) and double PZ

452 ($AZ_1AZ_2PZ_3PZ_4$) boosters, titers were reduced by 3.80-fold and 1.40-fold for BA.1, respectively, and
453 2.23-fold and 1.02-fold for BA.2, respectively. For BA.5, the reduction was 2.33-fold in the
454 $AZ_1AZ_2PZ_3$ group and 1.12-fold in $AZ_1AZ_2PZ_3PZ_4$ group. Clearly, the second booster with PZ vaccine
455 in these groups substantially reduced the difference in neutralization between different VOCs. These
456 slight reductions in neutralization for primary and secondary booster Delta, BA.1, BA.2, and BA.5
457 compared to ancestral virus are generally in line with the established literature for both wildtype virus
458 and pseudo typed virus is used for neutralization assays[39,40]. In primary vaccination regimens, much
459 more severe reductions were observed when pseudoviruses were used in neutralization assays.
460 For example, neutralization of a pseudovirus with BA.1 Spike protein reduced by 33-fold for PZ_1PZ_2
461 and 14-fold for AZ_1AZ_2[41]. In comparison, PRNT virus neutralization using infectious virus showed
462 7.5-fold reduction in $PZ_1PZ_2PZ_3$ cohort compared to ancestral virus[22]. This argues for remaining
463 need for virus-neutralization assays to more accurately estimate antibody responses.

464 **Antigen heterogeneity between prefusion locked and wild type vaccine Spike protein.**

465 Cryo-EM studies have indicated that the SARS-CoV-2 spike protein is exceptionally flexible and
466 displays multiple prefusion conformations[42], with its three RBDs assuming different positions: an
467 "up" state that allows receptor access, and a "down" state that prevents receptor access. The
468 Pfizer/BioNTech's BNT162b2 messenger RNA vaccine (PZ) encodes a mutant spike protein and is
469 formulated as an RNA–lipid nanoparticle of nucleoside-modified mRNA[3]. The Spike protein in
470 BNT162b2 vaccine is locked in a prefusion conformation by two proline substitutions at residues
471 986-987[43]. The Oxford/AstraZeneca's ChAdOx1-S non-replicating adenovirus-vectored vaccine
472 (AZ) expresses the SARS-CoV-2 spike protein gene with no mutations[4]. The HexaPro is an
473 engineered version of the Spike protein with six proline substitutions located at positions F817P,
474 A892P, A899P, A942P, K986P, V987P and a mutated furin cleavage site (RRAR to GSAG). These
475 changes have been shown to result in stable prefusion spike trimers[43,44]. It's important to note that
476 our IgG assays utilised plates coated with HexaPro proteins, engineered to stabilize the Spike
477 protein in the prefusion conformation. In contrast, the AZ vaccine encodes a Spike protein without
478 heterologous mutations that bias into the prefusion state, allowing for greater conformational
479 flexibility. This difference may lead to weaker or altered binding of IgGs elicited by the AZ vaccine to
480 HexaPro-coated assays, potentially influencing the observed differences in immunogenicity
481 between vaccine groups. Future studies could investigate the ratio of prefusion to postfusion specific
482 IgGs to provide a more wholistic understanding of the humoral immune response to different vaccine
483 types.

484 **Individual mutations in Spike protein contributing to antibody escape.**

485 The spike protein of SARS-CoV-2 has evolved to maintain a delicate balance between engaging the
486 ACE2 receptor for viral entry and mediating escape against neutralizing epitopes[14]. This dual
487 functionality of the spike protein underscores its central role in the ongoing evolution of SARS-CoV-
488 2 and its variants. In the spike protein of SARS-CoV-2 VOCs, several key amino acids have been
489 identified that mediate escape in neutralization. In the BA.5 variant, the L452R mutation is
490 associated with a reduction in neutralisation in both convalescent and immunised persons[45,46]. The
491 E484A mutation usually results in a high degree of resistance to several neutralising monoclonal
492 antibodies and polyclonal sera[13]. In the Delta variant B.1.617.2, the L452R and T478K mutations
493 are associated with moderate resistance with immune escape and enhanced viral entry into cells[47].
494 In the Omicron BA.1 and BA.2 variants, the E484A, N501Y, K417N and Δ69-70 mutations are
495 associated with immune escape[48,49]. The G446S mutations in BA.2 are thought to be primarily
496 responsible for its enhanced resistance to neutralizing antibodies[50]. Both S371L/F mutations in
497 BA.1/BA.2 are associated with immune escape[51]. The combinatorial mosaic of immune-resistant
498 residues between these groups in the most recent VOC we used in our study (BA.5) includes E484A,
499 N501Y, K417N, and L452R. While these changes may contribute to the antibody escape, our data
500 show that two immunizations with AZ vaccine followed by two additional boosters with PZ vaccine
501 elicit antibody response that is able to efficiently neutralise Omicron VOCs.

502 **Recombinant SARS-CoV-2 with deletion of all accessory genes as a tool allowing to conduct**
503 **research and serological testing under biosafety level 2 conditions.**

504 In this study, we successfully generated a recombinant, attenuated SARS-CoV-2 virus with deletions
505 in the accessory genes ORF 3, 6, 7, and 8 ($QLD02_{\Delta 3678}$). The $QLD02_{\Delta 3678}$ mutant exhibited delayed
506 and reduced replication on days 1 and 2 in Vero E6 cells and demonstrated reduced replication
507 across all days in the Caco2 cell line. Despite this attenuation, the $QLD02_{\Delta 3678}$ SARS-CoV-2
508 neutralization assay closely resembles the virus neutralization assays (VNA) from wild-type SARS-
509 CoV-2. This similarity allows the assay to generate neutralization results that closely approximate
510 the gold standard plaque reduction neutralization test (PRNT).

511 Our findings indicate that the $QLD02_{\Delta 3678}$ virus replicates robustly in Vero E6 cells, achieving viral
512 titres exceeding 10^6 FFU/mL. This high replication efficiency facilitates the large-scale production of
513 high-quality viral stocks, ensuring a sufficient supply and reducing costs for serological testing. At
514 the same time, demonstrated significant attenuation of such virus in human cells and in laboratory
515 animals[36] allows to conduct these assays under biosafety level 2 (BSL-2) conditions[38]. This presents
516 a significant advantage, as BSL-2 facilities are more widely available and easier to work with than
517 the higher containment BSL-3 laboratories. Additionally, almost all clinical laboratories operate at
518 BSL-2, thus broadening the accessibility and application of this assay.

519 Incorporating the Δ3678 mutant virus into serological testing workflows offers several other
520 significant advantages. Firstly, the attenuated Δ3678 SARS-CoV-2 closely resembles the authentic
521 virus regarding structural characteristics, maturation, and infection pathways. This ensures that the
522 neutralization results are reliable and comparable to those obtained using authentic SARS-CoV-2.
523 It is also important to maintain the remaining viral genetic backbone, i.e. the genome outside spike,
524 in neutralisation assays as it has been shown that, at least for BA.1, attenuating mutations outside
525 the spike protein i.e. NSP6[52] affect virus growth kinetics *in vitro,* which may confound neutralisation
526 assays.

527 It has been shown that IgG and neutralizing antibody responses, specifically ratio of IgG1/IgG3
528 positively correlate with neutralising ability for SARS-CoV-2 infection [53-55]. It has also been shown
529 that for BA.1, there is a significant reduction in the relationship between IgG and neutralisation
530 assays [56]. This further highlights the need for a workable virus-neutralization test, considered as the
531 gold standard for measuring neutralising antibodies against SARS-CoV-2 infection[57].

532 The flexibility of the Δ3678 platform and the updated CPER design allows for the potential
533 incorporation of reporter genes or scaling up to 96-well formats, which could significantly enhance
534 the throughput of virus neutralization assays. Additionally, our updated CPER format now includes
535 the spike gene on an individual fragment (F5), which allows for the rapid exchange of the spike gene
536 from different VOCs. While a similar ΔORF3678 recombinant virus system has already been
537 developed[36,38], generation of recombinant viruses with exchanged Spike genes from VOCs using
538 this approach requires a multistep process involving cloning and propagation of cloned fragments
539 in bacteria, followed by *in vitro* DNA ligation and *in vitro* transcription of a large viral RNA. In contrast,
540 our CPER approach does not require cloning, propagation in bacteria, and *in vitro* RNA transcription,
541 thus facilitating the rapid generation of recombinant viruses containing spike genes from future
542 VOCs.

543 This adaptability is particularly valuable for high-throughput screening in research and clinical
544 settings, enabling rapid and efficient testing of large numbers of samples. In summary, developing
545 a highly sensitive and robust live-attenuated $QLD02_{\Delta 3678}$ SARS-CoV-2 assay that can be used in
546 BSL-2 laboratories improves COVID-19 serological testing. This assay maintains high reliability and
547 accessibility, making it an invaluable tool for ongoing and future research and diagnostic efforts
548 against SARS-CoV-2.

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