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 (Name: *Severe acute respiratory syndrome-related coronavirus*, Species Betacoronavirus pandemicum, Genus: *Betacoronavirus*), 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;

139 EPI_ISL_407896). This QLD02 isolate differs from the Wuhan reference strain by the following
140 mutations: Spike P1143L, N S202N, and NS8 L84S (as classified by Pango v.4.3.1 PANGO-v1.23).
141 These mutations are maintained in the QLD02$_{\Delta 3678}$ backbone with the key difference being the
142 targeted deletion of accessory genes ORF 3, 6, 7, and 8 as illustrated in Figure 5A. Additional strains
143 included II) the Delta variant (B.1.617.2), specifically hCoV-19/Australia/QLD1893C/2021 collected
144 on 05/04/2021 (GISAID accession ID EPI_ISL_2433928); III) the Omicron BA.1, named hCoV-
145 19/Australia/QLD2639C/2021 collected on 14/12/2021 (GISAID accession ID EPI_ISL_8186598);
146 IV) the Omicron BA.2 variant named as hCoV-19/Australia/QLD-QIMR02/2022, collected on
147 12/05/2022 (GISAID accession ID: EPI_ISL_15671877.2); and V) an Omicron BA.5 sub lineage
148 BE.1 variant named hCoV-19/Australia/QLD-QIMR03/2022 (GISAID accession ID:
149 EPI_ISL_15671874.2) collected 19/07/2022. All working virus stocks were propagated and titred on
150 VE6-hTMPRSS2 cells using an immuno-plaque assay[29].

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

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

168 **Serum anti-Spike IgG ELISA**

169 Antigen-specific IgG titres were quantified using the indirect ELISA method as previously
170 described[32,33]. Briefly, high-bind protein plates (Cat: 655061, Greiner, Bio-One, Austria) were coated
171 with 1 µg/mL of the HexaPro S antigen in PBS overnight at 4°C. The following day, plates were
172 blocked with 5% KPL Milk Diluent/Blocking Solution (Cat: 5140-0011; SeraCare, USA) for 30 min at
173 37ºC. Sera samples were diluted in blocking buffer starting at a dilution of 1:10 and subsequent

174 serial dilutions at fivefold increments. A negative control of pooled pre vaccination baseline was
175 included with blocking buffer, while a human anti-Spike monoclonal (CR3022, 50 µg/mL)[34]. After
176 blocking 40 µL of each serial dilution was added to the ELISA plates and incubated at 37ºC for 1
177 hour. Plates were washed three times in PBS-Tween 20 (PBS-T, 0.1%). After primary incubation
178 and washing, 50 µL of goat anti-Human IgG Fc HRP antibody (0.5 µg/mL, Cat: A18829, Thermo
179 Fisher Scientific, USA) with blocking buffer was added to each well. The plates were incubated for
180 1 hour at 37°C. After five washes with 0.1% PBST, 50µL of 3,3',5,5'-tetramethylbenzidine (Cat:
181 002023, Life Technologies, USA) substrate was added. The plates were incubated at room
182 temperature for 5 minutes and the reaction was stopped by adding 1M sulfuric acid. Absorbance
183 was measured at 450 nm using a Varioskan LUX Microplate reader (Thermo Fisher Scientific, USA)
184 using the SkanIt™ Software v6.0.1 (Thermo Fisher Scientific, Waltham, MA, USA).

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

186 For the virus neutralization assays, $3x10^5$ VE6-hTMPRSS2 cells were seeded in 96-well plates in
187 complete puromycin-containing medium as previously described and incubated overnight at 37°C
188 with 5% CO_2. On the day of assay, serum samples were diluted in Gibco™ minimum essential
189 medium (MEM) supplemented with 2% (FCS) in sterile round-bottom 96-well plates. The initial
190 dilution was 1:10, and serial 1:2 dilutions were performed. The media control wells contained
191 MEM+2% FCS medium only. Each contained a media-only control and a pooled baseline sample
192 from non-infected and non-vaccinated individuals from the Mater cohort. SARS-CoV-2 viruses were
193 diluted in MEM+2% FCS and added to each well of the 96-well plates containing sera except control
194 (virus-negative) wells. The media control wells contained MEM+2% FCS only. The plates were then
195 incubated at room temperature for 1 hour. After incubation, 120µL of virus plus sera was transferred
196 to VE6-TMPRSS2 cells and incubated at 37°C for 1 hour. Following the incubation period, the
197 medium was aspirated, and 200 µL of MEM+2% FCS was added to each well. The plates were then
198 incubated for 48 hours at 37°C. After 48 hours, the medium was aspirated, and the plates were fixed
199 in ice-cold 80% acetone in PBS and placed at -20°C for 30 minutes before removal from the facility
200 and processing in a PC2 laboratory. After acetone removal from the plates, the plates are dried, and
201 PBS is added to flush out the remaining acetone. The plates were then blocked in 5% KPL solution
202 diluted in PBS-T for 1 hour at 37°C. For primary incubation, the human Fc-linked anti-SARS-CoV-2
203 nucleocapsid nanobody C2[35] was added at 1 µg/mL and incubated for 1 hour at 37°C. Plates were
204 then washed three times with PBS-T. Subsequently, 0.2 µg/mL anti-human IgG Fc Highly Cross-
205 Adsorbed Secondary Antibody (Cat: A18829, Thermo Fisher Scientific, USA) for 1 hour at 37°C. The
206 plates were incubated with TMB as previously described, and the reaction was stopped by adding
207 1M sulfuric acid. Absorbance was measured at 450 nm using a Varioskan LUX Microplate reader
208 (Thermo Fisher Scientific, USA).

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

210 Design of the attenuated recombinant SARS-CoV-2 QLD02$_{\Delta ORF3678}$ virus followed that of a live-
211 attenuated SARS-CoV-2 vaccine candidate with accessory protein deletions[36] with some minor
212 modifications. The virus was generated using the Circular Polymerase Extension Reaction (CPER)
213 as per our previously described method[27] with the following modifications (see Figure 4A). The
214 CPER amplicon design was refined to enable seamless exchange of the complete spike gene as
215 fragment 5, involving updated primer sets allowing for longer sequence overlaps in amplicons used
216 for CPER assembly (Table 2).

217 Table 2: Primers used for CPER assembly of QLD02$_{\Delta 3678}$ virus

Fragment	Primer name	Sequence (5' to 3')	Region
Fragment 1	F1-F	ATTAAAGGTTTATACCTTCCCAGGTAACAAACCAACCAACTTTCG	5'UTR-NSP3
	F1-R	GACATGTCCACAACTTGCGTATGTAGGTTAATGTTGTCTACTG	
Fragment 2	F2-F	AACAGTAGACAACATTAACCTACATACGCAAGTTGTGGACATGTC	NSP3-NSP6
	F2-R	TGATTGTTCTTTTCACTGCACTTTGGAAAGTAACACCTGAGCATT	
Fragment 3	F3-F	AATGCTCAGGTGTTACTTTCCAAAGTGCAGTGAAAAGAACAATCA	NSP6-NSP13
	F3-R	AATTTGTGGCCATTGAAATTTCATCAAAGACAACTATATCTGCTG	
Fragment 4	F4-F	CAGCAGATATAGTTGTCTTTGATGAAATTTCAATGGCCACAAATT	NSP13-NSP16
	F4-R	ACAACTCTGTTGTTTTCTCTAATTATAAGTCTACCTTTACTAAGA AGAGA	
Fragment 5	F5-F	ATCTCTTCTTAGTAAAGGTAGACTTATAATTAGAGAAAACAAC AGAGTTGT	NSP16-Spike
	F5-R	TTATGTGTAATGTAATTTGACTCCTTTGAGCACTGGCTCAGAGTC	
Fragment 6A	F6A-F	GACTCTGAGCCAGTGCTCAAAGGAGTCAAATTACATTACACA TAAGCACAAGCTGATGAGTACGAACTTATGTACT	ORF3 deletion-E-M
	F6A-R	TTACTGTACAAGCAAAGCAATATTGTCACTGCTACTGG	
Fragment 6B	F6B-F	CCAGTAGCAGTGACAATATTGCTTTGCTTGTACAGTAATTCGT GTTGTTTTAGATTTCATCTAA	ORF6-8 deletion-N-3'UTR
	F6B-R	GTCATTCTCCTAAGAAGCTATTAAAATCACATGGGGATAGCAC	
Linker	Linker-F	GTGCTATCCCCATGTGATTTTAATAGCTTCTTAGGAGAATGAC	3'UTR- spacer-CMV promoter-5'UTR
	Linker-R	CGAAAGTTGGTTGGTTTGTTACCTGGGAA	

218 Each purified SARS-CoV-2 and linker fragments were pooled in equimolar amounts (0.1 pmol) in a
219 50µL CPER reaction with 2 µL of PrimeSTAR GXL DNA Polymerase (Takara Bio, Japan). The cycling
220 conditions included: initial denaturation at 98°C for 1 minute, followed by 12 cycles of denaturation
221 at 98°C for 10 seconds, annealing at 60°C for 20 seconds, and extension at 68°C for 10 minutes,
222 followed by a final extension at 68°C for 10 minutes. Post-CPER assembly, the 50µL reaction
223 mixture was transfected into HEK293T-ACE2-TMPRSS2 cells with Lipofectamine LTX Plus reagent
224 (Invitrogen, USA) as per manufacturer's protocol. Twelve hours post-transfection, cells were
225 trypsinised and overlayed onto VE6-hTMPRSS2 cells. The cells were monitored daily for cytopathic
226 effect (CPE), and culture supernatant was tested using Rapid Antigen Tests (Medomics, China) until
227 a positive result was obtained. The recovered virus was titred on VE6-hTMPRSS2 cells using an
228 immuno-plaque assay[29].

229 **Statistical analysis**

Statistical analysis and visualisation were conducted with GraphPad Prism 10.0.2 (GraphPad Software Inc., San Diego, CA, USA). The IC_{50} value of sera was calculated by regression analysis (Sigmoidal, 4PL). The IC_{50} was used to indicate the IgG antibody titer. The neutralization index was calculated by the following equation: neutralization level = 1- [(|OD_s-OD_b |-OD_m)/OD_v-OD_m]%. (OD_s: absorbance of sample sera, OD_b: absorbance of baseline sera, OD_m: absorbance of media control, OD_v: absorbance of virus control). Nonlinear regression ($log_{inhibitor}$ vs. normalized response) was used to calculate the IC_{50} for neutralization assay. A linear regression analysis was undertaken to compare recombinant QLD02ΔORF3678 and QLD02 WT virus for viral neutralisation assays. For comparisons between VOCs in immunogenicity and neutralisation data, groups were first tested for normal distribution using the Kolmogorov-Smirnov test and then statistical significance between groups was determined using the Kruskal-Wallis test with Dunn's multiple comparisons test against QLD02 (Figure 2 and 3) and against all groups (Figure 4).

Results

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

The selection of healthcare workers from Mater Research Hospital and members from the University of Queensland provided complementary demographics for our study. Healthcare workers represent a priority vaccination group with higher risk of exposure to SARS-CoV-2, while the university cohort offered a broader age distribution including younger adults. This sampling strategy allowed us to evaluate vaccine responses across diverse demographics while utilizing established research networks for efficient recruitment and longitudinal follow-up. To assess the immune response elicited by vaccines or a hybrid immunity, blood samples were collected approximately six weeks after the double primary vaccinations AZ_1AZ_2 or PZ_1PZ_2 and heterologous booster vaccinations with PZ; $AZ_1AZ_2PZ_3$ and $AZ_1AZ_2PZ_3PZ_4$ (Figure 1A). We conducted longitudinal sampling for sera of individuals at two locations: Mater Hospital, Brisbane, Queensland, Australia (Mater) and the University of Queensland (UQ), Brisbane, Australia, from March 2021 to December 2022 (Figure 1A). Primary vaccinations of AZ/PZ coincided with the emergence and dominance of the Delta VOC (June 2021 – December 2021) (Figure 1B). Heterologous and homologous PZ for booster vaccination, coinciding with the dominance of the Omicron BA.1 lineage (January – February 2022). Subsequent Omicron sub-lineages BA.2 and BA.5 coincided with the double booster $AZ_1AZ_2PZ_1PZ_2$ cohort.

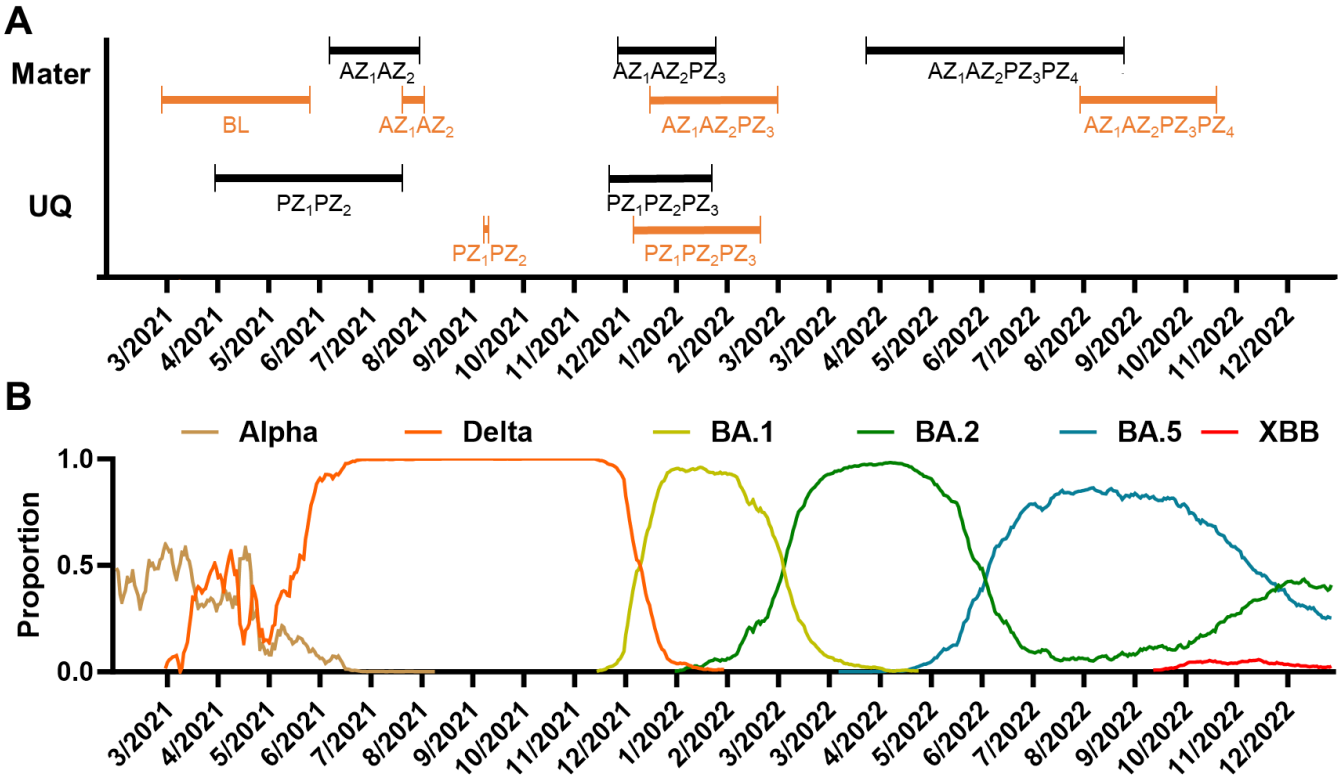

Figure 1: **Timescale of vaccination and sera collection for study cohorts and genetic epidemiology of SARS-CoV-2 VOC lineages in Australia.** A) The vaccination (black) and sera collection (orange) for AZ and PZ doses from the Mater and UQ cohorts with baseline (BL) collection indicated B) Genetic epidemiology 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.

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

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%)

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

IgG antibody responses after vaccinations and infection.

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

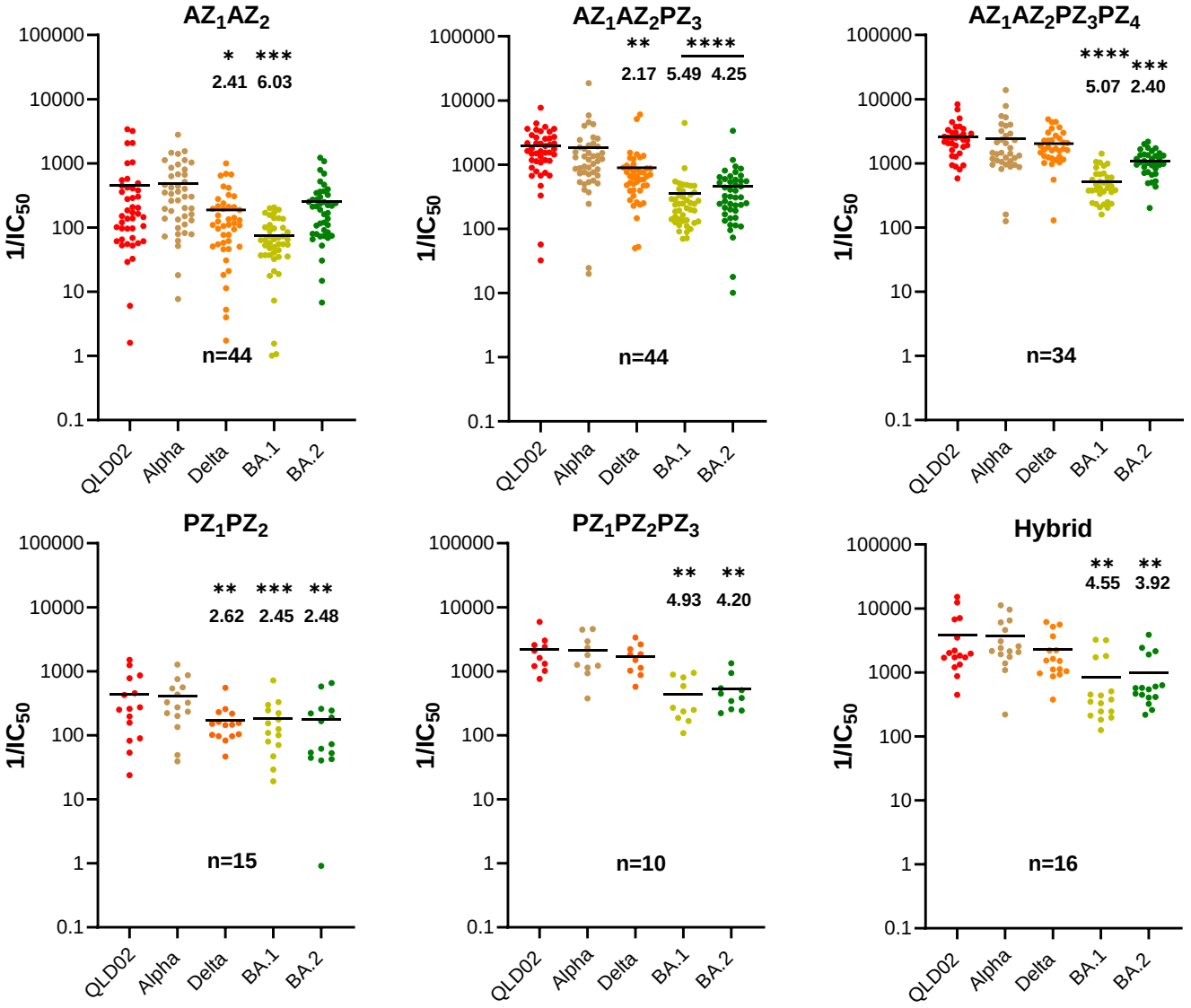

Figure 2: IgG antibody titres against purified Spike proteins from different SARS-CoV-2 VOCs. Average serum IgG antibody titres against HexaPro S protein derived from QLD02, Delta, and Omicron BA.1 and BA.2 variants measured by ELISA and shown by IC_{50} value. The decreased fold change compared with the ancestral virus QLD02 are shown in the figure. Statistical significance was determined using the Kruskal-Wallis test with Dunn's multiple comparisons test 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).

321 All vaccine groups demonstrated a consistent order of immunogenicity from highest to lowest
322 against the Spike proteins from ancestral QLD02 to Delta, BA.2, and then BA.1 VOCs, irrespective
323 of vaccine status. In the AZ_1AZ_2 group, IgG titres against Delta and Omicron BA.1 exhibited
324 decreases by 2.41- and 6.03-fold, respectively, compared to QLD02 while decrease against BA.2
325 was not significant. Within the $AZ_1AZ_2PZ_3$ cohort, the observed fold decreases were 2.17 for Delta,
326 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
327 in titres against VOCs, with a 5.07 for BA.1, and 2.40 for BA.2. The hybrid immunity group exhibited
328 a 4.55-fold decrease for BA.1 and 3.92-fold decrease for BA.2 while no significant decrease was
329 detected for Delta variant.

330 For the PZ-vaccinated cohorts, the PZ_1PZ_2 group demonstrated a 2.62-fold decrease in IgG titres
331 against Delta, 2.45 for BA.1, and 2.48 for BA.2 compared to QLD02. The $PZ_1PZ_2PZ_3$ group had a
332 4.93-fold decrease for BA.1 and 4.20 for BA.2, while no significant difference in antibody titres were
333 detected against the Delta variant.

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

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

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

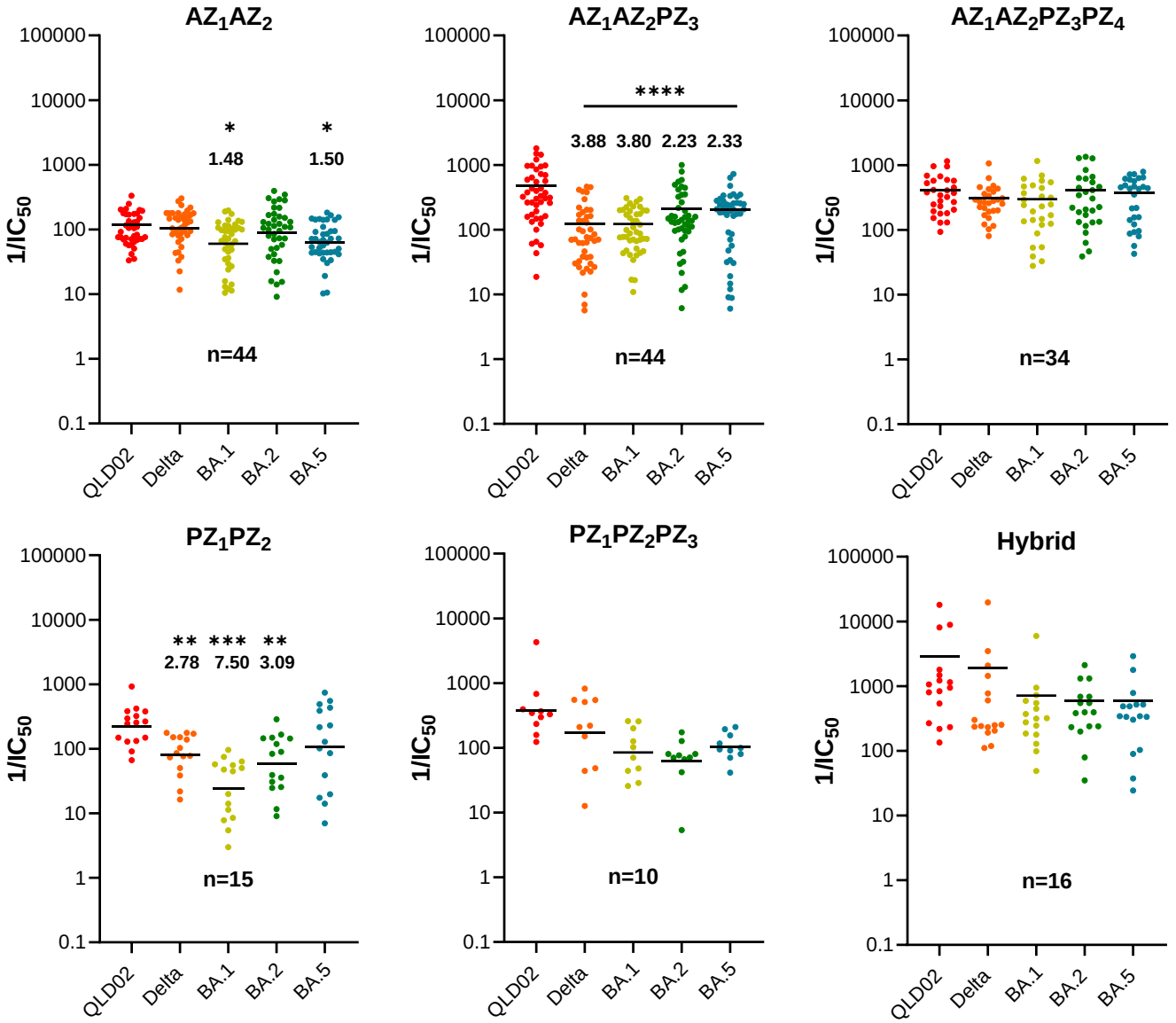

Figure 3: Virus-neutralizing antibody titres against SARS-CoV-2 VOCs. Sera neutralization antibody titres against QLD02, Delta, BA.1, BA.2, and BA.5 variants detected by microneutralization assay are shown by $1/IC_{50}$ values. Fold change reductions against VOCs compared with QLD02 ancestral virus are indicated. The mean and SEM are shown by the black bar. Statistical significance was determined using the Kruskal-Wallis test with Dunn's multiple comparisons 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).

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

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

Comparisons of antibody responses for each virus variant across different vaccination regimens.

To understand whether different vaccination regimens elicited varying levels of antibody responses, we compared IgG and virus-neutralizing antibody titers across all vaccination groups for each SARS-CoV-2 variant (Figure 4). When comparing IgG antibody responses against the ancestral QLD02 strain (Figure 4A), we observed a general trend toward higher antibody titers in groups that received three or four vaccine doses compared to those who received two doses, regardless of vaccine type (AZ or PZ). Similar patterns were observed for Alpha, Delta, and Omicron variants, although the differences between vaccination regimens were less pronounced for Omicron BA.1 and BA.2.

Interestingly, when comparing the two double-dose regimens (AZ_1AZ_2 vs. PZ_1PZ_2), no statistically significant differences in antibody titers were observed against any of the tested variants, suggesting comparable immunogenicity between the two vaccine platforms when administered as a primary series. Similarly, heterologous boosting ($AZ_1AZ_2PZ_3$) did not show significant advantages or disadvantages compared to homologous boosting ($PZ_1PZ_2PZ_3$) in terms of antibody titers against any variant.

For virus neutralization (Figure 4B), the patterns were largely consistent with the IgG results. The hybrid immunity group generally displayed the highest neutralization titers across all variants, although these differences were not always statistically significant, potentially due to the smaller sample size in this group. The four-dose regimen ($AZ_1AZ_2PZ_3PZ_4$) also showed robust neutralization against all variants tested, including the Omicron sublineages.

It is important to note that demographic differences between the cohorts, particularly age disparities between the Mater and UQ groups, may confound these comparisons. The UQ cohort (predominantly receiving PZ vaccines) had a younger mean age (30.5 ± 9.3 years) compared to the Mater cohort (predominantly receiving AZ vaccines initially, with mean age 46.6 ± 12.4 years), which might influence immune responses independent of vaccination regimen.

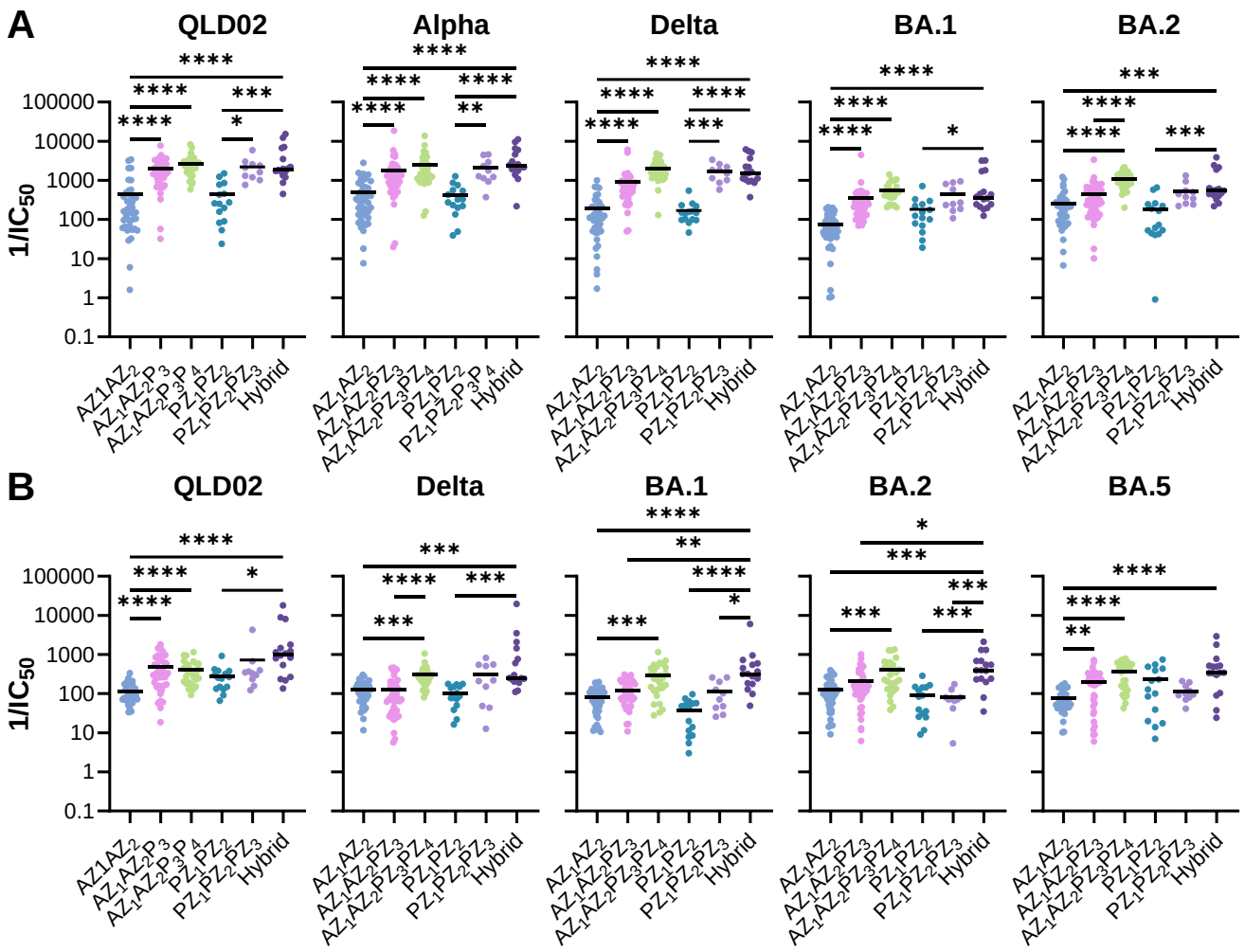

Figure 4: Comparison of antibody responses across different vaccination regimens against SARS-CoV-2 variants. A) Serum IgG antibody titers against HexaPro S protein derived from QLD02, Alpha, Delta, and Omicron BA.1 and BA.2 variants measured by ELISA, shown by $1/IC_{50}$ values. B) Virus-neutralizing antibody titers against QLD02, Delta, and Omicron BA.1, BA.2, and BA.5 variants detected by microneutralization assay, shown by $1/IC_{50}$ values. Each dot represents an individual sample, with black bars indicating mean values. Statistical significance was determined using the Kruskal-Wallis test with Dunn's multiple comparisons for evaluating differences between vaccine regimens for each variant. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$ and non-significant (ns). Number of participants in each group as per groups in Figures 2 and 3.

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

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

413 Herein, we employed our previously developed for SARS-CoV-2 circular polymerase extension
414 reaction (CPER)[27] to generate SARS-CoV-2 virus with deletion of ORFs 3, 6, 7 and 8 based on
415 ancestral QLD02 strain QLD02$_{\Delta ORF3678}$. To facilitate rapid adaptation of neutralization analysis with
416 future VOCs we reconfigured our previous CPER amplicon strategy to allow seamless swapping of
417 the complete Spike genes from any future VOCs in fragment 5 (Figure 5A). Using our new CPER
418 design, we recovered the QLD02$_{\Delta ORF3678}$ virus following transfection of CPER assembly mix into
419 HEK293-ACE2-TMPRSS2 cells which were co-cultured with VE6-hTMPRSS2 cells to recover and
420 amplify the virus yielding viral titre of 1.34 x 10^6 FFU/mL. RT-PCR from isolated viral RNA confirmed
421 ORF3678 deletion (Figure 5B). Immunoplaque assay showed that the virus produced smaller foci
422 compared to the parental QLD02 virus in Vero E6 cells (Figure 5C).

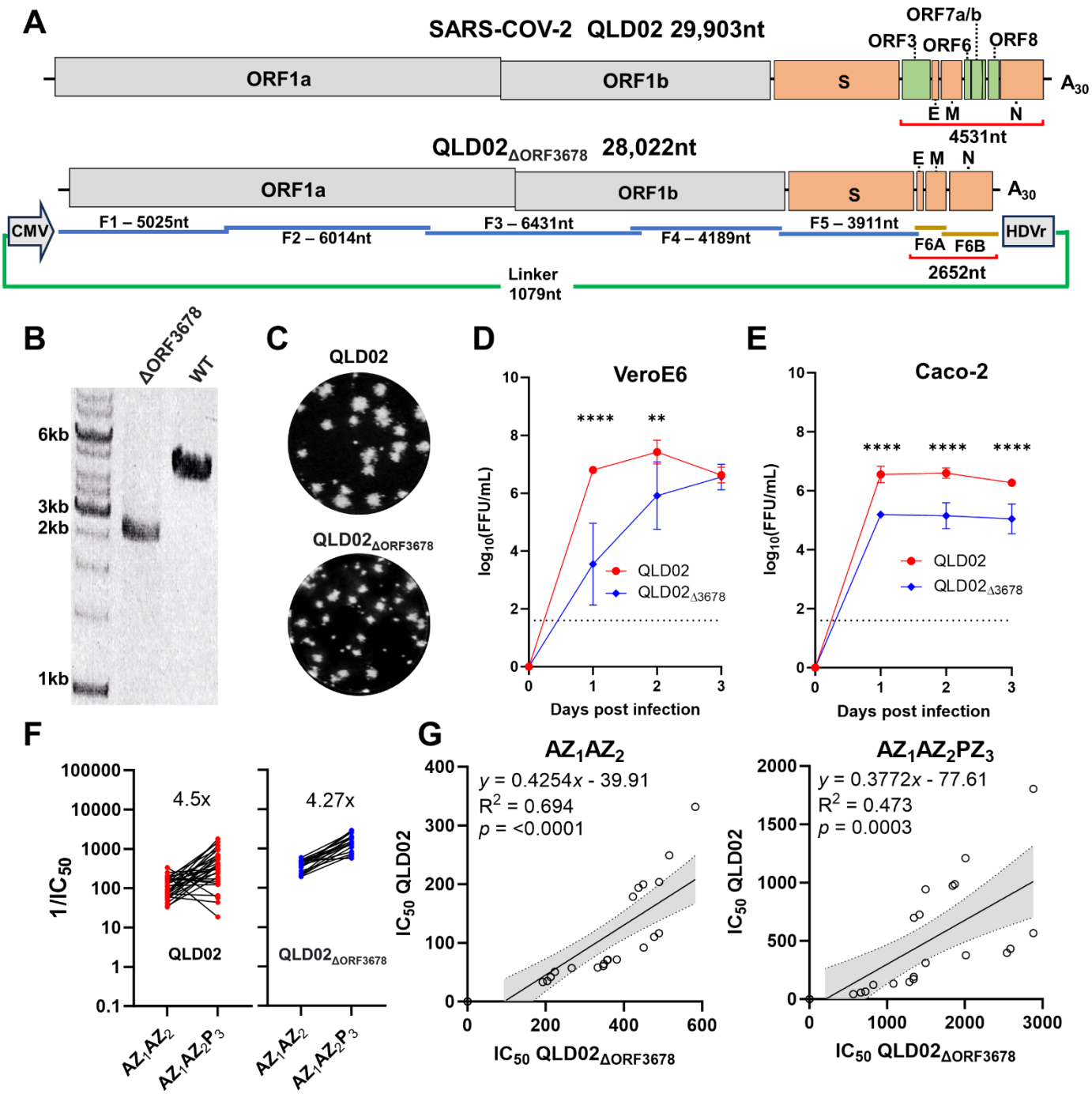

Figure 5: Generation and characterisation of an attenuated recombinant QLD02$_{\Delta ORF3678}$ virus as a substitute for native SARS-CoV-2 in virus-neutralization assay. A) Schematics of the SARS-CoV-2 QLD02 and the recombinant QLD02$_{\Delta ORF3678}$ virus genomes and amplicon fragments used for CPER assembly. Red lines with nucleotide sizes show RT-PCR amplicons of fragment 6 and the linker construct (green line) for the QLD02 and QLD02$_{\Delta ORF3678}$ viruses shown in B). Linker fragment is shown not to scale and contains the Cytomegalovirus (CMV) promoter and Hepatitis delta virus ribozyme (HDVr). 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, shown as fold reduction in viral load compared to control as IC_{50} values, for 20 matched samples from the

AZ_1AZ_2 and $AZ_1AZ_2PZ_3$ groups. G) 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). The linear regression function, coefficient of determination (R^2), and p-value for the function are given, with 95% confidence bands for the line of best fit.

We then compared the growth of WT QLD02 and QLD02$_{\Delta ORF3678}$ viruses in interferon-deficient Vero E6 cells and interferon-competent human Caco-2 cells at a MOI 0.01 over a three-day time course. Both viruses showed pronounced growth in Vero E6 cells (Figure 5D), with the QLD02$_{\Delta ORF3678}$ exhibiting delayed replication and significant differences between titres of the two viruses in the first two days (Day 1 $p<0.0001$, Day 2 $p=0.0020$, two-way ordinary ANOVA with Tukey's multiple comparisons test) while reaching comparable titres by day 3 ($p=0.9997$) (Figure 5D). In the interferon competent human Caco2 cell line, QLD02$_{\Delta ORF3678}$ grew to significantly lower viral titres than WT QLD02 at all time points (Figure 5E).

To examine if QLD02$_{\Delta ORF3678}$ virus could be used as a surrogate for virus-microneutralization assays, sera samples from 20 individuals that matched between AZ_1AZ_2 and $AZ_1AZ_2PZ_3$ groups were examined for virus neutralisation assays with the ΔORF3678 virus and compared with neutralization by WT QLD02 virus. The serum neutralization titres for the ΔORF3678 compared to the WT QLD02 virus were on average 3.29-fold higher for the AZ_1AZ_2 samples and 3.11-fold higher for the $AZ_1AZ_2PZ_3$ samples indicating higher assay sensitivity. In addition, the assay with QLD02$_{\Delta ORF3678}$ virus showed lower variability between individual samples than WT QLD02 virus while maintaining similarly increased neutralization between pre- and pos-booster immunization groups (Figure 5F).

To further examine the harmony between individual samples being assayed for IC_{50} using both viruses we plotted the relationship between IC_{50} values of the WT virus and ΔORF3678 virus for the same samples (Figure 5G). Three pre-immunisation sera samples were added to calibrate the baseline at IC_{50} 0. Generally, there was a strong linear relationship between WT QLD02 IC_{50} values and the ΔORF3678 virus for the AZ_1AZ_2 (R^2 = 0.694) and a slightly weaker relationship for the $AZ_1AZ_2PZ_3$ samples (R^2 = 0.473). There was also a significant linear relationship between IC_{50} values for the WT virus and ΔORF3678 virus for both pre- and post-booster immunization groups as indicated by the slope test (AZ_1AZ_2, p = <0.0001; $AZ_1AZ_2PZ_3$, p = 0.0003). The data therefore show that using the ΔORF3678 virus in virus neutralisation assays recapitulates results generated using WT QLD02 virus, and therefore an attenuated ΔORF3678 virus could be used as a suitable substitute for the WT virus in neutralization assays in BSL-2/PC2 facilities.

Discussion

Here we examined the total IgG and virus-neutralizing antibodies in the longitudinal sera from participants undergoing SARS-CoV-2 vaccinations with Vaxzevria (AstraZeneca: AZ) and Comirnaty (Pfizer: PZ) vaccines and some of them infected with SARS-CoV-2 in two cohorts in Queensland,

473 Australia. We show that similar to other published findings, Spike-specific IgG and virus-neutralising
474 antibody titres were reduced for VOCs Delta and Omicron sublineages. Neutralization assays with
475 natural isolates of SARS-CoV-2 revealed reductions in neutralizing antibody titers against Omicron
476 sublineages: in the AZ_1AZ_2 group with a follow up single PZ ($AZ_1AZ_2PZ_3$) and double PZ
477 ($AZ_1AZ_2PZ_3PZ_4$) boosters, titers were reduced by 3.80-fold and 1.40-fold for BA.1, respectively, and
478 2.23-fold and 1.02-fold for BA.2, respectively. For BA.5, the reduction was 2.33-fold in the
479 $AZ_1AZ_2PZ_3$ group and 1.12-fold in $AZ_1AZ_2PZ_3PZ_4$ group. Clearly, the second booster with PZ vaccine
480 in these groups substantially reduced the difference in neutralization between different VOCs. The
481 modest reductions we observed in neutralization against Delta and Omicron variants (BA.1, BA.2,
482 and BA.5) compared to the ancestral virus are consistent with previous reports using both wild-type
483 virus and pseudotyped virus neutralization assays[39,40]. In primary vaccination regimens, much more
484 severe reductions were observed when pseudoviruses were used in neutralization assays. For
485 example, neutralization of a pseudovirus with BA.1 Spike protein reduced by 33-fold for PZ_1PZ_2 and
486 14-fold for AZ_1AZ_2[41]. In comparison, PRNT virus neutralization using infectious virus showed 7.5-
487 fold reduction in $PZ_1PZ_2PZ_3$ cohort compared to ancestral virus[22]. This argues for remaining need
488 for virus-neutralization assays to more accurately estimate antibody responses.

489 Our study deliberately focused on the 2021-2022 timeframe to capture immune responses
490 temporally matched to the circulating variants experienced by our cohorts at the time. This approach
491 maintains the critical temporal relationship between vaccination/infection and variant exposure,
492 avoiding potential confounding factors in interpreting humoral IgG responses. The value of this
493 design lies in matching seropositivity measurements with contemporaneous variants, providing an
494 accurate snapshot of population immunity during this specific period.

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

496 Cryo-EM studies have indicated that the SARS-CoV-2 spike protein is exceptionally flexible and
497 displays multiple prefusion conformations[42], with its three RBDs assuming different positions: an
498 "up" state that allows receptor access, and a "down" state that prevents receptor access. The
499 Pfizer/BioNTech's BNT162b2 messenger RNA vaccine (PZ) encodes a mutant spike protein and is
500 formulated as an RNA–lipid nanoparticle of nucleoside-modified mRNA[3]. The Spike protein in
501 BNT162b2 vaccine is locked in a prefusion conformation by two proline substitutions at residues
502 986-987[43]. The Oxford/AstraZeneca's ChAdOx1-S non-replicating adenovirus-vectored vaccine
503 (AZ) expresses the SARS-CoV-2 spike protein gene with no mutations[4]. The HexaPro is an
504 engineered version of the Spike protein with six proline substitutions located at positions F817P,
505 A892P, A899P, A942P, K986P, V987P and a mutated furin cleavage site (RRAR to GSAG). These
506 changes have been shown to result in stable prefusion spike trimers[43,44]. It's important to note that
507 our IgG assays utilised plates coated with HexaPro proteins, engineered to stabilize the Spike

508 protein in the prefusion conformation. In contrast, the AZ vaccine encodes a Spike protein without
509 heterologous mutations that bias into the prefusion state, allowing for greater conformational
510 flexibility. This difference may lead to weaker or altered binding of IgGs elicited by the AZ vaccine to
511 HexaPro-coated assays, potentially influencing the observed differences in immunogenicity
512 between vaccine groups. Future studies could investigate the ratio of prefusion to postfusion specific
513 IgGs to provide a more wholistic understanding of the humoral immune response to different vaccine
514 types.

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

516 The spike protein of SARS-CoV-2 has evolved to maintain a delicate balance between engaging the
517 ACE2 receptor for viral entry and mediating escape against neutralizing epitopes[14]. This dual
518 functionality of the spike protein underscores its central role in the ongoing evolution of SARS-CoV-
519 2 and its variants. In the spike protein of SARS-CoV-2 VOCs, several key amino acids have been
520 identified that mediate escape in neutralization. In the BA.5 variant, the L452R mutation is
521 associated with a reduction in neutralisation in both convalescent and immunised persons[45,46]. The
522 E484A mutation usually results in a high degree of resistance to several neutralising monoclonal
523 antibodies and polyclonal sera[13]. In the Delta variant B.1.617.2, the L452R and T478K mutations
524 are associated with moderate resistance with immune escape and enhanced viral entry into cells[47].
525 In the Omicron BA.1 and BA.2 variants, the E484A, N501Y, K417N and Δ69-70 mutations are
526 associated with immune escape[48,49]. The G446S mutations in BA.2 are thought to be primarily
527 responsible for its enhanced resistance to neutralizing antibodies[50]. Both S371L/F mutations in
528 BA.1/BA.2 are associated with immune escape[51]. The combinatorial mosaic of immune-resistant
529 residues between these groups in the most recent VOC we used in our study (BA.5) includes E484A,
530 N501Y, K417N, and L452R. While these changes may contribute to the antibody escape, our data
531 show that two immunizations with AZ vaccine followed by two additional boosters with PZ vaccine
532 elicit antibody response that is able to efficiently neutralise Omicron VOCs.

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

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

542 Our findings indicate that the QLD02$_{\Delta 3678}$ virus replicates robustly in Vero E6 cells, achieving viral
543 titres exceeding 10^6 FFU/mL. This high replication efficiency facilitates the large-scale production of
544 high-quality viral stocks, ensuring a sufficient supply and reducing costs for serological testing. At
545 the same time, demonstrated significant attenuation of such virus in human cells and in laboratory
546 animals[36] allows to conduct these assays under biosafety level 2 (BSL-2) conditions[38]. This presents
547 a significant advantage, as BSL-2 facilities are more widely available and easier to work with than
548 the higher containment BSL-3 laboratories. Additionally, almost all clinical laboratories operate at
549 BSL-2, thus broadening the accessibility and application of this assay.

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

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

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

574 This adaptability is particularly valuable for high-throughput screening in research and clinical
575 settings, enabling rapid and efficient testing of large numbers of samples. In summary, developing

576 a highly sensitive and robust live-attenuated QLD02$_{\Delta 3678}$ SARS-CoV-2 assay that can be used in
577 BSL-2 laboratories improves COVID-19 serological testing. This assay maintains high reliability and
578 accessibility, making it an invaluable tool for ongoing and future research and diagnostic efforts
579 against SARS-CoV-2.

580 While multiple studies have examined vaccine responses against SARS-CoV-2 variants, our work
581 makes several unique contributions. Firstly, we provide valuable longitudinal data from Australian
582 cohorts during a critical vaccination period (2021-2022), with particular emphasis on heterologous
583 vaccination schedules (AZ followed by PZ boosters) that were common in Australia but less
584 extensively studied globally. Secondly, our attenuated QLD02Δ3678 virus system enables
585 neutralization studies in BSL2 facilities while maintaining concordance with wild-type virus results,
586 thus addressing a significant practical barrier in SARS-CoV-2 research. Thirdly, our CPER-based
587 system represents a significant methodological advancement for generating recombinant viruses
588 with VOC Spike genes, eliminating the need for time-consuming cloning and bacterial propagation
589 steps. Our CPER-based approach for generating QLD02Δ3678 offers advantages for incorporating
590 Spike genes from emerging VOCs. To incorporate a new VOC spike sequence, the spike gene from
591 the variant of interest would be amplified using primers F5-F and F5-R (Table 2), which contain
592 overlapping regions with adjacent fragments. This amplicon would replace Fragment 5 in the CPER
593 reaction, allowing integration of any VOC Spike gene into the QLD02 backbone with deletion of
594 ORFs 3, 6, 7, and 8. Finally, our comprehensive comparison of immunogenicity and neutralization
595 against multiple Omicron sublineages within the same cohorts provides important insights into
596 variant-specific responses across different vaccination regimens.

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604 Conflict of Interest statement

605 All authors declare that they have no conflicts of interest.

606 Author Contributions

607 R.H.P., A.A.K. conceptualization; R.H.P., Y.S., J.D.J.S. investigation; C.L.D.M., N.M., A.A.A., A.I.,
608 B.L., D.A.M., D.R., A.S., D.W. resources; S.A., S.K. administration; R.H.P., A.A.K. project
609 administration; A.A.A., L.D.B., A.A.K. funding acquisition. R.H.P., Y.S., J.D.J.S., C.L.D.M., N.M.,

A.A.A., A.I., B.L., D.A.M., D.R., A.S., S.A., S.K., D.W., L.D.B., and A.A.K. writing–review & editing; R.H.P., Y.S. writing–original draft.

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