Cross-reactivity of r*Pvs*48/45, a recombinant *Plasmodium vivax* protein, with sera from *Plasmodium falciparum* endemic areas of Africa

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Abstract (300/300)

Background: *Ps*48/45, a *Plasmodium* gametocyte surface protein, is a promising candidate for malaria transmission-blocking (TB) vaccine. Due to its relevance for a multispecies vaccine, we explored the cross-reactivity and TB activity of a recombinant *P. vivax Ps*48/45 protein (r*Pvs*48/45) with sera from *P. falciparum*-exposed African donors.

Methods: r*Pvs*48/45 was produced in Chinese hamster ovary cell lines and tested by ELISA for its cross-reactivity with sera from Burkina Faso, Tanzania, Mali, and Nigeria – In addition, BALB/c mice were immunized with the r*Pvs*48/45 protein formulated in Montanide ISA-51 and inoculated with a crude extract of *P. falciparum* NF-54 gametocytes to evaluate the parasite-boosting effect on r*Pvs*48/45 antibody titers. Specific anti-*rPvs*48/45 IgG purified from African sera was used to evaluate the *ex vivo* TB activity on *P. falciparum,* using standard mosquito membrane feeding assays (SMFA).

Results: r*Pvs*48/45 protein showed cross-reactivity with sera of individuals from all four African countries, in proportions ranging from 94% (Tanzania) to 40% (Nigeria). Also, the level of cross-reactive antibodies varied significantly between countries ($p<0.0001$), with a higher antibody level in Mali and the lowest in Nigeria. In addition, antibody levels were higher in adults (≥ 17 years) than young children (≤ 5 years) in both Mali and Tanzania, with a higher proportion of responders in adults (90%) than in children (61%) ($p<0.0001$) in Mali, where male (75%) and female (80%) displayed similar antibody responses. Furthermore, immunization of mice with *P. falciparum* gametocytes boosted anti-*Pvs*48/45 antibody responses, recognizing *P. falciparum* gametocytes in indirect immunofluorescence antibody test. Notably, r*Pvs*48/45 affinity-purified African IgG exhibited a TB activity of 61% against *P. falciparum* in SMFA.

Conclusion: African sera (exposed only to *P. falciparum)* cross-recognized the r*Pvs*48/45 protein. This, together with the functional activity of IgG, warrants further studies for the potential development of a *P. vivax* and *P. falciparum* cross-protective TB vaccine.

1. INTRODUCTION

Malaria is a human parasitic disease caused by *Plasmodium* species, including *Plasmodium falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* [1]. Currently, particular attention is given to controlling *P. falciparum* and *P. viva*x, responsible for over 90% of the 247 million malaria cases reported globally in 2021. Approximately 93% of these cases occurred in Africa, where *P. falciparum* causes 99% of clinical cases[2, 3]. In contrast, both species are endemic in Asia and America, where *P. vivax* accounts for ~74% of malaria cases[2, 3].

As a result of improved access to first-line treatment, rapid diagnostics tests, and vector control measures, a significant decrease (~54%) in malaria cases was observed worldwide between 2000 and 2015, stimulating malaria eradication efforts [2-4]. However, certain regions have shown a significant increase in malaria cases during the last few years [5], constituting an important obstacle to this goal. This appears to be due to the emergence of resistance to first-line anti-malarial drugs like artemisinin derivatives[2] [6-9] and mosquito resistance to insecticides, both of which appear to be rapidly disseminating across the globe [10-13]. Furthermore, the vast presence of asymptomatic and sub-microscopic infections also contributes to malaria transmission levels [14-16]. Therefore, efforts must be intensified to develop novel tools and strategies, such as vaccines, to strengthen malaria control and elimination efforts.

Vaccines are considered a highly cost-effective method for combating infectious diseases and are garnering increasing interest within the malaria public health agenda [17-21]. Following extensive evaluations in a phase III clinical trial with children across several African countries, [21-29], *P. falciparum* RTS, S and R21, based on the circumsporozoite (CS) protein fused to the Hepatitis B surface antigen (HBsAg) and expressed in yeast, have emerged. RTS,S is the first malaria vaccine to be recommended by the World Health Organization (WHO) for widespread use among children in sub-Saharan Africa, and other regions which display moderate to high *P. falciparum* transmission rates[30, 31]. However, RTS,S/AS01 exhibits modest efficacy, 39% against clinical malaria and 29% against severe malaria during a median of 48 months follow-up period [21]. Nevertheless, other *P. falciparum* vaccine candidates like R21/Matrix-M has exhibited a remarkable protective efficacy of 75% in children living in areas with high malaria seasonal transmission rates, proving to be safe, highly immunogenic, and promising in terms of efficacy [32-34]. Additionally, a *Pf*SPZ candidate based on whole cryopreserved parasites is also being evaluated in clinical trials in African countries [32, 35-38].

98 Although *P. vivax* vaccine research lags behind *P. falciparum*, several vaccine-candidate
99 antigens from different parasite development stages are also being investigated in preclinical
100 and clinical phases. A *P. vivax* CS synthetic vaccine formulation has reached phase 1 and 2
101 clinical evaluations [39-41]. At the same time, antigens like *Pvs*48/45, *Pvs*25, and *Pvs*230,
102 which are expressed in sexual parasite forms [42-49], as well as several antigens from asexual
103 parasite stages containing coiled-coil motifs [50, 51], are among the most relevant *P. vivax*
104 vaccine candidates [50, 52-54].
105 *Pvs*48/45 is an orthologous protein to *P. falciparum* and is currently being investigated as a TB
106 vaccine candidate. Previous studies have shown that individuals from malaria-endemic areas
107 harbor antibodies specific to this protein, with *ex-vivo* TB activity [55, 56]. Generally, *Pv*s48/45
108 genes are highly conserved and display an overall sequence homology of ~56% between *P.*
109 *falciparum* and *P. vivax* [57-59]. Furthermore, the low genetic polymorphism does not appear
110 to influence the tertiary structure or the antigenic cross-reactivity [60]. Importantly, the
111 functional analysis of the Ps*48/45* gene in *P. berghei* established its crucial role in the fertility
112 of male gametes [47, 48, 58].
113 In the case of *P. vivax,* recent studies have shown that communities endemic with *P. vivax* or
114 *P. falciparum* transmission exhibit high recognition of the *Pvs*48/45 protein and demonstrate
115 TB activity in *ex vivo* DMFA [61, 62]. In these studies, human samples were collected from
116 both *P. vivax* and *P. falciparum* endemic areas, so cross-reactivity in humans could not be
117 studied. Furthermore, in mouse immunization studies, it had been observed that mice
118 immunized with *Pvs*48/45 displayed strong cross-reactive antibodies to *Pfs*48/45 and showed
119 that *Pfs*48/45 and *Pvs*48/45 antigens were able to cross-boost each other in cross-boosting
120 experiments [55, 63]. In this study, we used sera from Africa where only *P. falciparum* was
121 present to check the natural antibody cross-reactivity and TB activity in humans.

122 2. MATERIALS AND METHODS

123 2.1 Ethics, consent, and permissions

124

125 This study was conducted as part of a research protocol on malaria immunity in Mali (Protocol
126 # 08-I-N120). All serum samples were anonymized archived and stored at -80°C, and the same
127 samples used in our previous or collaborative studies [64-69]. Samples from Burkina Faso (BF)
128 were collected in 1998 in the village of Goundry, 30km from Ouagadougou (the capital city of

129 Burkina Faso. In 1998, no authorization was required for research study in BF. For Mali (ML),
130 samples were collected from 2009 to 2011 in Kenieroba village located in Bancoumana district,
131 73 km from Bamako (the capital city), and in Dangassa village in Kourouba town, 80 km from
132 Bamako. Approval was obtained from the Ethical committee (EC) of Faculty of Medicine,
133 Pharmacology and Odonto-Stomatology (FMPOS), University of Bamako, Mali
134 (0840/FMPOS) . For Tanzania (TZ), samples were selected from those collected from 1982 to
135 1984 during a large-scale community-based study undertaken in Ifakara village in the
136 Kilombero District in Morogoro. The authorization was obtained from the Commission for
137 Science and Technology (UTAFITI NSR/RCA 90). Samples from Nigeria (NIG) were
138 collected on March 2nd of 2007, from donors living in Lagos, southwest, Ethical approval was
139 obtained from the Lagos State University Teaching Hospital (LASUTH) ethical review
140 committee [70]. Sera from healthy Swiss adults were from those who gave their informed
141 consent (IC) to participate in malaria vaccine research in 2012 (code: NCT01605786de a).
142 Written IC for collection of iRBCs for IFAT and sera for ELISA were obtained from all adults.
143 Informed assent (IA) was obtained from children in addition to IC from their parents or legal
144 guardians. The animal studies were approved by the Research Ethics Committee of the School
145 of Health, Universidad del Valle (Cali-Colombia) (Code: 031-015).

146 **2.2 Blood samples**

147

148 Blood samples were collected from adults (≥17-year-old) and children (≤5-year-old) donors
149 living in four different malaria-endemic countries of Africa: Burkina Faso (BF; adults N=35),
150 Mali (adults N=62 and children N=97), Tanzania (TZ; adults N=83 and children N=63) and
151 from Nigeria (NIG; adults N=10). Whole blood was collected in EDTA tubes, and then the sera
152 were extracted by centrifugation and stored at -80°C prior to the different tests. Samples from
153 Burkina Faso (BF) and Nigeria (NIG) were collected from donors living in urban settings. In
154 contrast, samples from TZ and Mali were obtained from rural settings where exposure to
155 malaria is potentially higher than in urban settings. In BF, samples were collected in 1998 from
156 Ouagadougou (the capital city), and those of Mali were collected from 2009 to 2011 from
157 Kenieroba, Bozokin, and Fourda, villages located in the Bancoumana district, about 55 km from
158 Bamako (the capital city), and Dangassa, a village in the Kourouba District, 80 km from
159 Bamako. Samples from Tanzania (TZ) were collected from 1982 to 1984 during a large-scale
160 community-based study undertaken in Ifakara (village in the Kilombero District in Morogoro).

161 Samples from NIG were obtained from adult donors living in Lagos city, southwest NIG.
162 Anonymized sera from 10 healthy Swiss adults non-exposed to malaria (who had no malaria
163 history) who gave their ICs to participate in malaria vaccine research (2012, study
164 NCT01605786) were used as negative controls.

165 **2.3 BALB/c mice immunization and bleeding**

166 In a previous study [55], the immunogenicity of CHO-r*Pvs*48/45 protein emulsified in
167 Montanide ISA-51 was evaluated in twelve male and female, 6-8 weeks old BALB/c mice (six
168 experimental and six control). After three doses of 20 µg (subcutaneously, s.c.) of the protein
169 (days 0, 20, and 40), all animals of the experimental group seroconverted and reached ELISA
170 titers up to 1:10^6 [55]. Control mice were immunized with saline solution formulated in
171 Montanide ISA 51. In this study, when specific anti-CHO r*Pvs*48/45 antibodies had waned to
172 baseline (day 260), a lysate of 5x10^5 extract of mix gametocytes (~80% mature forms) from *P.*
173 *falciparum* NF54 parasite cultures were formulated in Montanide ISA 51 and inoculated
174 intramuscularly (i.m.) to assess the potential boosting effect of *P. falciparum* gametocytes on
175 anti-CHO r*Pvs*48/45 antibody titers. Four weeks after, mice were bled from the submandibular
176 veins (~100 µL), and specific anti-*rPvs*48/45 antibodies were analyzed by ELISA.

177 **2.4 *Pvs*48/45 protein sequence and its homology analysis with *Pfs*48/45**

178

179 The primary sequence of *Pvs*48/45 presented 6-Cys domains with 15 Cys residues, the N-
180 terminal signal peptide, and the C-terminal GPI anchor predicted by the Signal P 3.0 and the
181 GPI-SOM servers. Sequences of the *Pvs*48/45 (PlasmoDB accession number ALS19583.1) and
182 *Pfs*48/45 (PlasmoDB accession number CAA57308.1) orthologous proteins were determined
183 using the Salvador I genome database (PlasmoDB). The two sequences were then compared for
184 homology using Blastp (protein-protein BLAST) (Suppl. figure 1) [57, 59].
185

186 **2.5 Recombinant CHO-r*Pvs*48/45 protein production, purification, and analysis**

187

188 The full-length CHO-*rPvs*48/45 protein was produced by Transient Gene Expression
189 (Excellgene SA, Monthey, Switzerland) with sufficient viable cell culture biomass of
190 suspension-adapted CHO-Express™ cells as described before [62, 71]. Briefly, the full-length

191 *Pvs48/45* codon harmonized gene was produced in CHO cell lines. All production cultures
192 (post-transfection) were performed in serum-free, animal-protein-free medium (low protein
193 content). A total of 150 mg of this antigen was produced after a single purification step on
194 IMAC-FPLC. Protein identity was confirmed using SDS-PAGE analysis of CHO-r*Pvs*48/45
195 protein under reducing (0.05 mol/L dithiothreitol) and non-reducing conditions together with
196 immunoblot and mass spectrometry (LC-MS/MS) (Suppl. figure 2) [55].

197 **2.6 ELISA assays**

198

199 ***Indirect ELISA*** was performed using Maxisorp 96-well microtiter plates (Thermo Scientific,
200 Ref. 442404) coated with 50 µL of a 2 µg/mL solution of the r*Pvs*48/45 protein solution
201 overnight at 4°C. The plates were incubated (blocked) for 1 hour at RT with phosphate buffered
202 saline (PBS) containing 3% non-fat milk powder (PBSx1- milk 3%), then incubated for 2 hours
203 at room temperature (RT) with human sera at a dilution of 1:200 in PBS containing 3% milk
204 and 0.05% Tween 20 (PBS-T). The plates were then washed four times with PBS-T. Goat anti-
205 human IgG conjugated with horseradish peroxidase (HRP) was used as the secondary antibody
206 at a dilution of 1:2000 (Life technologies, Ref H10307) in PBS-T- milk for 1 hour at RT. After
207 four time washing with PBS-T, the signals were revealed using TMB substrate reagent (BD
208 OptEIA, cat 555214) for 25 min in the dark at RT followed by a second stage blocking using
209 1M sulphuric acid (Merck, 1.00731.1000). Optical density (OD) was measured at 450 nm/630
210 nm using a TECAN Nano Quant Infinit M200 PRO spectrophotometer. ELISA was considered
211 positive when a sample's ODs was higher than the mean OD + 3 SD of negative controls (naïve
212 human sera, NHS from Swiss naïve donors) diluted to 1:200.
213 ***Competition ELISA*** was performed by incubating sera from BF (BF40 and BF70) at a dilution
214 of 1:200 with r*Pvs*48/45 at 10-fold serial dilutions from starting at 300 µg/mL for 1 hour at RT
215 prior to transferring the mixture to wells coated with the same r*Pvs*48/45. Plates were then
216 incubated for 30 min at RT, and the reactivity was determined as previously described [72].
217 Each test was performed in duplicate. The percentage of inhibition was thus calculated as:
218 (mean of Ab OD with competitor protein /mean antibody OD without competitor protein) x100.
219

220 **2.7 Affinity purification of anti-*Pvs*48/45 antibodies**

221 A serum pool collected from TZ adult donors with high anti- CHO-r*Pvs*48/45 antibody titers
222 was used for IgG purification, as described previously [72, 73]. To prepare the antigen-
223 Sepharose conjugate, CNBr-sepharose 4B (Amersham Bioscience AB, Uppsala, Sweden) was
224 activated with 1 mM HCl. Then, 5 mg of the CHO-r*Pvs*48/45 protein was dissolved in 1 mL of
225 coupling buffer (0.1 M NaHCO3 containing 0.5 M NaCl, pH 8.0). The sera were diluted 5-fold
226 with PBS (1x) containing 0.5 M sodium chloride and were mixed with the antigen-sepharose
227 conjugate and stirred O/N gently at 4°C. The antigen-sepharose beads were then washed with
228 5 mL of TRIS (20 mM containing 0.5 M NaCl, pH 8.0) and then with 5 mL of TRIS (20 mM,
229 pH 8.0). The bound antibody was eluted with a solution containing glycine (0.1 M, pH 2.5).
230 The fractions (F1, F2, F3) were collected in TRIS solution (1 M, pH 8.0) to instantly neutralize
231 the solutions before dialyzing them against phosphate buffer (0.1M, pH 7.0). The antibody
232 (IgG) concentration of each fraction was determined by the absorbance of the solution at 280
233 nm [72, 73]. In addition, ELISA was used to determine the recognition of r*Pvs*48/45 by each of
234 the purified antibody fractions.

235 **2.8 Indirect Immunofluorescence Antibody Test (IFAT)**

236 Cross-recognition of *P. vivax* and *P. falciparum* was determined by IFAT using sera from mice
237 immunized with CHO-r*Pvs*48/45. To this end, *P. vivax*-infected red blood cells (iRBC) were
238 obtained from infected patients (code CECIV 1506-2017). White blood cells were separated
239 using a 45% percoll gradient centrifugation at 5,000 rpm for 10 min, and an enriched *P. vivax*
240 gametocytes fraction was obtained [74] and used to prepare 12-well glass microscope slides.
241 For *P. falciparum,* mature gametocytes were obtained from *in vitro* culturing of the *Pf*-NF-54
242 parasite isolate, which were used to prepare IFAT glass microscope slides as described before
243 [75]. IFAT slides were kept at -70ºC prior to use. For IFAT reaction, slides were incubated with
244 a pool of serum samples (at a 1:200 dilution) obtained from mice immunized with r*Pvs*48/45
245 and with a pool of control sera from naïve mice (at a 1:20 dilution) in PBS-Evans blue for 30
246 min. After PBS washing, slides were incubated with fluorescein isothiocyanate (FITC)
247 conjugated anti-mouse IgG antibody at 1:100 dilution. Slides were examined under an
248 epifluorescence microscope, and antibody titers were determined as the reciprocal of the
249 endpoint dilution that showed positive fluorescence.

250 **2.9 Transmission-Blocking Assays**

251 The functional cross-reactivity of human anti-*Pvs*48/45-specific IgG against *P. falciparum*
252 parasites was evaluated by SMFA, as described previously [43, 48, 76]. Briefly, 163 µg/mL of
253 the test IgG was mixed with 0.15% - 0.2% stage V gametocytemia of *P. falciparum* NF54 strain
254 and then fed to 3-6 day/old female *Anopheles stephensi* in the presence of human complement.
255 The mosquitoes (N=20 per sample) were maintained for 8 days and dissected to count the
256 number of oocysts in each midgut. A group of mosquitoes (N=40 per sample) were fed with
257 normal human or normal mouse Protein-G purified antibodies and was uses as a negative
258 control.

259 **2.10 Statistics**

260

261 All ELISA data are presented as an average optical density (OD) value from triplicate wells.
262 The Mann-Whitney test was utilized for comparing two groups, and a Kruskal-Wallis test,
263 followed by Dunn's multiple comparison test, was used for the comparison of more than two
264 groups. A Fischer exact test was used to compare the relative proportion of responding sera
265 between two groups, and a chi-square test was performed for more than two groups. If the chi-
266 square test shows a significant difference among groups, Fischer's exact test was used to
267 compare two groups at a time, and Bonferroni corrected p-values were calculated. GraphPad
268 Prism software, version 5.0, was used for the analysis. A descriptive statistical analysis of
269 median OD, quartile 1 (Q1), and Q3 was used to measure the variation in OD values. The
270 percent reduction of the mean oocyst intensity (TRA) was calculated using the formula: $[(Xc -$
271 $Xa)/Xc)] \times 100$, where X is the arithmetic mean oocyst intensity in control (c) and test (a) IgG.
272 The 95% confidence interval and *p*-value for TRA (either from a single assay or two assays)
273 were calculated using a zero-inflated negative binomial model as previously described [76].

274 **3. RESULTS**

275 **3.1 Protein purity and cross-reactivity of r*Pvs*48/45 protein with *P. falciparum* adult**
276 **immune sera from the four African country donors**

277

278 After CHO-r*Pvs*48/45 expression, the protein was purified by affinity chromatography, and
279 purity was confirmed by MS/MS [55]. The sequences alignment of full-length *Pvs*48/45 and

280 *Pfs*48/45 proteins confirmed an overall homology of (60.8%) [55], with an even higher
281 homology (> 80%) in the carboxyl region (aa 284-428) (Supl. figure). Sera samples from BF
282 (adults), Mali (adults and children), TZ (adults and children), and from NIG (adults) were
283 analyzed by ELISA for their recognition of the CHO-r*Pvs*48/45. Analysis of adult's samples
284 indicated a high recognition of CHO-r*Pv*s48/45 proteins in individual sera across the four
285 participant countries, with a proportion of positive responders ranging from 40-94%. Indeed,
286 the responder's proportion was high in TZ (94%), Mali (90%), and BF (90%), whereas in NIG,
287 the responder's proportion was lower (40%) ($p < 0.0001$; **Figure 1A**). Furthermore, significant
288 variations were observed in OD values among the different countries ($p < 0.0001$; **Figure 1B**).
289 We observed that OD values were more similar between adult groups from Mali (with a median
290 OD (Q1; Q3) of 0.310 (0.170; 0.450)) and TZ (with a median (Q; Q3) of 0.252 (0.183; 0.342),
291 but these were slightly higher than BF (with a median OD of 0.205 (0.169; 0.302)) which in
292 turn, was significantly higher ($p < 0.0001$) than NIG with a median OD of 0.045 (0.033; 0.086).
293

294 **3.2 Cross-reactivity of r*Pvs*48/45 protein with *P. falciparum* African immune sera with**
295 **regard to the age and gender**

296
297 Moreover, we analyzed the cross-reactivity of r*Pvs*48/45 against the individual sera with
298 regards to the age of donors (adult *vs*. children) in Mali and TZ. In Mali, adults demonstrated a
299 significantly higher responder proportion ($p < 0.0001$) to r*Pvs*48/45 and showed a drastically
300 higher median OD of 0.310 (0.170; 0.450) than children (0.151 (0.100; 0.245) (**Figure 2A** and
301 **B**). For individual sera from TZ, adults and children presented comparable responder rates ($p >$
302 0.05; **Figure 2A**) and comparable antibody levels with median OD of 0.252 (0.183; 0.342) and
303 0.224 (0.176; 0.305) ($p < 0.05$; **Figure 2B**), respectively. However, sera from children in TZ
304 showed a significantly higher responder proportion ($p < 0.001$) and higher OD ($p < 0.0001$) than
305 those from Mali (**Figures 2A** and **B**) while the responder proportion and antibody levels
306 remained comparable in adult donors between the two countries. Of the 97 samples collected
307 from Malian children, gender information was available only for 41 samples, thus we evaluated
308 the gender effect for them. No significant difference was observed in the antibody responder
309 proportion between male (75%; N=16) and female (80%; N=25) young children from Mali
310 (**Figure 3A**), nor in the antibody level between these two groups (**Figure 3B**).
311

312 **3.3 Cross-reactivity of r*Pvs*48/45 protein with African immune sera of *P. falciparum***
313 **in competitive ELISA and specific antibody purification**

314 To further characterize the cross-reactive binding of antibodies toward r*Pvs*48/45, the
315 r*Pvs*48/45 protein was inhibited with itself (**Figure 4A**) in competition ELISA in the presence
316 of the two best responder sera from BF adult donors, BF40 and BF70. The competitor r*Pvs*48/45
317 inhibited binding of anti-r*Pvs*48/45 antibodies to the adsorbed r*Pvs*48/45 protein on the ELISA
318 plate by ~80% (**Figure 4A**) for both BF40 and BF70 at 300 µg/mL. This suggests that there is
319 no difference or change in conformation of the r*Pvs*48/45 protein between the liquid and the
320 ELISA plate.

321 Twelve adult samples from Tanzania (TZ) with the highest response against r*Pvs*48/45
322 (**Figures** 1 and **2**) were further screened at serial dilutions by ELISA (**Figure 4B**), and three of
323 samples with the strongest responses were then pooled to purify specific IgG against r*Pvs*48/45.
324 Anti-r*Pvs*48/45 responses in each elution fraction from the purification was evaluated by
325 ELISA (**Figure 4C**). As expected, fraction 1 (F1) yielded the highest protein concentration of
326 IgG and the strongest recognition of r*Pvs*48/45 of all the purified fractions and demonstrated a
327 considerably stronger recognition of r*Pvs*48/45 than naive human sera (NHS, as negative
328 control) (**Figure 4C**). Fraction 1 IgG was then later used for the *ex vivo* TB activity assay.
329 For functional cross-reactivity evaluation, purified r*Pvs*48/45-specific IgG from pooled sera
330 from TZ adults were tested by SMFA against *P. falciparum* parasites. The *ex-vivo* SMFA was
331 carried out using *P. falciparum* gametocytes against fraction F1 of purified IgG (**Table 1**). The
332 purified r*Pvs*48/45-specific IgG showed a 61% inhibition of oocyst intensity (95% CI, 31 to 79
333 %; p=0.003) at 163 µg/mL (the highest concentration used based on the available IgG).
334

335 **3.4 *Mice immunization with P. falciparum* gametocytes boost anti-*Pvs*48/45 antibody**
336 **responses which recognize *P. falciparum* gametocytes in IFAT**

337
338 In ELISA, a vigorous anti-CHO-r*Pvs*484/45 antibody response was observed in BALB/c mice
339 that were immunized with CHO-r*Pvs*484/45 protein (round symbols) on days 0, 20 and 40 (thin
340 arrows) and not seroconversion were observed in the control group (square symbols). The
341 antibody responses of the experimental group declined subsequently, and reached to baseline
342 levels on day 260. When the mice were inoculated with a single dose of *P. falciparum*
343 gametocytes (5 x10^5), they produced a significant boost of the anti r*Pvs*48/45 ELISA antibody

344 titers (**Figure 5A**). Additionally, IFAT carried out using the pooled sera collected on day 320
345 (60 after *P. falciparum* boost) showed strong reactivity with enriched *P. falciparum* gametocyte
346 preparation (**Figure 5B,** bottom panel). The pooled sera collected on day 120 (before *P.*
347 *falciparum* boost) reacted strongly with *P. vivax* homologous antigens (upper panel).
348

349 **4. DISCUSSION**

350

351 *Pvs*48/45 is a protein expressed on the surface of *P. vivax* gametocytes, known to be involved
352 in parasite fertilization [47, 48, 58]. Both *P. falciparum* and *P. vivax* 48/45 proteins are well-
353 established as targets of natural antibody responses to parasitic sexual stages, which have shown
354 important TB activity in *ex vivo* assays. Consequently, they are currently being pursued as TB
355 vaccines candidates [45, 46, 77-79].
356 This study indicates that sera from a significant proportion of donors (40-94%) living in *P.*
357 *falciparum*-endemic areas of Africa cross-recognize the r*Pvs*48/45 protein. The high and
358 consistent recognition of r*Pvs*48/45 by sera from different endemic regions of Africa, with no
359 *P. vivax* transmission at the time of sera collection, is remarkable. Unlike previous studies in
360 endemic areas of Latin America, where both parasites coexist and tend to induce cross-boosting
361 of the antibody responses in natural conditions and in mice immunization [63, 80, 81], the
362 present study clearly demonstrates cross-reactivity of *Pvs*48/45 against samples from *P.*
363 *falciparum*-endemic areas. This cross-reactivity is most likely explained by the significant
364 amino-acid sequence homology (~ 60,8%) between *P. vivax* and *P. falciparum Ps*48/45 [57,
365 59]. This feature makes this protein a promising target for the development of an effective cross-
366 species vaccine.
367
368 The communities analyzed in this study have been historically exposed to variable *P.*
369 *falciparum* transmission intensities, according to the 2018 WHO report [82] . The r*Pvs*48/45
370 recognition intensity, as determined by the level of specific anti-r*Pvs*48/45 and the percentage
371 of positive responders in each of the four endemic countries, may be correlated with the relative
372 transmission intensity and episodes history of malaria in these countries. Further research is
373 now necessary to test these hypotheses. Furthermore, despite a high proportion of positive
374 serological responses, our research highlights significant differences in antibody levels (OD
375 value) based on demographics. While cross-reactive antibody levels were more comparable and
376 higher for Mali and TZ where samples were collected from rural sites, than those from BF or

377 NIG, collected from urban sites. These findings support the argument that populations living in
378 rural communities and small villages are more likely to be exposed to malaria vectors than those
379 living in metropolitan areas [83].
380
381 In addition to location, age played an important role in antibody response. Adults made up a
382 greater proportion of responders and demonstrated higher antibody levels than children, mostly
383 in Mali. This suggests that cross-reactive immune responses to the r*Pvs*48/45 protein may
384 increase with age, which is argued to be a result of adults being exposed for longer periods of
385 time, as a function of their age. Hence, these cross-reactive anti-r*Pvs*48/45 antibodies are likely
386 acquired early in childhood and are then boosted throughout the donors' life, most likely in
387 response to subsequent *P. falciparum* infections. This age-related increasing trend of the
388 specific immune response in the naturally exposed population has also been demonstrated with
389 other malaria antigens, although such immunity was due to antigens specific to *Plasmodium*
390 species [53, 77, 84-86]. Moreover, recent sero-epidemiological studies, performed with sera
391 from malaria-endemic regions where both *P. vivax* and *P. falciparum* are co-transmitted,
392 suggest that frequent exposure to *P. falciparum* infections results in the maintenance of anti-*P.*
393 *vivax* antibodies [45, 55, 56, 63, 79]. However, this current study did not determine which
394 Ps48/45 segments were cross-reactive, or demonstrate which conserved protein domains are
395 prone to be restricted despite the recognition of heterologous parasites that was observed [62,
396 87, 88]. One of the limitations of this study is also that these sera were not tested with the
397 recombinant *Pf*s48/45 protein. Although, in Africa, where *Pf* is present, it has been shown that
398 a naturally acquired antibody response to the recombinant *Pf*s 48/45 protein is clearly detected
399 [89-91]. Another limitation of the study is that the gender effect was assessed only in a part of
400 Malian children samples, where genter information was available. Further study is required
401 whether there is no gender difference in other populations.
402 Altogether, the consistent reactivity of antibodies against r*Pvs*48/45 protein in both *P.*
403 *falciparum* and *P. vivax* parasites under natural conditions in distant continents with significant
404 epidemiological differences, as well as in animal models, correlates with the protein sequence
405 conservation. The results of populations naturally exposed to *P. falciparum* are also in
406 agreement with the cross-reactivity and cross-boosting effects observed in mice experimentally
407 immunized with r*Pfs*48/45 and r*Pvs*48/45 *E. coli* recombinant products [55, 63]. Moreover, the
408 anti-r*Pvs*48/45 response appears to be consistent with the high proportion of antibodies against

409 *Pvs*48/45 reported in adults from malaria-endemic areas of Latin America, where malaria
410 transmission is significantly lower [62, 92].
411
412 More importantly, the significant *ex-vivo* reduction by 61.2% of *P. falciparum* oocyst
413 development in *An. stephensi* fed with *P. falciparum* gametocytes by affinity-purified anti-
414 r*Pvs*48/45 IgG is of interest and encourages investing further efforts into characterizing the
415 functional domains to model multispecies TB vaccine development. Although the cross-species
416 *ex vivo* TB activity is suboptimal, the likelihood of inducing robust TB in both species through
417 vaccination is highly likely.
418
419 The recognition of the native proteins of the two species in IFAT assays, and the *P. falciparum*
420 *ex-vivo* TB activity and the *P. falciparum* gametocyte boosting of anti-CHO r*Pvs*48/45
421 antibodies in mice, represent solid foundations that support r*Pvs*48/45 as a target for a TB
422 vaccine. Moreover, the current epidemiological data, together with the ELISA cross-species
423 reactivity and the TB capacity of r*Pvs*48/45-specific antibodies purified from *P. falciparum*
424 semi-immune individuals against, support the further development of a cross-species TB
425 vaccine.
426

427 **5. Conflict of Interest**

428 The authors declare that the research was conducted in the absence of any commercial or
429 financial relationships that could be construed as a potential conflict of interest.

430 **6. Author Contributions**

431 SB, GP, SH and MAH designed the experiment. SB, KM, IA, DK, NCI, VA, SMH and CL
432 performed most experiments, tests, and analyses. SB, KM, GP, SMH, SH and MAH wrote the
433 manuscript. MAG, SAD, SO, IN, AVK and MD contributed to antigen and sample processing,
434 and manuscript revisions. All authors read and approved the submitted version.
435

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9. Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

452
453 **Table 1: Transmission-blocking activity of anti-r*Pvs*48/45-specific IgG from African**
454 **donors.**

	1st SMFA		2nd SMFA				
Sample name	Mean oocyst	Mosquitoes[c]	Mean oocyst	Mosquitoes[c]	**% inhibition**	**95% CI[d]**	**p-value**
Control IgG[a]	9.1	36/40	15.3	31/40			
Anti-rPvs48/45 IgG[b]	4.3	13/20	4.9	11/20	61.2	31.2 to 78.6	0.003

455
456 [a] In the first assay, normal human IgG at 3750 µg/mL was used as a control and normal
457 mouse IgG at 750 µg/mL was used in the second assay
458 [b] The anti-r*Pvs*48/45-specific IgG from Tanzania adults was tested at 163 µg/mL in both
459 assays
460 [c] Number of infected mosquitoes (mosqs) / Number of dissected mosquitoes
461 [d] 95% confidence interval

462

463

464 ## Figure Legends

465

466 **Figure 1: Distribution of cross-reactive antibody responses against r*Pv*s48/45 in P.**
467 ***falciparum naturally exposed* populations from different African endemic areas**
468 Adult sera from Mali, Burkina Faso (BF), Tanzania (TZ) and Nigeria (NIG) were collected and
469 tested by ELISA against r*Pvs*48/45 at dilution of 1/200. Naïve human sera (NHS) from Swiss
470 donors were used as a negative control. **A**) The proportion of responder samples among Mali,
471 BF and TZ were comparable but significantly higher as compared to NIG. **B)** Global analysis
472 of samples (responder and non-responder) shows that antibody levels (median OD shown as a
473 horizontal black line in the dot plots) for r*Pv*s48/45 were similar among Mali, TZ and BF but
474 significantly higher than NIG. The table shows the median OD, quartile 1 (Q1) and quartile 3
475 (Q3) of antibody responses. Chi-square test was used to compare responder proportions (A) and
476 Kruskal-Wallis test, followed by Dunn's multiple comparison test was applied to compare OD
477 values (B). ****p≤ 0.0001; ns; not significant; n, number of sera donors .
478

479 **Figure 2: Distribution of cross-reactive antibodies against r*P*vs48/45 protein in adults**
480 **and children from Mali and Tanzania**
481 In addition to adult samples from Mali and TZ as described in Figure 1, ELISA also tested
482 additional sera from young children (≤ 5 years) for r*Pvs*48/45 protein recognition at the same
483 dilution of 1:200. **A)** The proportion of responders against r*P*vs48/45 was significantly higher
484 for adults than children in Mali, whereas adults and children from TZ showed similar levels.
485 However, children from TZ were more likely to be responders than those from Mali. **B)** Levels
486 of cross-reactive antibodies (median OD shown as a horizontal black line in the dot plots)
487 between adults and children varied significantly in Mali, while antibody levels remained similar
488 between the two age groups in TZ, and between adults from the two countries. The table shows
489 the median, quartile 1 (Q1), and quartile 3 (Q3) OD's of antibodies against r*Pvs*48/45 protein.
490 Chi-square test was used to compare responder proportions (A) and Kruskal-Wallis test,
491 followed by Dunn's multiple comparison test was applied to compare OD values (B). ***p≤
492 0.001; ****p≤ 0.0001; ns; not significant; N, number of donors.
493

494 **Figure 3: Distribution of cross-reactive antibody responses against r*Pvs*48/45 with**
495 **regards to the gender of young children in Mali**
496 Cross-reactive antibody responses for r*Pvs*48/45 in young children (≤ 5-year-old) from Mali
497 was further analyzed according to gender (male and female). **A)** Proportion of responder
498 samples for r*Pvs*48/45 remained similar between the male and female. **B)** Cross-reactive
499 antibody levels (median OD shown as a horizontal black line in the dot plots) remained also
500 comparable between male and female children. Fisher's exact test was performed to compare
501 the proportion of responders between the two gender groups (A) and the Mann-Whitney test
502 was applied to compare the variation of OD's between male and female individuals (B). N,
503 number of male or female; ns: not significant.
504

505 **Figure 4: Antibody affinity evaluation and titration curve of cross-reacting sera and**
506 **purified anti-r*Pvs*48/45 IgG.**
507 **A)** Two adult best responder sera from BF (BF40 and BF70) in Figure 1 were used for
508 competition ELISA at dilutions of 1:400 and 1:200, respectively. The 1:400 and 1:200 dilutions
509 gave 50% of the maximum signal in indirect ELISA for each sample. **B)** Adult best responder
510 sera from TZ donors (N=12; F6, F10,…F37) in Figure 1 were tested by ELISA at serial dilutions
511 for their recognition of r*Pvs*48/45. **C)** A pool of the three best responder sera was then used for
512 IgG purification. ELISA (titration curve) studied purified IgG from different eluted fractions

513 (F1, F2 and F3) for r*Pvs*48/45 protein recognition. The inserted table shows the IgG protein
514 concentration in each fraction as measured by nanodrop spectrophotometer. Negative control
515 (neg.C), i.e. naïve human sera, NHS from Swiss naïve donors.
516
517 **Figure 5: Mice immunized with *P. falciparum* gametocytes boost the anti-Pvs48/45**
518 **antibody responses which recognized *P. falciparum* gametocytes in IFAT.**
519 **A**) Groups of experimental and control mice were immunized on days 0, 20, and 40 (thin
520 arrows) with r*Pvs*48/45 protein (round symbol) or placebo (square symbol), respectively, then
521 boosted with 5 x 10^5 *P. falciparum* NF-54 gametocytes emulsified in Montanide ISA-51 on day
522 260 (bold arrow). ELISA determined the antibody titers in individual mice at different time
523 points. Mean and standard deviation in log-transformed antibody titers are shown. **B)** A pooled
524 mouse sera collected on day 120 (before *P. falciparum* boost) were tested by IFAT with *P.*
525 *vivax* (upper panel), and another pooled sera collected on day 320 (60 after *P. falciparum* boost)
526 were tested with *P. falciparum* gametocytes (bottom panel). Parasites were seen under light
527 (left) or epifluorescence (right) microscopy with a 100X objective lens are shown. Picture scale
528 238 μm.
529
530 **Supplemental figure 1. Sequence homology between the *Pv*s48/45 and *Pf*s48/45 proteins**
531 The amino acid (aa) sequences alignment of full-length *Pvs*48/45 and *Pfs*48/45 proteins were
532 obtained using PlasmoDB database, and sequences matched with Blastp (protein-protein
533 BLAST; https://bit.ly/3C0hPpK). *Pfs*48/45 and *Pvs*48/45 share ~56% identity (238 out of 423)
534 and ~78% similarity in their protein sequences. Conserved cysteine residues are identified by
535 C letter in black and bold. In the red sequence, identical amino acid residues are identified by
536 (letter|), the similar residues by (+) and the different residue by ($_*$).
537

538 **Supplemental figure 2: Recombinant CHO-rPvs48/45 protein analysis in western Blott**
539 CHO-rPvs48/45 protein identity was confirmed using 12% SDS-PAGE gel in western bloot.
540 Analysis was carried out under reducing (0.05 mol/L dithiothreitol, DTT) and non-reducing
541 conditions (wt) [55].

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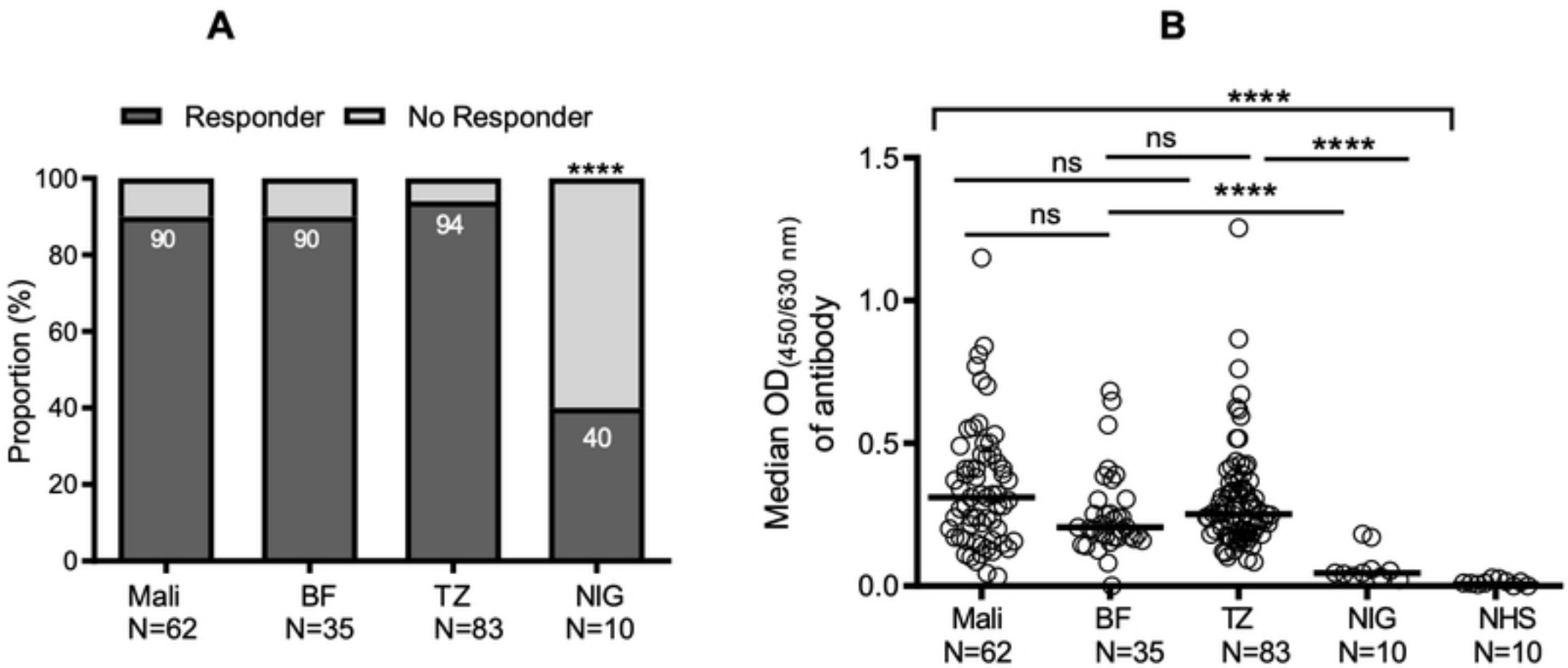
840

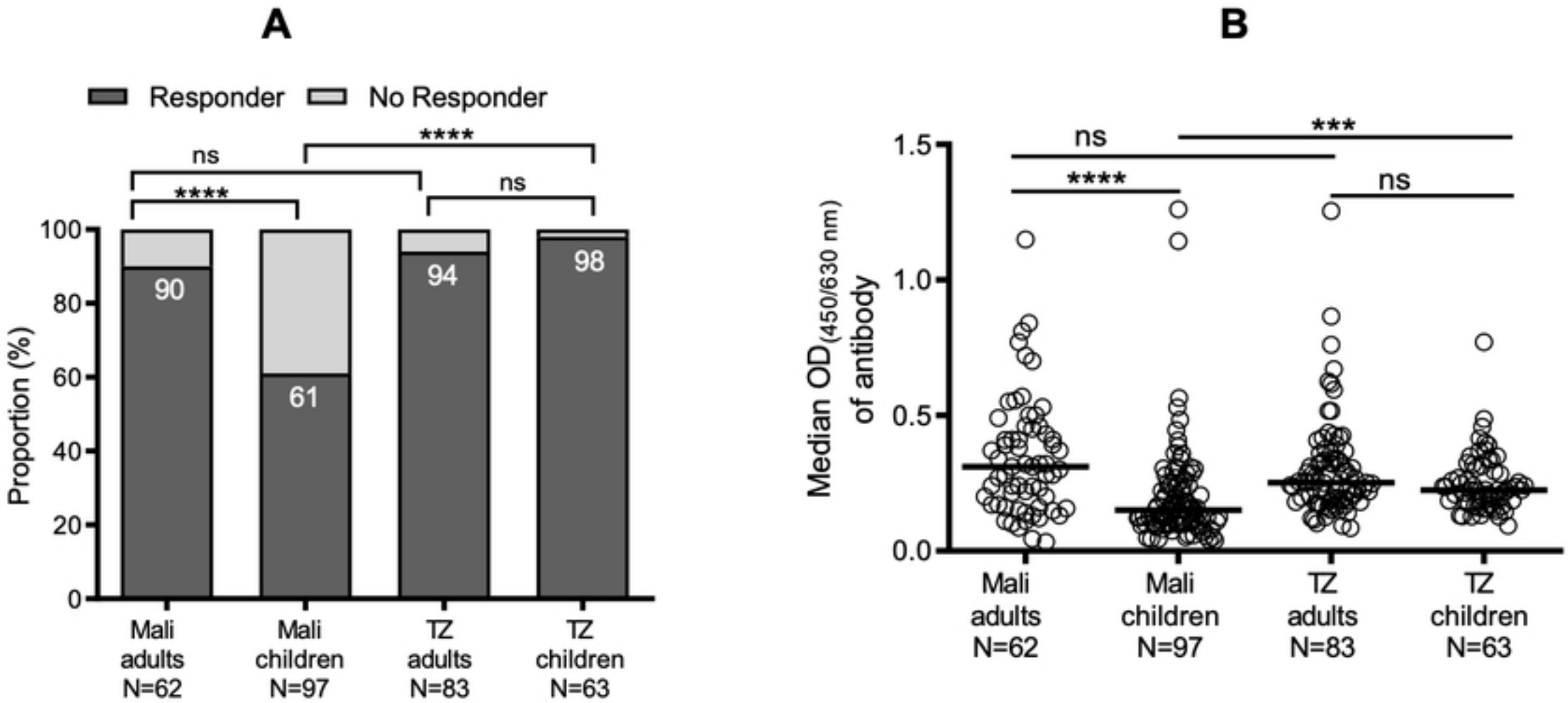
Figure 1



Country of origin	N	Median OD (Q1; Q3) of antibody
Mali	62	0.310 (0.170; 0.450)
Burkina Faso (BF)	35	0.205 (0.169; 0.302)
Tanzania (Tz)	83	0.252 (0.183; 0.342)
Nigeria (NIG)	10	0.045 (0.033; 0.086)

Figure

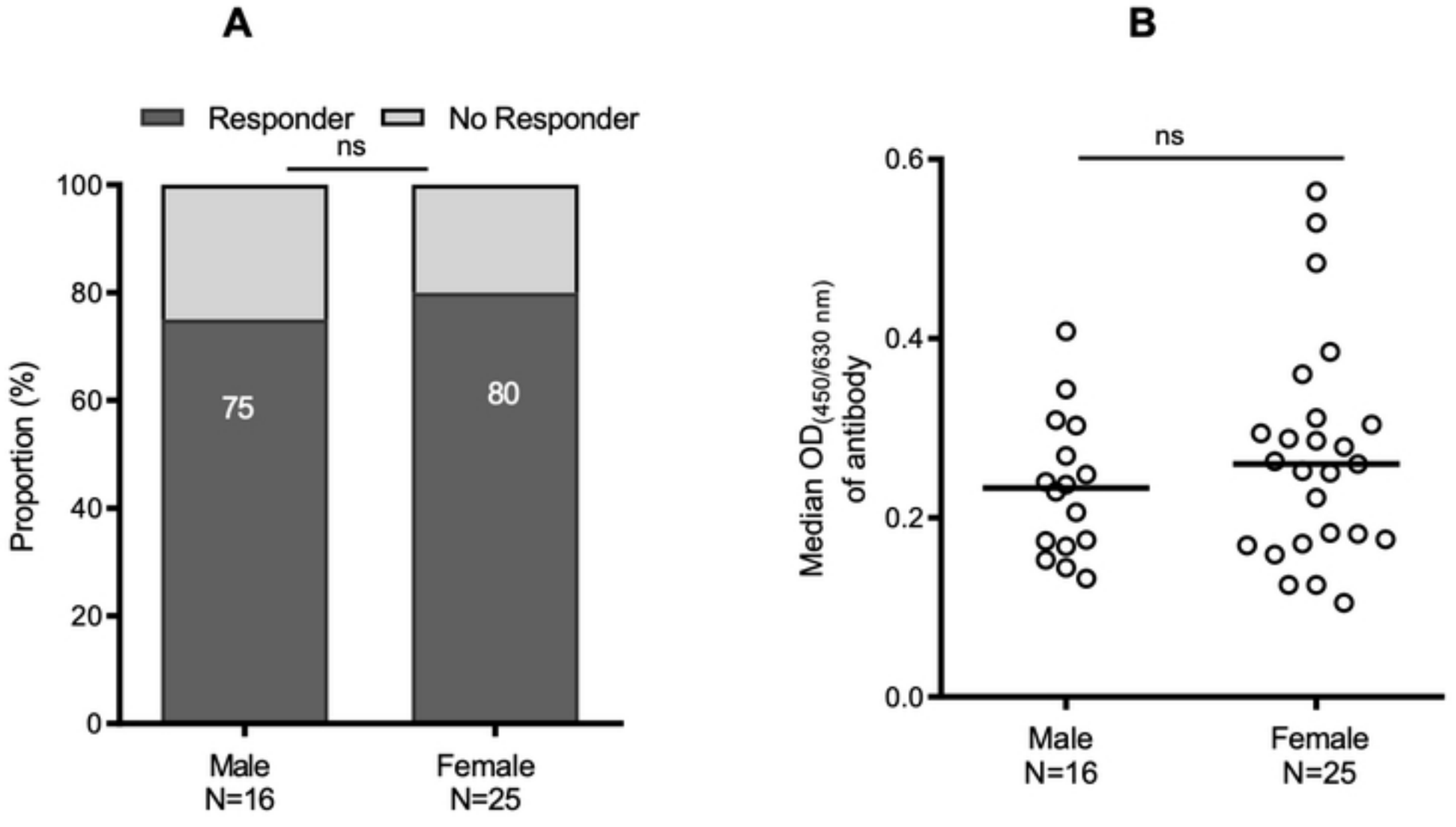
Figure 2



Country of origin	Age group	N	Median OD (Q1; Q3) of antibody
Mali	Adults	62	0.310 (0.170; 0.450)
	Children	97	0.151 (0.100; 0.245)
Tanzania (Tz)	Adults	83	0.252 (0.183; 0.342)
	Children	63	0.224 (0.176; 0.305)

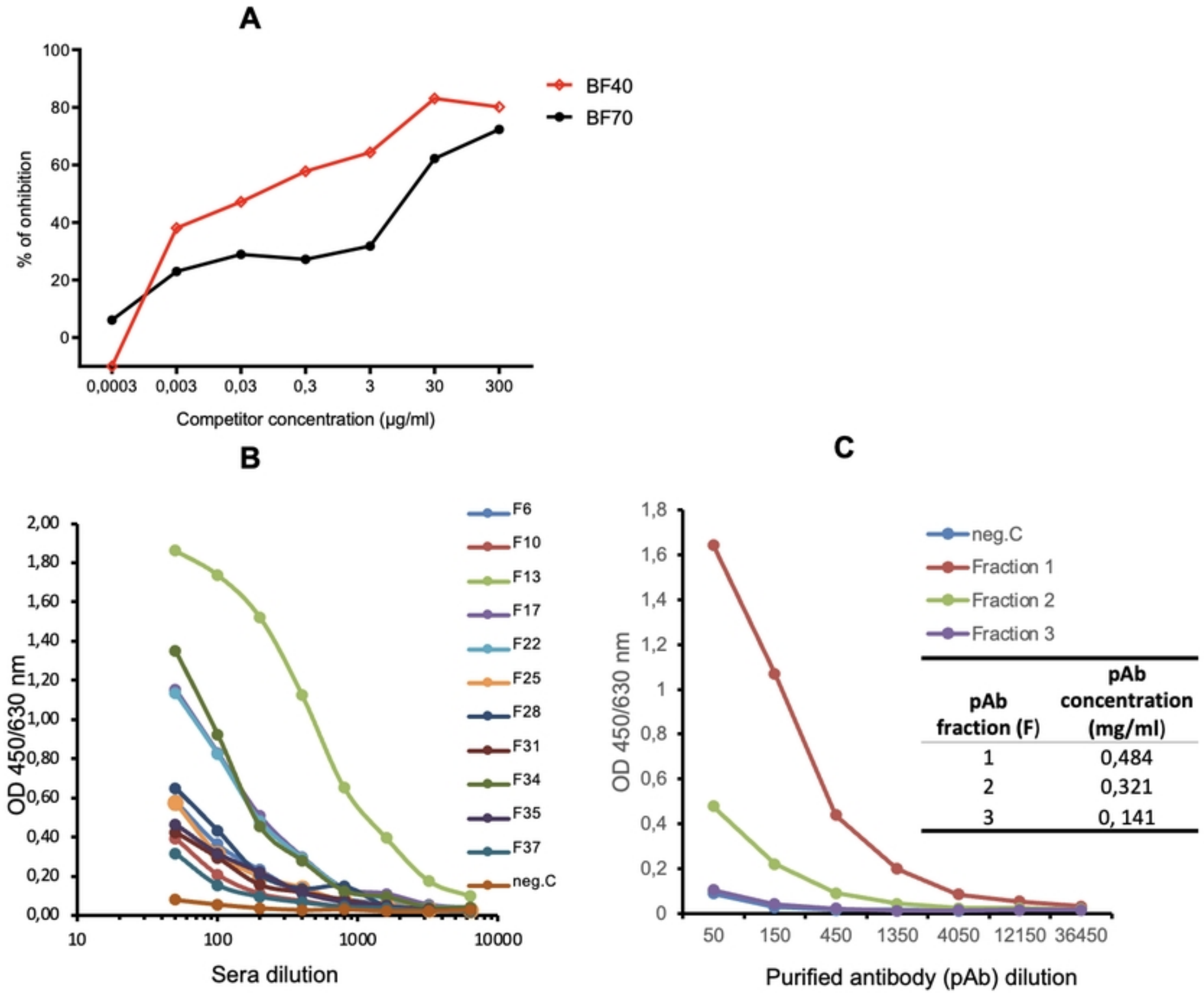
Figure

Figure 3



Figure

Figure 4

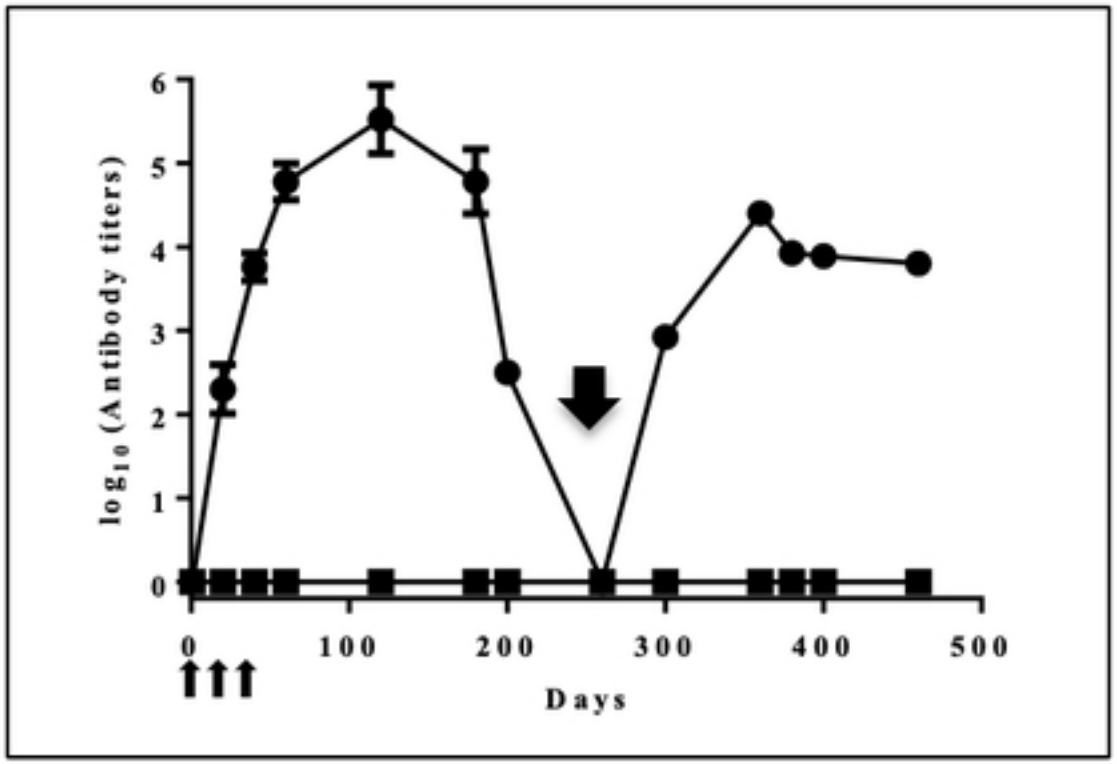


pAb fraction (F)	pAb concentration (mg/ml)
1	0,484
2	0,321
3	0, 141

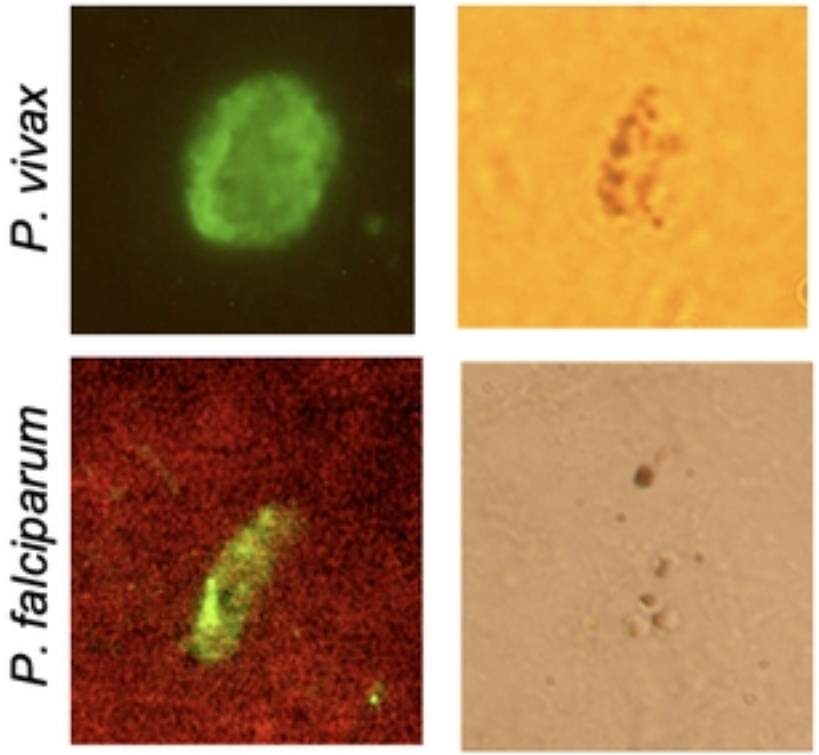
Figure

Figure 5

A



B



Figure