

Short-Term Cryopreservation and Thawing have Minimal Effects on Plasmodium Falciparum Ex Vivo Invasion Profile

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1 **Short-term cryopreservation and thawing have minimal effects on *Plasmodium***
2 ***falciparum ex vivo* invasion profile**

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Abstract

4 *Ex vivo* phenotyping of *P. falciparum* erythrocyte invasion diversity is important in the
5 identification and down selection of potential malaria vaccine targets. However, due to
6 the lack of appropriate laboratory facilities in remote areas of endemic countries, direct
7 processing of *P. falciparum* clinical isolates is usually not feasible. Here, we
8 investigated the combined effect of short-term cryopreservation and thawing
9 processes on the *ex vivo* invasion phenotypes of *P. falciparum* isolates. *Ex-vivo* or *in*
10 *vitro* invasion phenotyping assays were performed with *P. falciparum* clinical isolates
11 prior to or following culture adaptation, respectively. All isolates were genotyped at Day
12 0 for parasite clonality. Subsequently, isolates that were successfully culture-adapted
13 were genotyped again at Days 7, 15, 21, and 28-post adaptation. Invasion phenotyping
14 assays were performed in isogenic isolates revived at different time points (3, 6, and
15 12 months) post-cryopreservation and the resulting data were compared to that from
16 *ex-vivo* invasion data of matched isogenic parental isolates. Our findings indicate that
17 natural *P. falciparum* infections mostly occur as polyclonal infections. We also show
18 that short-term culture adaptation selects for parasite clonality and could be a driving
19 force for variation in invasion phenotypes as compared to *ex vivo* data where almost
20 all parasite clones of a given isolate are present. Interestingly, our data show little
21 variation in the parasites' invasion phenotype following short-term cryopreservation.
22 Altogether, our data suggest that short-term cryopreservation of uncultured *P.*
23 *falciparum* clinical isolates is a reliable mechanism for storing parasites for future use.

24 **Key words:** *Plasmodium falciparum*, Cryopreservation, thawing protocols, culture-
25 adaptation, invasion phenotype
26

27 Introduction

28 *Plasmodium falciparum* uses complex mechanisms for efficiently invading human
29 erythrocytes and evading the host immune response^{3,4}, therefore, a better
30 understanding of these mechanisms is critical for developing effective vaccines.
31 Studies with *P. falciparum* field isolates are important for providing a clinically relevant
32 elucidation of the molecular mechanisms involved in host-parasite interactions and
33 pathogenesis of malaria^{1,2}. One of the key approaches used in investigating molecular
34 interactions at the host-parasite interface is phenotyping parasites for their erythrocyte
35 invasion pathways. Invasion phenotyping generates data that are more representative
36 of the parasite's natural biology when these assays are conducted on parasites *ex*
37 *vivo*, or during their first replicative cycle in culture. However, direct processing of
38 clinical isolates requires advanced laboratory equipment and technical capacities,
39 which are not always available in the clinics in the remote areas of endemic countries.
40 Therefore, parasites isolates collected from patients at point-of-care usually need to
41 be processed for cryopreservation, storage and transportation to laboratories with the
42 requisite facilities for phenotyping¹. These frozen parasites are then thawed using
43 optimized protocols that minimize erythrocyte lysis and ensure maximal parasite
44 survival. Therefore, it is important to determine the impact of any, of the
45 cryopreservation and thawing processes on the parasites' biology in general and their
46 invasion phenotypes.

47 In the last decade, many laboratories reported on the invasion phenotypes of *P.*
48 *falciparum* clinical isolates. However, cross-study comparison of these data is
49 challenged by the use of different approaches to characterize such phenotypes¹. The
50 bulk of these phenotypic data were collected from cryopreserved parasites⁵⁻⁸ while
51 only a few were from *P. falciparum* clinical isolates that were directly assayed after
52 collection⁹⁻¹¹. Moreover, in some cases parasites were allowed to grow *in vitro* for at
53 least five cycles prior to assay set up^{5,6,8}, therefore, providing room for clonal selection,
54 if any. Unlike laboratory strains, *P. falciparum* clinical isolates are usually
55 representative of a population of different clones, presenting intrinsic characteristics
56 that could determine their *in vitro* adaptability. Given that different clones of the same
57 isolate express distinct versions of surface antigens¹², it could be that different clones
58 use distinct invasion pathways and only the dominant clones of a given isolate will be
59 reflected during invasion phenotyping experiments.

60 Alongside the cryopreservation, different laboratories may use different thawing
61 protocols prior to culturing the parasites. Therefore, the consequences of variations in
62 cryopreservation and thawing protocols in *P. falciparum in vitro* culture need to be
63 assessed, especially with respect to parasite adaptation and subsequent invasiveness
64 during *ex vivo* and *in vitro* phenotyping assays.

65 Previous literature has reported similar *ex vivo* adaptation rates in pre-cryopreserved
66 isolates¹⁴ as compared to fresh clinical isolates¹⁵. However, these conclusions were
67 drawn from studies using isolates of different isogenic backgrounds, which were
68 phenotypically and functionally distinct. In this study, parasites from the same isogenic
69 backgrounds were used to investigate the effect of freeze-thaw protocols on *P.*
70 *falciparum ex vivo* invasion phenotypes and early *in vitro* adaptation.

71 **Materials and methods**

72 **Sample collection and processing**

73 *P. falciparum* clinical isolates were collected from 25 symptomatic children, aged 2 to
74 14 years old, visiting the LEKMA Hospital, in Accra between February 2017 and
75 January 2018. The study was approved by the Institutional Review Board of the
76 Noguchi Memorial Institute for Medical Research, University of Ghana (IRB00001276)
77 and the Ghana Health Service Ethical Review Committee (GHC-ERC: 005/12/2017).
78 All guidelines and principles contained in the approved protocol were duly followed in
79 the execution of the project. For all children, informed consent forms were endorsed
80 by the parents or legal guardians. Venous blood samples were collected in ACD
81 vacutainers (BD Biosciences) and transported to the laboratory for processing within
82 two hours after collection. At the laboratory, the infected erythrocytes were separated
83 from the leucocytes through centrifugation at 2000 rpm and washed twice with
84 RPMI1640 medium (Sigma). About 200 μ L of packed erythrocytes were put straight in
85 culture, while the remaining of the sample was resuspended in glycerolyte in ~500 μ L
86 vials following the standard protocol¹⁶ and stored in liquid nitrogen. Frozen vials were
87 thawed at different time intervals using two distinct sodium chloride-based protocols.
88 Vials were thawed using either a two-step protocol (12% NaCl and 1.6% NaCl) or a
89 three-step protocol (12% NaCl, 1.8% NaCl and 0.9% NaCl supplemented with 0.2 %
90 Glucose) as per standard procedure¹⁶. To minimize the effect of possible confounders,
91 all reagents used in this study were prepared from single batches and stored as single-
92 use aliquots.

93 ***Plasmodium falciparum* in vitro culture**

94 *P. falciparum* clinical isolates were cultured as per standard protocols¹⁶. In brief,
95 isolates were maintained at 37^o C in RPMI1640 medium (Sigma), supplemented with
96 5% Albumax (Gibco), 2 mg/ml sodium bicarbonate, 50 μ g/ml gentamycin (Sigma) and
97 2% AB⁺ heat-inactivated normal human serum (PAN Biotech, UK). All cultures were
98 adjusted to 4% hematocrit using O⁺ erythrocytes from a single donor and incubated in
99 an atmosphere of 2% O₂, 5% CO₂ and balanced with Nitrogen. For isolates cultured
100 upon arrival to the lab, the parasites multiplication rate (PMR), defined as the ratio of
101 the parasitemia before and after re-invasion, was monitored for the first two *in vitro*
102 cycles and fresh erythrocytes were only added after 96 hours in culture, while fresh
103 erythrocytes were immediately added upon thawing of cryopreserved isolates.
104 Following the addition of fresh erythrocytes, growth tests were performed to assess
105 the PMR every 48 hours for the first three *in vitro* replicative cycles for the
106 cryopreserved isolates and for up to 12 cycles for freshly culture adapted isolates. After
107 each cycle, cultures were diluted to 0.5% parasitemia for the next cycle. Sample
108 aliquots, taken following each replicative cycle were stained with 1 μ M of Hoechst
109 33342 dye (Sigma Aldrich, UK) and the resulting parasitemia was assessed using flow
110 cytometry. The growth test was considered successful only when the resulting PMR,
111 measured after 48 hours, was greater than one (>1) (Figure 1). The median PMR of
112 successful growth tests after twelve successive replicative cycles following the addition
113 of fresh erythrocytes were considered as PMR of culture-adapted isolates.

114 ***Plasmodium falciparum* genotyping**

115 *P. falciparum* genomic DNA (gDNA) was extracted from filter paper spots using the
116 QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and eluted in 30 µL elution
117 buffer following the manufacturer's instructions. The concentration and purity of the
118 eluted gDNA were estimated using a NanoDrop One (Thermo Fisher Scientific,
119 Madison, WI, USA). For each isolate, the presence of single or multiple parasite clones
120 was assessed using a nested PCR approach as described earlier. The assays were
121 performed using primers targeting the highly polymorphic regions of *msp1* (block 2)
122 and *msp2* (block 3). All isolates that grew successfully during culture adaptation were
123 genotyped after 7, 15, 21 and 28 days in culture and the number of clones was
124 compared to that of the fresh isogenic isolate. The expected heterozygosity (H_E)
125 defined as the probability of being infected by at least two distinct alleles at a given
126 locus was calculated as follows: $H_E = [n / (n-1)] [(1-\sum p_i^2)]$, with n being the number of
127 isolates and p_i the allele frequency at a given locus¹⁷.

128 **Enzyme treatment and erythrocyte invasion phenotyping assays**

129 Invasion phenotyping assays were performed using enzyme-treated erythrocytes
130 (targets) from a single donor. Target erythrocytes were treated with either 250 mU/mL
131 of neuraminidase, 1 mg/mL of trypsin or 1 mg/mL of chymotrypsin for 1 hour at 37° C
132 with gentle shaking and washed thrice with RPMI1640 medium. The efficiency of
133 enzyme treatment was assessed as previously described¹⁸. Treated erythrocytes were
134 then labelled with 20 µM of carboxyfluorescein diacetate, succinimidyl ester (CFDA-
135 SE) (Thermo Fisher Scientific) for two hours at 37° C with gentle shaking and protected
136 from light exposure. For each isolate, schizont-infected erythrocytes were adjusted to
137 2% parasitemia and co-incubated with an equal volume of target erythrocytes in 96
138 well plates. All assays were performed in triplicates in a total volume of 100 µL at 2%
139 haematocrit and incubated at 37° C for 24 hours. Parasitemia were adjusted to 1% for
140 all isolates and invasion assays were considered successful only when invasion
141 efficiency into control erythrocytes (untreated) was at least two-fold greater than the
142 starting parasitemia. Plates were removed from the incubator and span at 2,000 rpm
143 for 3 minutes, after which the supernatant was discarded, replaced with a solution of 1
144 mM Hoechst 33342 to label the parasites' DNA and incubated for an hour at 37° C.
145 Plates were subsequently washed with 1X PBS and flow cytometry analyses were
146 performed on a BD LSR Fortessa X-20 cytometer (BD Biosciences, Belgium). Invasion
147 into target erythrocytes was determined by analysis of the proportion of Hoechst
148 positive erythrocytes in 50,000 counted CFDA-SE positive cells. Percent invasion into
149 enzyme-treated erythrocytes was expressed as a percentage of the invasion efficiency
150 into labelled untreated erythrocytes.

151 **Results**

152 ***P. falciparum* clinical isolates show different growth patterns during early *in vitro*** 153 **culture adaptation**

154 Samples used in this study were collected from Ghanaian children with uncomplicated
155 malaria visiting the LEKMA hospital in Accra. To monitor the *in vitro* growth patterns of
156 freshly collected *P. falciparum* clinical isolates, parasites were initially allowed to grow
157 in the patient-derived erythrocytes for a minimum period of 96 hours after which freshly
158 washed O⁺ erythrocytes (from a single donor)¹⁸ were added and growth tests were

159 performed for isolates that successfully grew. Of the 25 isolates used in this study, 19
160 (76%) yielded detectable parasitemia 48 hours following *in vitro* adaptation. As
161 measured by flow cytometry, *P. falciparum* clinical isolates showed different growth
162 patterns during the first 96 hours of *in vitro* adaptation (Figure 2A). Monitoring of the
163 PMR during these first two *in vitro* cycles revealed that most of the isolates had less
164 than two-fold increase in parasitemia from one cycle to another. Isolates with
165 successful growth patterns were further diluted with fresh erythrocytes and the
166 parasitemia was used to assess the parasite multiplication rate through successive
167 growth tests for a maximum period of twelve successive replication cycles. Overall, all
168 isolates showed a minimum of 8 successful growth tests (66.67% success rate) with a
169 median PMR of 1.77 (range 1.13 – 2.43) (Figure 2B).

170 ***P. falciparum* clinical isolates represent genotypically diverse parasite** 171 **populations**

172 Unlike laboratory strains, *P. falciparum* clinical isolates frequently harbour multiple
173 parasite clones, which could modulate the parasite's *in vitro* adaptability. Here, we
174 assessed the presence of multiple parasite clones in each of the tested isolates using
175 the highly polymorphic regions of *msp1* and *msp2* genes. All 25 isolates were shown
176 to have a number of alleles detected was 74 and 44 for *msp1* and *msp2*, respectively
177 (Table 1). For *msp1*, the allele frequencies were 36.47% (27/74), 32.43% (24/74) and
178 31.10% (23/74) for K1, MAD20 and RO33, respectively, while those for the *msp2* allelic
179 families were 54.54% (24/44) and 45.46% (20/44) for 3D7 and FC27, respectively. The
180 prevalence of multiple infections was respectively 16 and 14 for *msp1* and *msp2*, while
181 the multiplicity of infections was 2.6 and 1.76, respectively for the two genes (Table 1).
182 Taking *msp1* and *msp2* genes together, the overall multiplicity of infection was 4.4.
183 Besides, the H_E was 0.35 and 0.52 for *msp1* and *msp2*, respectively. However, there
184 was no relationship between the number of genotypes per isolate and the PMR (data
185 not shown).

186 **Short-term culture adapted isolates harbour lower number of parasite clones**

187 To measure the effect of short-term culture adaptation on the parasites' genotypes, ten
188 of the culture-adapted isolates were genotyped at days 7, 15, 21 and 28 post-
189 adaptation using the *msp1* and 2 allelic families. Overall, there was little variation in
190 the proportion of allelic families across the different time points (Figure 3). However,
191 our analysis showed a reduction in the number of clones per isolate at day 28 as
192 compared to day 0 (Table 2). The maximum number of clones per isolate was reduced
193 from 4 to 1 for the *msp1* gene, while that of *msp2* was reduced from 2 to 1 (Table 2).
194 Of all *msp1* allelic families, K1 was the most predominant at both day 0 and day 28,
195 with a percentage of 41.94% and 58.33%, respectively (Table 2). MAD20 and RO33,
196 which were present at the same proportion at day 0 (29.03%, each), represented
197 respectively 16.67% and 25.00% of the total number of alleles (Table 2). For *msp2*,
198 there were little changes in the proportions of the respective allelic families, with 3D7
199 being the most predominant allele representing 63.16% and 61.55% at day 0 and 28,
200 respectively (Table 2). Out of the eight isolates that harboured the MAD20 allelic family
201 at day 0, only two were detected with a copy of the allele at day 28, while the RO33
202 allele which was initially present in nine isolates at day 0, was detected in only three
203 isolates at day 28. K1, initially detected in all ten isolates, was still present in seven of
204 them at day 28. Of all three *msp1* allelic families, only K1 and RO33 were

205 simultaneously detected in the same isolates at day 28, while MAD20 was only
206 detected as single infections (Table 2).

207 For the *msp2* gene, out of the seven isolates harbouring the FC27 allelic family at day
208 0, only four persisted at day 28, while the 3D7 allelic family was initially present in all
209 ten isolates at day 0 but was detected in eight of them at day 28. As for *msp1*, both
210 allelic families of *msp2* were present in three isolates as co-infections, while two and
211 four isolates presented single infections of FC27 and 3D7, respectively at day 28.
212 However, there was no specific dominant combination of *msp1* and *msp2* detected in
213 our isolates at day 28. Moreover, aside those detected at day 0, there were no newly
214 detected alleles in our isolates at day 28, therefore suggesting the absence of
215 detectable cross-contamination during culture adaptation.

216 **Differences in thawing protocols does not affect early *in vitro* culture adaptation** 217 **of short-term cryopreserved *P. falciparum* clinical isolates**

218 Culture-adaptation of *P. falciparum* clinical isolates has been reported as more labor-
219 intensive than immediate *ex vivo* processing. Moreover, there are earlier reports of *P.*
220 *falciparum* clonal selection during *in vitro* adaptation¹⁹. To ascertain the effect of
221 cryopreservation on *P. falciparum* early *in vitro* adaptation, we compared the PMR of
222 short-term cryopreserved clinical isolates to that of their freshly cultured isogenic
223 counterparts. Two vials of the same isolate were simultaneously revived using two
224 distinct NaCl-based thawing protocols. Parasitemia were adjusted to 0.5-1% using
225 erythrocytes from a single donor and the PMR was monitored during the first three
226 asexual replicative cycles. Successful monitoring of the PMR of isolates prior to and
227 following cryopreservation (one-year interval) revealed differences in PMR between
228 cryopreserved isolates and the matched freshly culture-adapted counterparts. The
229 median PMR was 1.62 for fresh isolates while that of cryopreserved isolates was 1.12
230 and 1.27 following two-step and three-step thawing, respectively (Figure 4A). However,
231 the difference in PMR was only significant when comparing fresh isolates and
232 cryopreserved parasites thawed using a two-step protocol ($P = 0.03$; Figure 4A), while
233 no significant difference was observed between isogenic isolates thawed using distinct
234 protocols (Figure 4A-B). Flow cytometric analysis of the DNA content of revived
235 cryopreserved parasites revealed different fluorescence peaks following Hoechst
236 33342 staining suggesting the presence of different parasite stages after 48-, 96- and
237 144-hours post-incubation. However, no significant difference was observed in the
238 proportions of the different parasite stages when comparing the parasites' growth after
239 thawing using different protocols (Figure 4C).

240 **Short-term culture adaptation has minimal effect on *P. falciparum* invasion** 241 **phenotype**

242 *P. falciparum* invasion phenotyping has mostly been conducted following short-term
243 culture-adaptation⁵⁻⁸. To assess the effect of short-term culture adaptation on *P.*
244 *falciparum* invasion phenotype, we compared the *ex vivo* invasion phenotype of four
245 isolates to that obtained after 28 days in culture. Of the four isolates tested, only one
246 (ACC015) had a significant change in the sensitivity to enzyme treatment following
247 culture adaptation (Figure 5), while significant changes were only observed in ACC003 and
248 ACC014 following treatment with neuraminidase and trypsin, respectively.

249 Moreover, changes in invasion profile, defined as the combination of sensitivity to the
250 three enzymes, was observed in two isolates (ACC014: NrTsCs → NsTsCs and
251 ACC015: NrTsCr → NrTsCs), where N: neuraminidase, T: trypsin, C: chymotrypsin, s:
252 sensitive and r: resistant; while → depicts the changes in profile). Interestingly,
253 although all three msp1 alleles were initially present in these two isolates, only K1
254 persisted at Day 28-post adaptation, while both the 3D7 and FC27 alleles of msp2
255 persisted throughout the 28-day period post adaptation.

256 **Cryopreservation has minimal effect on *P. falciparum* invasion phenotype**

257 Cryopreserved isolates were thawed at different time intervals (from 3 to 12 months
258 after cryopreservation) and assayed for their ability to invade enzyme-treated
259 erythrocytes as compared to their fresh uncultured counterparts. Given the challenges
260 associated with the parasites' *in vitro* adaptability during the first rounds of asexual
261 replication, and to avoid clonal selection following long-term culture adaptation, all
262 assays were set during the first two replicative cycles. Of the 25 isolates collected in
263 this study, seven had sufficient numbers of cryopreserved vials (six vials) to be thawed
264 at all the time-points and successfully phenotyped over the course of one-year post
265 cryopreservation. All isolates showed a sialic acid independent phenotype with the
266 invasion of neuraminidase treated erythrocytes greater than 50% relative to that of
267 untreated control erythrocytes (Figure 6). Overall, there was no significant change in
268 sialic acid dependency in the parasites after cryopreservation relative to fresh
269 uncultured isolates (Figure 6). The most common invasion profile was neuraminidase
270 resistant, trypsin sensitive and chymotrypsin sensitive (NrTsCs). However, three out of
271 the seven isolates showed changes in invasion profiles in trypsin and chymotrypsin
272 treated erythrocytes following cryopreservation with the apparition of three novel
273 phenotypes (NrTrCs, NrTsCr and NrTrCr) after six months post cryopreservation
274 (Figure 6). Nevertheless, none of the novel phenotypes persisted after twelve months
275 post cryopreservation, suggesting a technical or random effect associated with these
276 changes.

277 **Discussion**

278 The phenotypic diversity of *P. falciparum* clinical isolates has widely been reported in
279 the last two decades ¹. However, conducting such assays with uncultured clinical
280 isolates has been precluded by the lack of appropriate laboratory settings in remote
281 areas where the highest malaria burden occurs. Consequently, the majority of the
282 pioneering works were conducted in cryopreserved *P. falciparum* clinical isolates and
283 in some cases after short-term culture adaptation following parasite thawing ⁵⁻⁸,
284 although the effect of such procedures in the parasite's invasion phenotype has never
285 been reported.

286 In this study, we investigated the effect of cryopreservation and different thawing
287 protocols on *P. falciparum* *in vitro* adaptation and invasion phenotyping assays. We
288 showed that *P. falciparum* clinical isolates show specific growth patterns during the
289 early *in vitro* culture adaptation, while most of the isolates did not recover following
290 culture dilution with fresh erythrocytes. Previous studies have reported such findings,
291 mainly attributed to unreported antimalarial drug use prior to presentation at hospital ⁹.

292 Given that *P. falciparum* *in vitro* adaptation could also be influenced by the number of
293 parasite clones present in a given isolate, our genotyping analysis revealed that all *P.*

294 *falciparum* clinical isolates used in this study presented multiple parasite clones, with
295 an overall MOI of 4.4, which is slightly higher than previously reported in data from
296 Burkina Faso²⁰, Ghana²¹, Republic of Congo²², Nigeria and Senegal²³. However, the
297 predominance of K1 and 3D7 allelic families of *m*sp1 and *m*sp2 reported here is in
298 agreement with previous reports from Burkina Faso^{20,24}, Ethiopia²⁵, Ghana²⁶, Nigeria
299 and Senegal²³, but contrary to studies from Uganda and Sudan which reported the
300 predominance of RO33 and FC27, respectively for the two genes^{25,27}.

301 In this study, the median PMR of short-term culture adapted isolates was 1.77,
302 consistent with previous reports¹¹, however, there was no relationship between the
303 observed PMR and the number of parasite clones per isolate. Furthermore, of the 19
304 culture-adapted isolates tested in this study, 10 were successfully genotyped at day
305 28 post culture inoculation and our data show an apparent clonal selection following
306 short-term culture adaptation. This is in agreement with previous data that also
307 reported culture adaptation of *P. falciparum* clinical isolates as a modulator of the
308 parasite's susceptibility to a wide range of drugs as compared to their fresh uncultured
309 counterparts²⁸.

310 Our data also show the persistence of K1 at day 28 in almost all isolates that harboured
311 this allelic family on day 0, while MAD20 prevalence significantly decreased at day 28.
312 Furthermore, MAD20 was outgrown by K1 in almost all isolates harbouring mixed
313 infections (8/10 isolates), consistent with reports by Sondo and colleagues where
314 MAD20 was outcompeted by K1 in natural malaria infections²⁰.

315 For some of the isolates in this study, the invasion phenotype following culture
316 adaptation was different from the parasites' *ex vivo* phenotype. This, therefore,
317 suggests a possible effect of short-term culture adaptation and/or clonal selection on the
318 parasite invasion phenotype. However, this could also result from technical variations
319 during invasion assay set up, and given the small number of isolates tested for this
320 experiment (only four isolates), there is a need for further confirmation with a larger
321 number of isolates and possibly a longer culture adaptation time.

322 The present study also investigated the effect of cryopreservation and thawing
323 protocols in the parasites' early *in vitro* adaptation and invasion phenotype as
324 compared to their freshly cultured isogenic counterparts. Our data show that
325 cryopreserved *P. falciparum* clinical isolates used in this study have a lower
326 multiplication rate during the first *in vitro* cycles as compared to their fresh isogenic
327 counterparts. However, this difference in PMR was only significant when freshly
328 cultured isolates were compared to those revived using a two-step NaCl protocol. This
329 could be because of a simple artefact during culture adaptation or due to the fitness of
330 the different parasite clones in each isolate. It is therefore possible that the parasite-
331 induced stress during the thawing process will accentuate the low fitness of certain
332 clones, while this effect could be minimal when parasites are thawed using a three-
333 step protocol.

334 Another potential factor that could affect the early *in vitro* adaptation of revived
335 cryopreservation is the addition of fresh erythrocytes following parasites revival. Given
336 the difference between the freshly added erythrocytes and the patient-derived
337 erythrocytes initially present in the freshly cultured parasites, there is a possibility that
338 the lower multiplication rate observed post-cryopreservation is solely due to the
339 parasite adaptation to the new erythrocytes. However, given the observed differences

340 in the PMR post cryopreservation, and to minimize the effect of clonal selection
341 invasion phenotyping assays were only performed in isolates that yielded appropriate
342 parasitemia during the first *in vitro* replicative cycles.

343 As a result, our data show that most of the isolates assayed following cryopreservation
344 maintained a relatively stable invasion phenotype regardless of the length of
345 cryopreservation or the thawing protocol used for the revival of the parasites. To our
346 knowledge, this is the first study to investigate the effect of short-term culture
347 adaptation and cryopreservation on *P. falciparum* invasion phenotype using clinical
348 isolates from the same isogenic backgrounds. Altogether, these results suggest that
349 short-term culture adaptation could influence the invasion phenotype of *P. falciparum*
350 clinical isolates due to clonal selection during *in vitro* culturing, but this needs further
351 confirmatory studies with a larger number of isolates.
352

353 **Figure legends**

354 **Figure 1: Schematics of *in vitro* culture-adaptation and growth test of freshly**
355 **collected *P. falciparum* clinical isolates**

356 **Figure 2: Early *in vitro* adaptation of *P. falciparum* clinical isolates.** (A) The initial
357 parasitemia (at H0) of each sample was recorded upon arrival from the field and the
358 parasitemia of *ex vivo*-cultured isolates were monitored for 96 hours. The medium in
359 the culture flasks was changed daily and supplemented with fresh erythrocytes only
360 after 96 hours *in vitro*. (B) Parasite multiplication rates of successfully culture-adapted
361 parasites following culture dilution with fresh erythrocytes. Depicted on the graph are
362 the mean and standard errors of the PMRs of parasites with successful growth tests
363 over a period of 28 days *in vitro*, and the red dotted line represents the median PMR
364 (1.77).

365 **Figure 3: Proportions of different parasite clones during culture-adaptation.**
366 Parasites were all genotyped at Day 0 upon collection and successfully culture-
367 adapted ones were further genotyped at Days 7, 15, 21 and 28-post adaptation.
368 Represented are the proportions of individual alleles for the *msp 1* and *2* genes.

369 **Figure 4: Multiplication rates of *P. falciparum* clinical isolates before and after**
370 **cryopreservation.** A-B: Box and whiskers plots showing the PMR of fresh cultured *P.*
371 *falciparum* clinical isolates (green) or thawed with a two-step (red) or three-step (blue)
372 protocol following cryopreservation. Kruskal Wallis test was conducted to compare the
373 PMR of fresh versus cryopreserved isolates after three *in vitro* replicative cycles (A) or
374 to compare the PMR of isolates thawed with different protocols after each replicative
375 cycle (B). Represented in (C) are the proportions of different parasites stages after 1,
376 2 and 3 cycles following thawing with either protocol.

377 **Figure 5: Invasion phenotypes of *P. falciparum* clinical isolates before and after**
378 **short-term culture adaptation.** The *ex vivo* phenotype of freshly collected isolates
379 (blue bars) obtained during the first asexual replicative cycle upon arrival at the
380 laboratory was compared to that obtained after a short-term *in vitro* adaptation of about
381 28 days (red bars). The Mann Whitney U test was used to assess the differences in
382 invasion efficiency between the different time points. The dotted lines depicted the
383 sensitivity threshold for each treatment.

384 **Figure 6: Invasion phenotypes of *P. falciparum* clinical isolates before and after**
385 **short-term cryopreservation.** The assays were set up between 24- and 36-hours
386 following sample processing and the parasites were incubated for another 18 to 24
387 hours prior to flow cytometric analysis. For each isolate, the invasion phenotype of
388 freshly culture adapted parasites (black bars) was compared to that obtained after
389 three months (dark grey bars), six months (light grey bars) and twelve months (white
390 bars) post cryopreservation. Kruskal Wallis was conducted to test for statistical
391 differences in invasion efficiency of fresh versus cryopreserved isolates. Red stars
392 denote significant differences associated with changes in invasion profile of a given
393 treatment and black stars depict significant differences with no changes in the invasion
394 profile.
395

396 **Author Contributions**

397 LGT, YA and GAA conceived the study; LGT and FA performed the experiments LGT,
398 FA, MN, YA and GAA analyzed the data and drafted the manuscript; YA, GAA, and
399 MN supervised the study. All authors critically reviewed and edited the manuscript.

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404 **Declaration of Conflicting interests**

405 The authors declare no competing interest

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Figures

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Figure 1 :

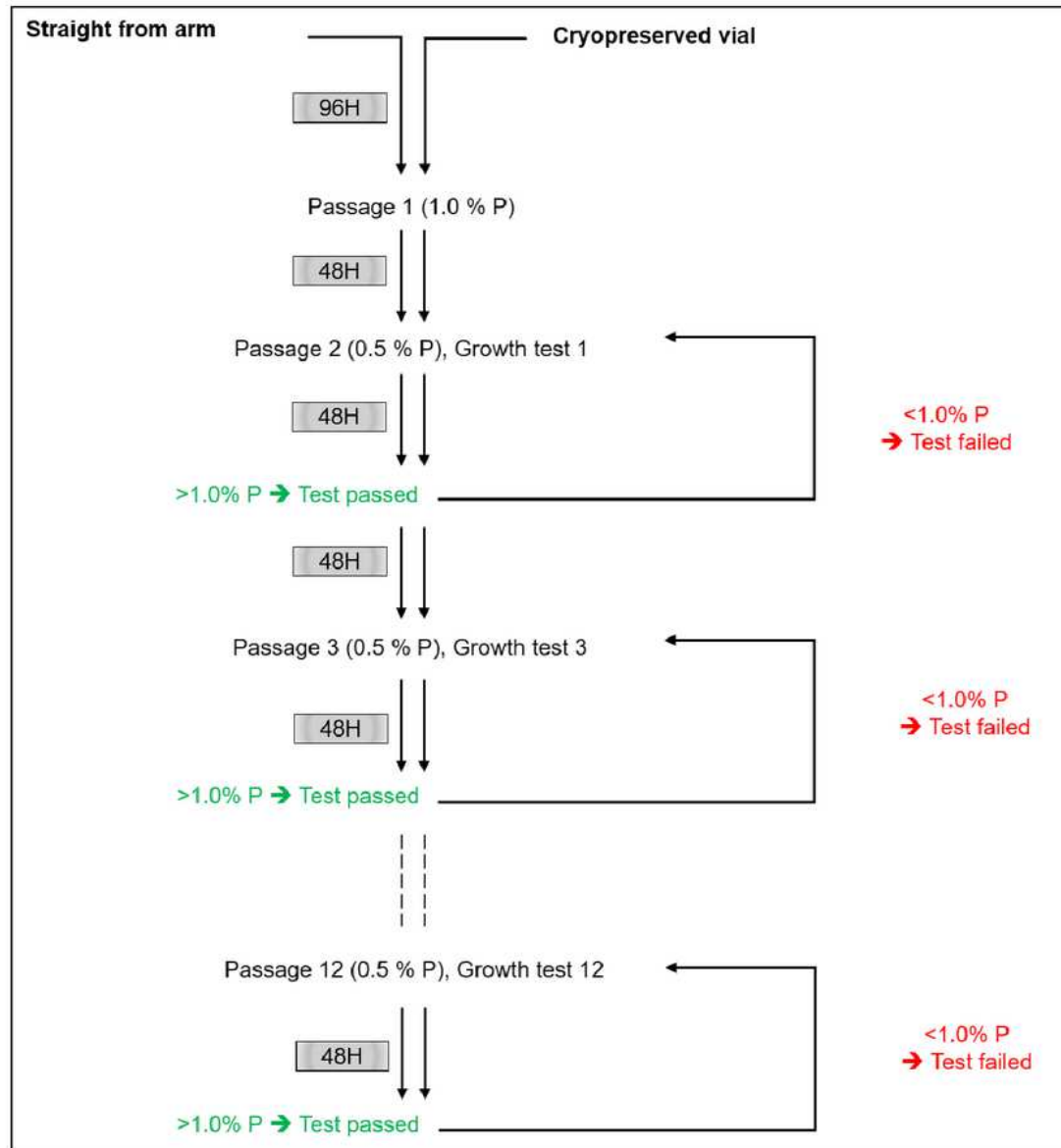


Figure 1

Schematics of in vitro culture-adaptation and growth test of freshly collected *P. falciparum* clinical isolates

Figure 2 :

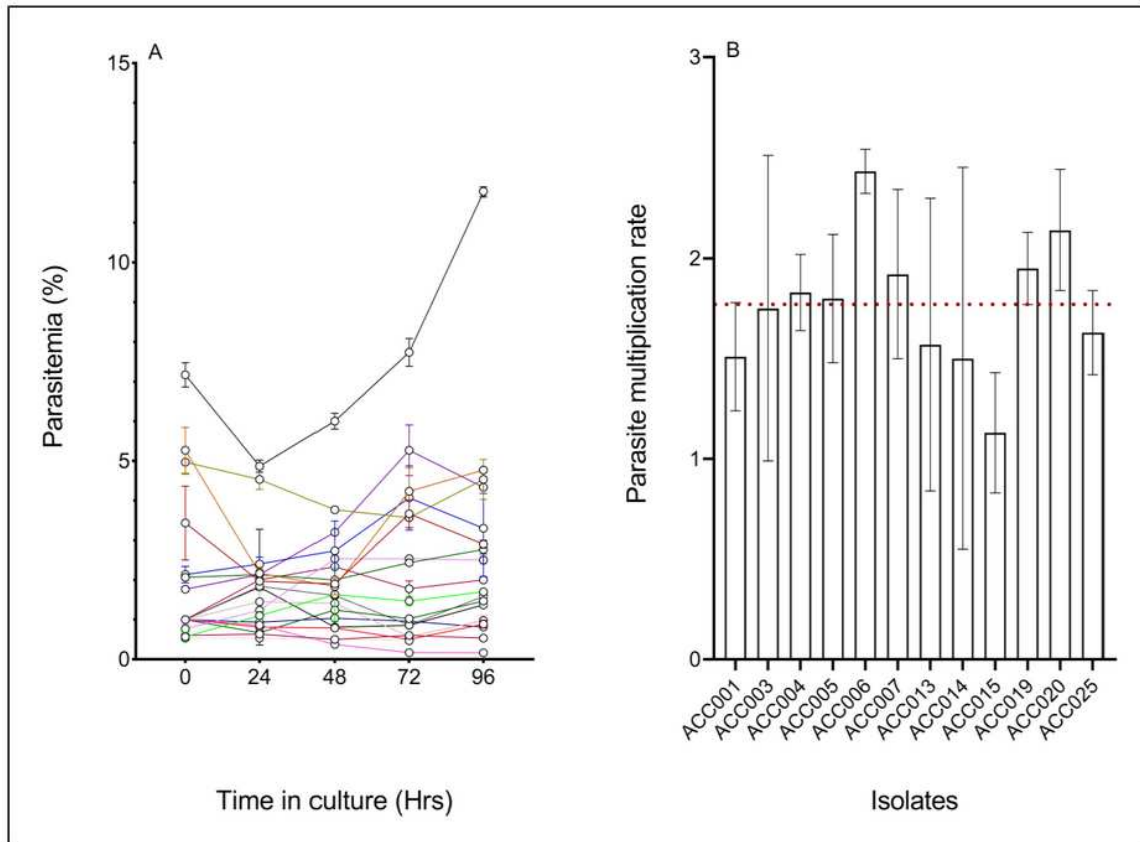


Figure 2

Early in vitro adaptation of *P. falciparum* clinical isolates. (A) The initial parasitemia (at H0) of each sample was recorded upon arrival from the field and the parasitemia of ex vivo-cultured isolates were monitored for 96 hours. The medium in the culture flasks was changed daily and supplemented with fresh erythrocytes only after 96 hours in vitro. (B) Parasite multiplication rates of successfully culture-adapted parasites following culture dilution with fresh erythrocytes. Depicted on the graph are the mean and standard errors of the PMRs of parasites with successful growth tests over a period of 28 days in vitro, and the red dotted line represents the median PMR (1.77)

Figure 3 :

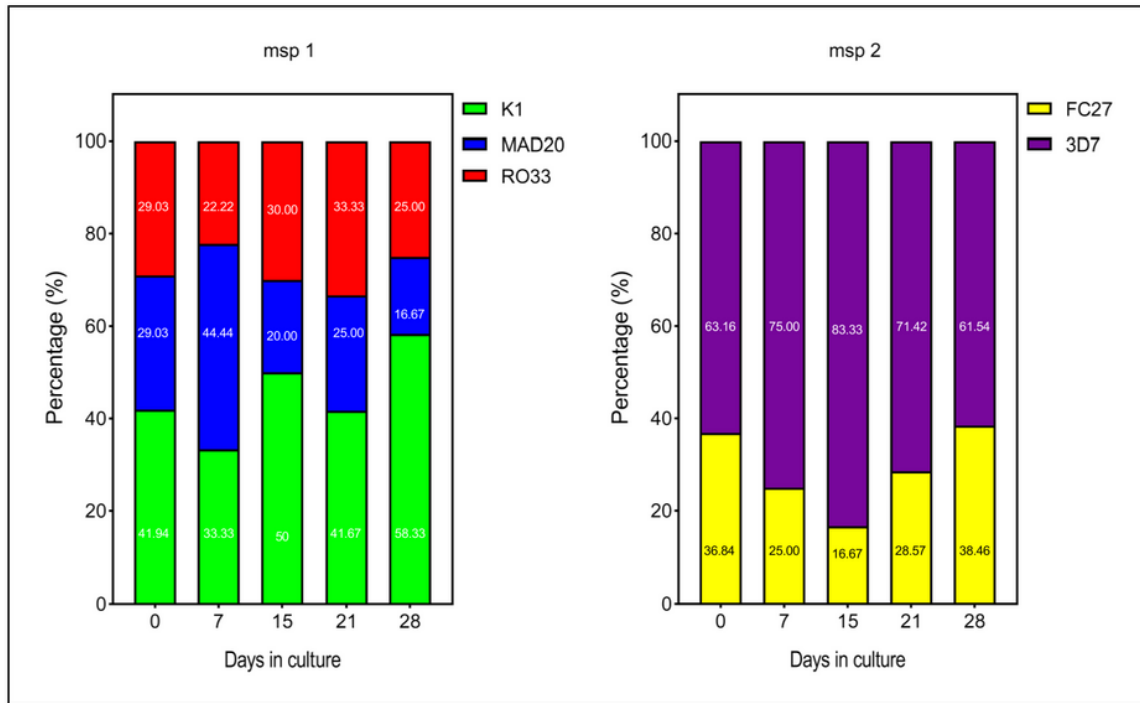


Figure 3

Proportions of different parasite clones during culture-adaptation. Parasites were all genotyped at Day 0 upon collection and successfully culture adapted ones were further genotyped at Days 7, 15, 21 and 28-post adaptation. Represented are the proportions of individual alleles for the msp 1 and 2 genes.

Figure 4

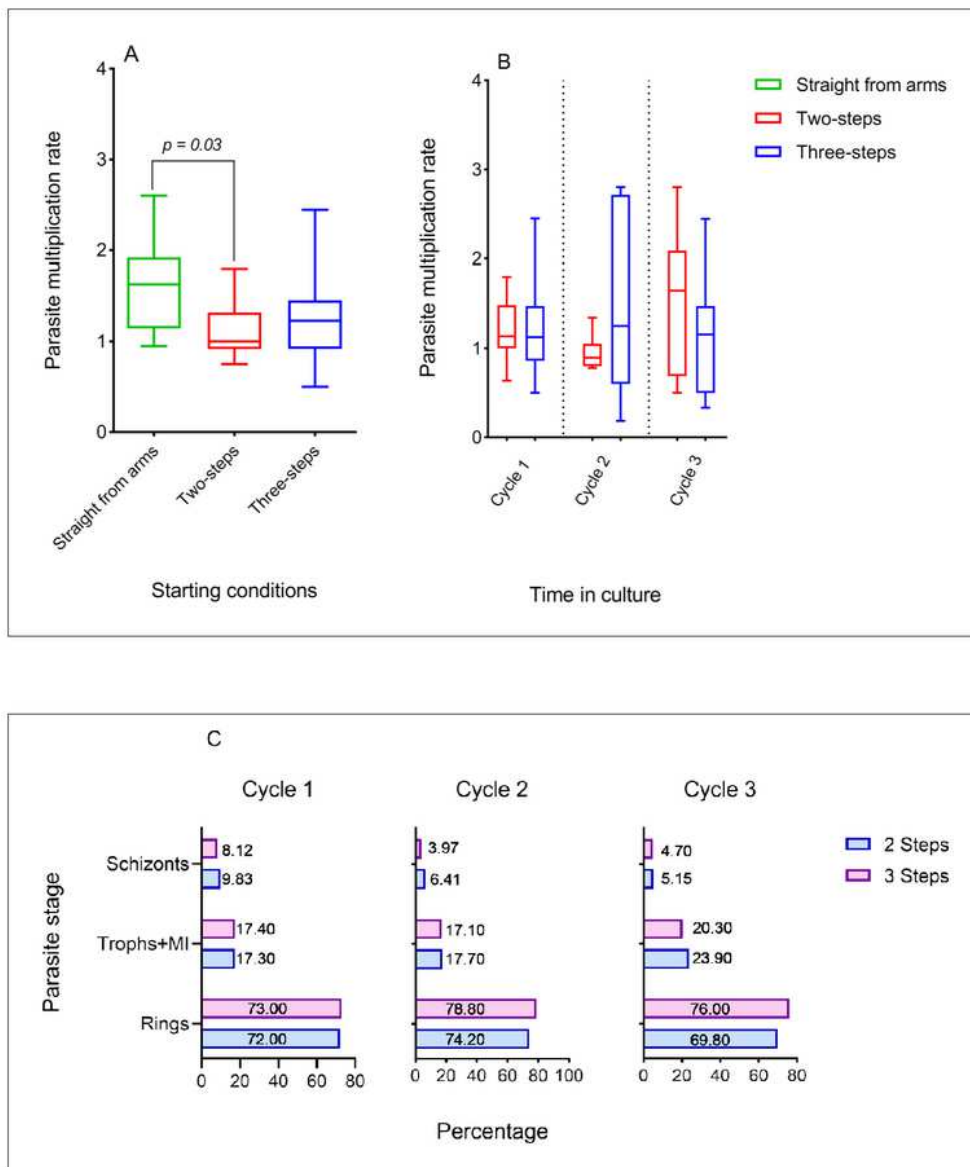


Figure 4

Multiplication rates of *P. falciparum* clinical isolates before and after cryopreservation. A-B: Box and whiskers plots showing the PMR of fresh cultured *P. falciparum* clinical isolates (green) or thawed with a two-step (red) or three-step (blue) protocol following cryopreservation. Kruskal Wallis test was conducted to compare the PMR of fresh versus cryopreserved isolates after three in vitro replicative cycles (A) or to compare the PMR of isolates thawed with different protocols after each replicative cycle (B). Represented

in (C) are the proportions of different parasites stages after 1, 2 and 3 cycles following thawing with either protocol.

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Figure 5 :

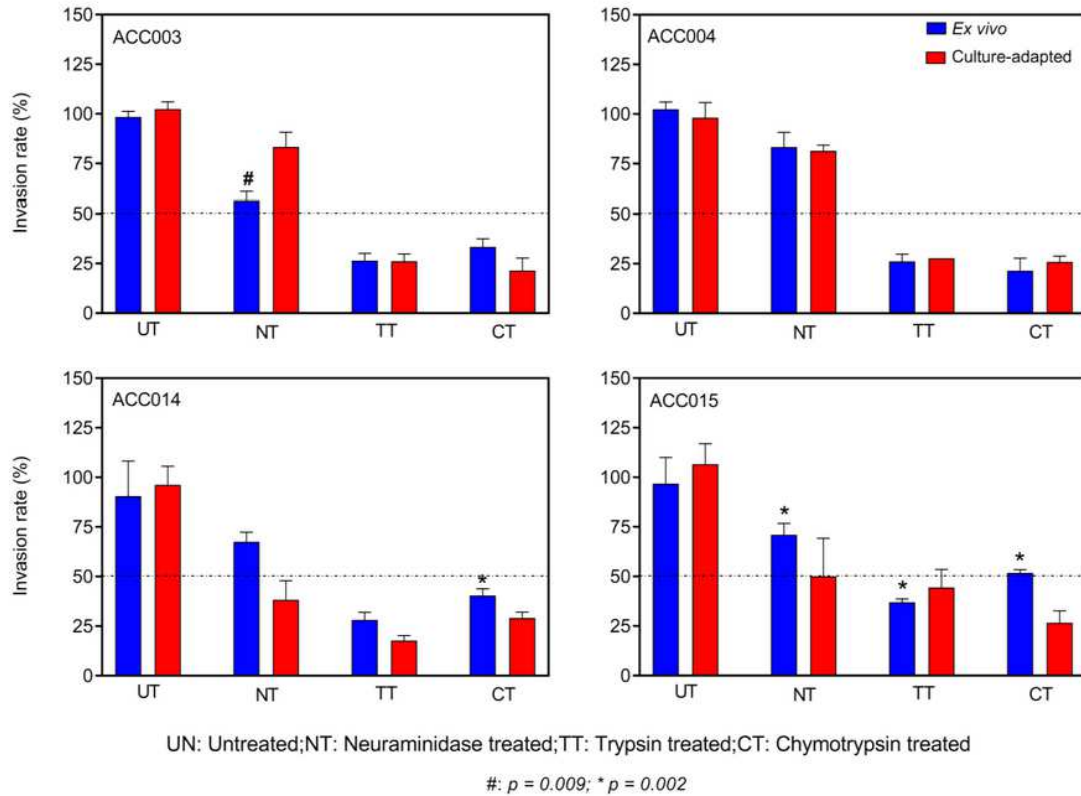


Figure 5

Invasion phenotypes of *P. falciparum* clinical isolates before and after short-term culture adaptation. The ex vivo phenotype of freshly collected isolates (blue bars) obtained during the first asexual replicative cycle upon arrival at the laboratory was compared to that obtained after a short-term in vitro adaptation of about 28 days (red bars). The Mann Whitney U test was used to assess the differences in invasion efficiency between the different time points. The dotted lines depicted the sensitivity threshold for each treatment.

Figure 6 :

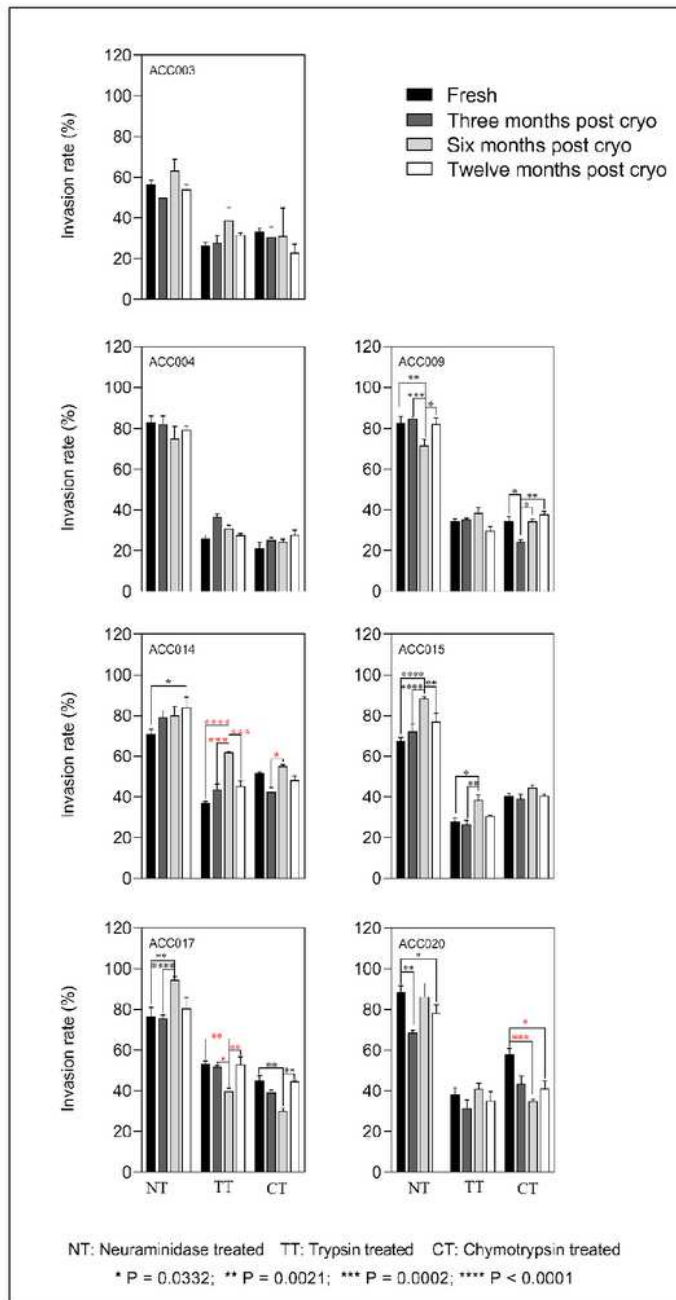


Figure 6

Invasion phenotypes of *P. falciparum* clinical isolates before and after short-term cryopreservation. The assays were set up between 24- and 36-hours following sample processing and the parasites were incubated for another 18 to 24 hours prior to flow cytometric analysis. For each isolate, the invasion phenotype of freshly culture adapted parasites (black bars) was compared to that obtained after three months (dark grey bars), six months (light grey bars) and twelve months (white bars) post

cryopreservation. Kruskal Wallis was conducted to test for statistical differences in invasion efficiency of fresh versus cryopreserved isolates. Red stars denote significant differences associated with changes in invasion profile of a given treatment and black stars depict significant differences with no changes in the invasion profile.

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