1    RH: SHORT COMMUNICATIONS

2    **Long-Term Storage of *Cryptosporidium parvum* for In Vitro Culture**

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7    Abstract: The long-term storage of *Cryptosporidium* life-cycle stages is a prerequisite for in

8    vitro culture of the parasite. *Cryptosporidium parvum* oocysts, sporozoites and intracellular

9    forms inside infected host cells were stored for 6 to 12 mo in liquid nitrogen utilizing

10    different cryoprotectants (dimethyl sulfoxide [DMSO], glycerol and fetal calf serum [FCS]),

11    then cultured in vitro. Performance in vitro was quantified by estimating the total

12    *Cryptosporidium* copy number using qPCR in 3- and 7-day-old cultures.  While only few

13    parasites were recovered either from stored oocysts or from infected host cells, sporozoites

14    stored in liquid nitrogen recovered from freezing successfully. More copies of parasite DNA

15    were obtained from culturing those sporozoites than sporozoites excysted from oocysts kept

16    at 4 C for the same period. The best performance was observed for sporozoites stored in

17    RPMI with 10% FCS and 5% DMSO, which generated 240% and 330% greater number of

18    parasite DNA copies (on days 3- and 7-post-infection, respectively) compared to controls.

19    Storage of sporozoites in liquid nitrogen is more effective than oocyst storage at 4 C and

20    represents a more consistent approach for storage of viable infective *Cryptosporidium*

21    aliquots for in vitro culture.

22        In vitro culture is an essential tool to study the apicomplexan gut parasite

23    *Cryptosporidium* (Karanis and Aldeyarbi, 2011), but despite recent advances in culture

24    systems (Morada et al., 2016), long-term storage of isolates continues to depend on

25    maintenance of oocysts at 4 C in a refrigerator. Production of oocysts in culture may be

26    sparse (Karanis and Aldeyarbi, 2011) and there are no methods for long-term storage of

27    sporozoites or other short-lived stages. We present here observations on long-term (6 mo and

28    1 yr) preservation of *Cryptosporidium* life cycle stages in liquid nitrogen, which offer an

29    alternative approach to storage of this intractable pathogen.

30    *Cryptosporidium* first came to attention some 35 yr ago as an important human gut

31    pathogen causing severe diarrhea (Bird and Smith, 1980), and is best known for the infection

32    of several hundred thousand individuals in the Milwaukee incident in 1993 (MacKenzie et

33    al., 1995). The huge number of oocysts produced by a diarrheic infected host, the apparent

34    long life span of oocysts and the possibly severe consequences of infection, in particular in

35    immunocompromised individuals, make this a highly significant pathogen, recognized by its

36    classification by Centers for Disease Control and prevention as a potential biowarfare agent

37    (www. emergency.cdc.gov/bioterrorism).

38    Calves are the natural hosts of *C. parvum*, while *C. hominis* has no natural host apart

39    from humans and is therefore only available from clinical isolates. Neonatal or SCID mouse

40    models for these pathogens require infectious doses 10-100 times higher than their natural

41    hosts (see e.g., Fayer et al., 1991; Zambriski et al., 2013). With concerns over the validity of

42    these experimental models, and the welfare and logistic issues surrounding maintenance in

43    animal hosts, we are dependent on in vitro culture to advance our understanding of

44    *Cryptosporidium* biology. Long-term storage is an essential adjunct of in vitro culture, partly

45    because of the propensity for genetic drift in cultures or animal hosts, but also because oocyst

46    production in culture is not yet as prolific as that from natural hosts (Karanis and Aldeyarbi,

47    2011), although long-term maintenance with substantial oocyst production has recently been

48    described (Morada et al., 2016; DeCicco RePass et al., 2017). In general, in vitro cultures are

49    initiated from oocysts stored at 4 C in PBS supplemented with antibiotics as collected from

50    animal hosts, on the assumption that these stages are resistant and can survive for many

51  months, despite the experimental observation that oocyst survival declines sharply after 3 mo

52  in these conditions (Liang and Keeley, 2012; Paziewska-Harris et al., 2016). Other

53  apicomplexans, such as *Plasmodium*, are routinely cultured in vitro from isolates stored in

54  liquid nitrogen for many months or years. Here we present encouraging results on the storage

55  of *Cryptosporidium parvum* in liquid nitrogen for 6 mo and 12 mo using an alternative

56  infectivity assay, which suggests that long-term storage in liquid nitrogen could become an

57  important tool in *Cryptosporidium* in vitro culture.

58        Oocysts of *C. parvum* IOWA strain (Waterborne Inc., New Orleans, Louisiana) were

59  stored prior to use at 4 C in PBS supplemented with 100U/ml penicillin, 100 µg/ml

60  streptomycin, 10 mg/ml gentamicin, 0.25 µg/ml Amphotericin B and 0.01% Tween 20. The

61  oocyst batch used was 4 mo old (post shedding by calves and purification) when storage

62  experiments began. The concentration of supplied oocysts was initially calculated using a

63  hemocytometer (W. Schreck,Hofheim/TS, Germany), and samples of appropriate numbers of

64  oocysts for experiments were generated by dilution. Human colon adenocarcinoma (HCT-8;

65  ATCC CCL 244) cells were maintained as described previously (Paziewska-Harris et al.,

66  2015), and grown in 6-well plates (Thermo Fisher Scientific, Landsmeer, The Netherlands;

67  9.6 cm$^2$ per well) at 37 C in an atmosphere containing 5% $CO_2$ until they reached 90%

68  confluence. Before storage or infection of host cells (in case of stored oocysts, see below),

69  oocysts were excysted (Hijjawii et al., 2001) by incubation for 30 min at 37 C in a 0.25%

70  trypsin (from bovine pancreas, Sigma, cat. no. T1426, Zwijndrecht, The Netherlands)

71  solution (pH 2.5, adjusted with 1M hydrochloric acid), followed by centrifugation at $2000 \times g$

72  for 5 min, after which the trypsin solution was replaced by *Cryptosporidium* maintenance

73  medium (CMM; Hijjawi et al., 2001 as modified by Paziewska-Harris et al., 2015) containing

74  200 µg/ml of bile salts (from bovine and ovine pancreas, Sigma, cat. no. B8381) and

75  incubated for a further 2.5 hr at 37 C. Released sporozoites were not separated from non-

3

76  excysted oocysts as dead/non-infective parasite stages were washed from cell monolayers 12

77  hr after addition to the cultures (Paziewska-Harris et al., 2015). Released sporozoites were

78  used either for infection (see below) or aliquoted for storage in an excess of freezing medium

79  (sporozoites in CMM: storage medium, 1:9). Four different storage media were used: I)

80  RPMI with 20% fetal calf serum (FCS) and 12% glycerol; II) RPMI with 20% FCS and 12%

81  dimethyl sulfoxide (DMSO); III) RPMI with 10% FCS and 5% DMSO (the medium

82  normally used for long term frozen storage of HCT-8 cells); IV) RPMI with 20% FCS, 10%

83  of glycerol and 10% of DMSO. All media were supplemented with 100 U/ml penicillin and

84  100 µg/ml streptomycin (Gibco, provided by Thermo Fisher Scientific, Waltham,

85  Massachusetts).

86        Oocysts, sporozoites and infected host cells were stored in each of the 4 different

87  media. Samples were cooled to -70 C at the rate of -1 C/min in a mechanical freezer (Mr.

88  Frosty Freezing Container, Thermo Fisher Scientific) before transfer to liquid nitrogen. Two

89  replicates of each combination of *Cryptosporidium* sample and storage medium were stored

90  for each time point (replicates A-B for 6 mo, C-D for 12 mo storage). Thawing after storage

91  in liquid nitrogen was performed at approximately 100 C/min, achieved by 1 min exposure to

92  room temperature followed by 1 min incubation in a water bath at 37 C, a rate compatible

93  with both protozoan survival (e.g., Miyake et al., 2004) and the recovery of host HCT-8 cells.

94  Preparation of different *Cryptosporidium* samples and their processing after freezing was as

95  follows (see also Table I for a summary of study design):

96  (i)   Oocysts (17,300 per replicate) were stored in freezing media for 6 mo. After thawing

97       they were excysted as described above, then divided and the 2 aliquots added to near-

98       confluent HCT-8 cells;

99  (ii)  Oocysts (17,300 per replicate) were excysted and released sporozoites divided into

100      freezing media (4 replicates per medium type). After 6 or 12 mo of storage 2 replicates

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101      stored in each medium were thawed, divided into 2 aliquots and added to fresh HCT-8

102      cells;

103  (iii) Newly excysted sporozoites from oocysts pre-treated with trypsin and bile salts as

104      described above (17,300 per replicate) were added to fresh HCT-8 monolayers in 25 cm$^2$

105      culture flasks. Infected cells (containing intracellular parasite stages) were harvested on

106      day 3-post-infection and divided between 16 tubes with different freezing media (4

107      replicates per medium). After storage (6 or 12 mo) they were thawed and each replicate

108      divided and seeded into 2 fresh culture plates containing HCT-8 medium.

109  For all samples the medium was changed after host cell infection (12 hr post infection) or

110  seeding of infected cells and prior to sampling (12 hr before first sampling). Samples were

111  then collected 3-or 7-days post infection/seeding (d.p.i/d.p.s.); these time points were chosen

112  to allow for any lag in development following freezing. Extracellular parasite stages were

113  recovered from the supernatant (centrifugation at 2,000 $g$ for 5 min) and intracellular stages

114  from host cells following trypsin treatment and centrifugation at 2,000 $g$ for 5 min. Following

115  centrifugation, pelleted extracellular or intracellular stages were suspended in 950 µl of lysis

116  buffer (Boom et al., 1990).

117      Additionally, different types of controls were included in the experiment. At the time

118  of freezing, 3 replicates of both intact and pre-treated oocysts were kept as controls to

119  estimate the total number of viable sporozoites and oocysts frozen (control 1). Excystation

120  rate was estimated as described (Paziewska-Harris et al., 2016): after standard excystation

121  using trypsin and bile salts, DNA from parasites was extracted (Boom et al.,1990) and qPCR

122  performed. This method allows estimation of the number of excysted parasites, as DNA is not

123  purified from intact oocysts (Paziewska-Harris et al., 2016). To estimate the quality of

124  excystation and culturing protocols, positive controls were used at every time point (control

125  2). These were cell monolayers infected with sporozoites excysted from fresh oocysts (less

5

126    than 2-mo-old with more than 75% excysting; see Paziewska-Harris et al., 2016). Negative

127    controls (HCT-8 cells without parasites, control 3) were also included. Positive and negative

128    controls were set up and harvested at the same time as experimental samples. To test the

129    differences in infectivity after standard storage in 4 C and storage in liquid nitrogen, oocysts

130    kept for 10 mo at 4 C (control 4) were used to compare with the experimental samples kept at

131    4 C for 4 mo and then 6 mo in liquid nitrogen. Sub-culturing of cells infected with

132    *Cryptosporidium* was also performed (control 5): HCT-8 cells were infected with sporozoites

133    and sub-cultured 3 days post infection into fresh plates. Samples were then collected on day 3

134    and day 7 post sub-culturing. To assess the excystation ability of oocysts frozen with

135    different cryoprotectants, triplicates of 1,000 4-mo-old oocysts were stored at -20 C for 1 wk,

136    and after thawing they were subjected to excystation followed by DNA isolation (control 6).

137    Three samples of unfrozen oocysts from the same batch were used as a control.

138         DNA was isolated as described by (Boom et al., 1990) and qPCR used as rapid

139    method bulk approach to estimate 'zoites' (Paziewska-Harris et al., 2016) rather than semi-

140    quantitative slide-based methods such as immunofluorescence microscopy. This method

141    measures only DNA from living sporozoites as the 12 hr rinse of cultures effectively removes

142    dead sporozoites and DNA released from lysed stages (Paziewska-Harris et al., 2015), giving

143    confidence that only DNA from excysted sporozoites which had remained viable for at least

144    12 hr was being measured. A standard curve was constructed using a 10-fold dilution series

145    of sporozoites from 20,000 fresh oocysts/µl, which excysted with an efficiency of between 90

146    to 100% (estimated as in Paziewska-Harris et al., 2016). The highest concentration was

147    therefore assumed to represent 80,000 DNA parasite copies/µl (as each oocyst contains 4

148    haploid sporozoites). PCR sensitivity was estimated as 0.2 oocyst/µl, which translates to 4

149    copies of 18S rDNA (as each sporozoite has 5 copies of 18S rDNA gene in the genome;

150    Abrahamsen et al., 2005). At each time point only 2 or 3 samples of each replicate (recovered

151   from different media or controls) were analyzed, precluding statistical analysis and the results

152   are therefore shown as arithmetic means.

153   The mean number of excysting oocysts estimated by qPCR for the 4-mo-old pre-

154   treated input controls (control 1) was 1,540 (of a total of 17,300) per replicate, suggesting a

155   maximum viability for sporozoites of 9%. Positive controls (cells infected with sporozoites

156   from 2 mo-old oocysts with greater than 75% excystation) contained a mean of about 25,000

157   parasite stages after 3 d.p.i. and 18,000 after 7 d.p.i. (control 2). None of the negative controls

158   gave a signal using PCR targeting *Cryptosporidium* DNA (control 3). In all experimental

159   samples and controls the majority of parasite DNA was present in the extracellular phase

160   (data not shown), as also noted by Paziewska-Harris et al. (2015). The results are presented as

161   the total number of parasites recovered, combining both extracellular and intracellular stages.

162   We also confirmed that parasites could be recovered from infected cells harvested on day 3

163   p.i. and sub-cultured to new culture plates (average number of parasites in infected cells

164   while sub-culturing: 275 per sample) (control 5). After 3 days of sub-culturing the mean

165   number of recovered parasites was 320, while after 7 days 310 parasites were recovered.

166   Oocysts stored in liquid nitrogen for 6 mo lost their infectivity regardless of the

167   medium used for storage (see Table II). The total number of *Cryptosporidium* stages

168   recovered from cell cultures infected with sporozoites from 10 mo old oocysts which had

169   been kept in liquid nitrogen for 6 mo did not exceed 50, only 7% and 22% (3 d.p.i. and 7

170   d.p.i., respectively) of the number released from oocysts kept throughout at 4 C (control 4).

171   Previous attempts to freeze oocysts had shown that there is no apparent increase in survival

172   of oocysts frozen with cryoprotectants at -20 C, -80 C or in liquid nitrogen (Fayer et al.,

173   1991; Kim and Healey, 2001). Short-term freezing was more successful (control 6): oocysts

174   frozen for a week in -20 C using different storage media (I-IV) showed viability of 2%-74%

175   as compared to refrigerated controls (maximum of 927 live sporozoites estimated from

176 oocysts stored in medium II vs. 1,246 from the control samples) (Table II). It appears that the

177 thick oocyst wall prevents cryoprotectants from penetrating the cells, leading to a loss of

178 viability over longer periods at lower temperatures. Parasites stored within host cells also lost

179 viability and only very small numbers (or none) were recovered after 6 and 12 mo storage in

180 liquid nitrogen. Only 1 replicate (cells stored in medium IV for 6 mo and cultured for 7 days)

181 with better parasite recovery from cells than from frozen oocysts (Table II) was noted. This

182 may relate to the small proportion of human cells that recover after freezing.

183       Excysted sporozoites stored in liquid nitrogen retained viability much better than

184 either oocysts or parasites in host cells; after 6 mo storage those sporozoites could be

185 recovered from all storage media. The highest signal on day 3 p.i. was observed for parasites

186 stored in medium IV, and on day 7 p.i. for those kept in medium III (Fig. 1). The infectivity

187 of sporozoites from oocysts stored at 4 C for 4 mo and then in liquid nitrogen for 6 mo was

188 greater than that of sporozoites from oocysts kept at 4 C for the full 10 mo (control 4). The

189 number of *Cryptosporidium* stages recovered after combination of storage at 4 C and in liquid

190 nitrogen ranged from 1.15× (3 d.p.i.) to 3.3× (7 d.p.i.) the number recovered from cultures

191 using 10-mo-old oocysts stored at 4 C (control 4) (Fig. 2). After 12 mo of storage in liquid

192 nitrogen, the best recovery of parasites 3 and 7 days p.i. came from medium III (Fig. 3), with

193 only few parasites recovered after 12-mo-storage in media I and II. The potential for

194 successful infection by sporozoites stored in liquid nitrogen decreased with time (Fig. 4), but

195 this decline was smallest using medium III; between 45% (7 d.p.i.) and 65% (3 d.p.i.) of

196 sporozoites retained infectivity after 12 mo storage relative to 6 mo storage in this medium.

197 Frozen storage of sporozoites has been tried previously with *Cryptosporidium* (Sherwood et

198 al., 1982; Rossi, 1990; Fayer et al., 1991; Rhee and Park, 1996; Kim and Healey, 2001), but

199 as oocysts did not retain infectivity for neonatal or immunosuppressed mice, the approach

200 was dropped, and it is clear that freezing kills oocysts. Fayer et al. (1991) also observed a

201   lack of infectivity of rectally-intubated *C. parvum* sporozoites for neonatal BALB/c mice

202   following frozen storage.  However, mice are a poor host for *C. parvum*, requiring a much

203   higher inoculum than the 10 oocysts needed to infect calves (Zambriski et al., 2013), and

204   there is no doubt that frozen storage does reduce viability of sporozoites. It may therefore be

205   that in these experiments viability may have been reduced to below the level at which

206   infections could take place. Cryopreservation with simple cryoprotectants may also render

207   sporozoites uninfective in animal models, since the presence of FCS in frozen storage media

208   can potentially trigger the sporozoite-trophozoite transition (Edwinson et al., 2016)

209   prematurely. Nevertheless, we would argue that cryopreservation can provide effective long-

210   term storage medium for sporozoites which is then more consistent than storage of oocysts

211   for equivalent periods at 4 C. Given the overwhelming importance of in vitro studies (e.g.,

212   Vinayak et al., 2015; Edwinson et al., 2016; Morada et al., 2016; DeCicco RePass et al.,

213   2017) for understanding the biology of *Cryptosporidium*, and for large scale drug-screening,

214   a potential lack of infectivity of frozen sporozoites in animal models is less significant.

215   Further experiments will certainly fine-tune the method and adapt it for particular

216   experimental needs, but even based on the experimental data provided here it can be

217   concluded that cryopreservation of sporozoites is a viable approach to long-term storage of

218   *Cryptosporidium*. Indeed, even using the methodologies described here, freezing of excysted

219   aliquoted sporozoites is likely to represent a more consistent and possibly more efficient

220   means of keeping isolates than the recommended storage of oocysts at 4 C.

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278    Figure 1. Number of parasites (mean of 2 replicates) recovered from host cell cultures

279    infected with sporozoites stored in different media for 6 mo. Parasites were harvested 3 days

280    post infection (d.p.i.) (black bars) and 7 d.p.i. (white bars). Storage media used: I) RPMI with

281    20% FCS and 12% glycerol; II) RPMI with 20% FCS and 12% DMSO; III) RPMI with 10%

282    FCS and 5% DMSO; IV) RPMI with 20% FCS, 10% of glycerol and 10% of DMSO.

283    Figure 2. Differences in proportion of parasites recovered from cultures after storage in liquid

284    nitrogen in different media for 6 mo as compared to controls kept at 4 C. Parasites were

285    harvested 3 days post infection (d.p.i.) (back bars) and 7 d.p.i. (white bars). All the

286    calculations are based on 2 replicates of each condition. Storage media used: I) RPMI with

287    20% FCS and 12% glycerol; II) RPMI with 20% FCS and 12% DMSO; III) RPMI with 10%

288    FCS and 5% DMSO; IV) RPMI with 20% FCS, 10% of glycerol and 10% of DMSO.

289    Figure 3. Number of parasites (mean of 2 replicates) recovered from host cell cultures

290    infected with sporozoites stored in different media for 12 mo. Parasites were harvested 3 days

291    post infection (d.p.i.) (black bars) and 7 d.p.i. (white bars). Storage media used: I) RPMI with

292    20% FCS and 12% glycerol; II) RPMI with 20% FCS and 12% DMSO; III) RPMI with 10%

293    FCS and 5% DMSO; IV) RPMI with 20% FCS, 10% of glycerol and 10% of DMSO.

294    Figure 4. Differences in proportion of parasites recovered from host cell cultures infected

295    with sporozoites stored in liquid nitrogen in different media for 12 mo as compared to 6 mo.

296    Parasites were harvested 3 days post infection (d.p.i.) (back bars) and 7 d.p.i. (white bars). All

297    the calculations are based on 2 replicates of each condition. Storage media used: I) RPMI

298    with 20% FCS and 12% glycerol; II) RPMI with 20% FCS and 12% DMSO; III) RPMI with

299    10% FCS and 5% DMSO; IV) RPMI with 20% FCS, 10% of glycerol and 10% of DMSO.

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Table 1

Table I. Experimental design: all samples stored in liquid nitrogen for every freezing medium for each time period; d.p.i.- day post infection, d.p.s.- day post seeding.
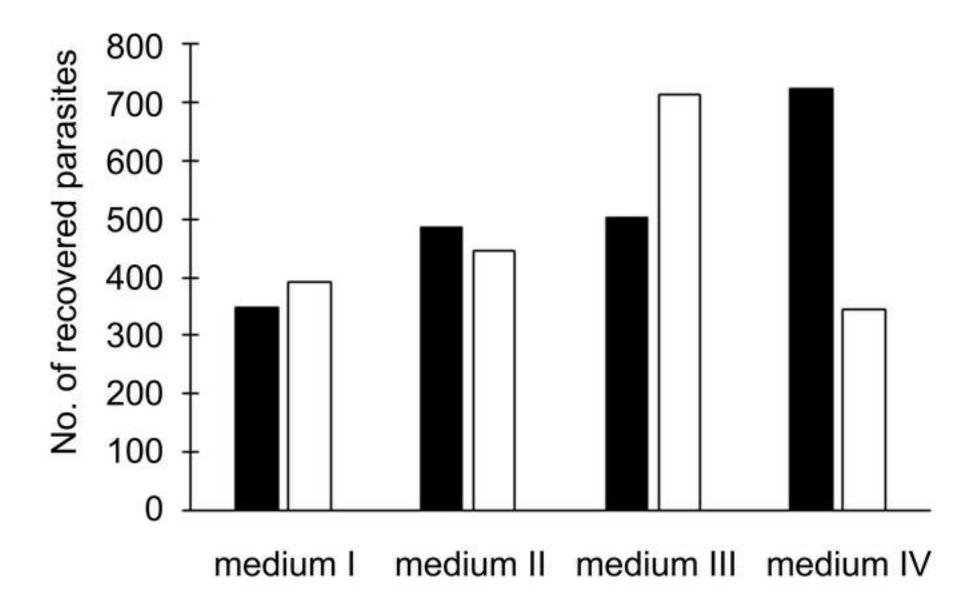
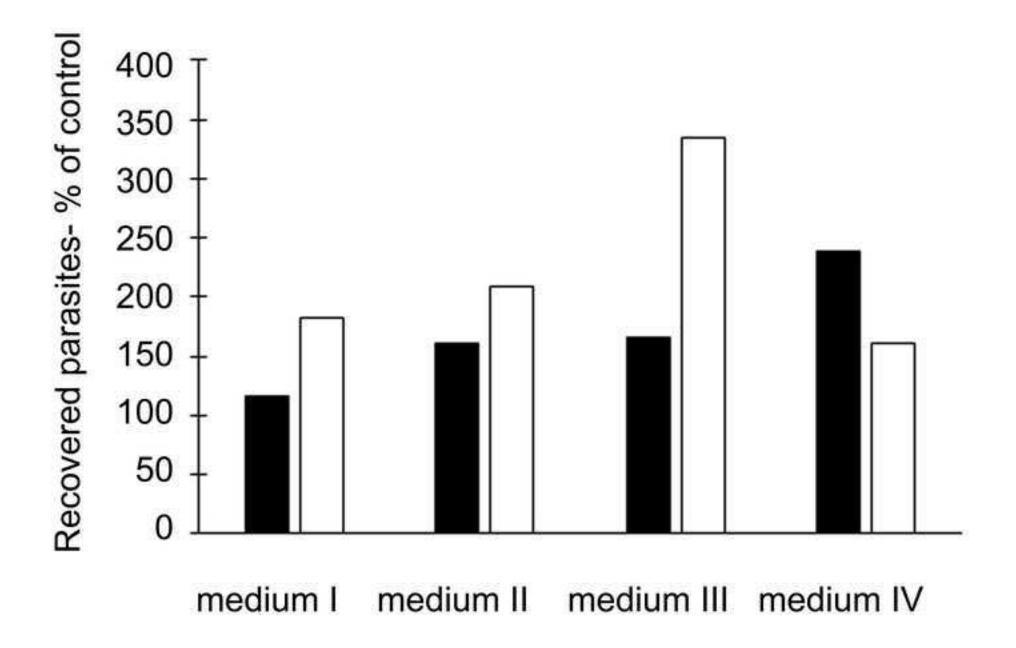| | Oocysts | Sporozoites | Infected cells |
|---|---|---|---|
| Treatment before storage | None | Excystation | Excystation, host cell infection, harvesting on 3 d.p.i. |
| Oocysts used per replicate | 17300 | 17300 | 17300 |
| Total number* of stored parasites per replicate | 69200 | 69200 | 6920† |
| Number of replicates | 2 | 4 | 4 |
| Storage time | 6 months (replicates A, B) | 6 months (replicates A, B) and 12 months (replicates C, D) | 6 months (replicates A, B) and 12 months (replicates C, D) |
| Treatment after storage | Each replicate excysted, aliquoted (aliquots 1-2) | Each replicate aliquoted (aliquots 1-2) | Each replicate aliquoted (aliquots 1-2) |
| Culturing | Each aliquot added to fresh HCT-8 monolayer, harvested on 3 d.p.i. (1) or 7 d.p.i. (2) | Each aliquot added to fresh HCT-8 monolayer, harvested on 3 d.p.i. (1) or 7 d.p.i. (2) | Each aliquot seeded on the plate, harvested on 3 d.p.s. (1) or 7 d.p.s. (2) |

*Based on 4 sporozoites per oocyst.

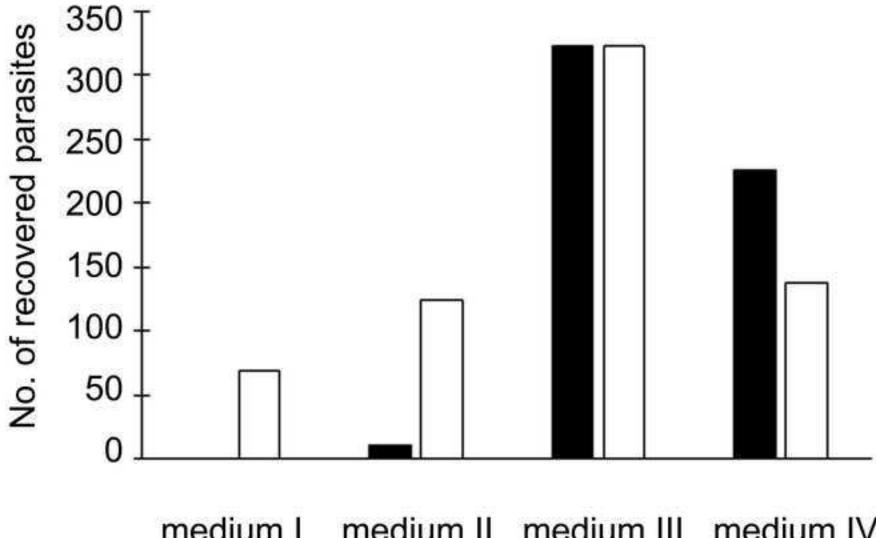†Based on 1% recovery at the time of harvesting infected cells on 3 d.p.i. estimated based on experiments using oocysts of the same age as in the study (data not published).

Table 2

Table II. Numbers of parasites recovered from cultures after 3 or 7 days post infection/seeding. Oocysts used in the experiments were stored for 4 mo at 4 C. Then either oocysts or HCT-8 cells infected with parasites derived from these oocysts were stored in different freezing media (I-IV, see text for details) in liquid nitrogen (LN; for input numbers see text and Table 1) or at -20 C (1,000 oocysts) for different time periods; d.p.i. - day post infection, d.p.s. - day post seeding; NA- not applicable- only viability was tested.

| | Oocysts | | | Infected cells | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Storage conditions | 1 wk at -20 C | 6 mo in LN | | 6 mo in LN | | 12 mo in LN | |
| D.p.i./d.p.s. | NA | 3 | 7 | 3 | 7 | 3 | 7 |
| Medium I | 493 | 16 | 0 | 0 | 0 | 0 | 0 |
| Medium II | 927 | 0 | 29 | 0 | 0 | 6 | 0 |
| Medium III | 147 | 7 | 47 | 0 | 0 | 0 | 0 |
| Medium IV | 27 | 20 | 8 | 0 | 256 | 42 | 0 |

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4