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Aly S Muadica , Sooria Balasegaram , Kazim Beebeejaun, Pamela C Köster , Begoña Bailo, Marta Hernández-de-Mingo, Alejandro Dashti, Elena Dacal, José M Saugar, Isabel Fuentes, David Carmena .

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4 Aly S. Muadica[1,2§], Sooria Balasegaram[3§], Kazim Beebeejaun[3], Pamela C. Köster[1], Begoña

5 Bailo[1], Marta Hernández-de-Mingo[1], Alejandro Dashti[1], Elena Dacal[1], José M. Saugar[1],

6 Isabel Fuentes[1], David Carmena[1, *]

7

8 [1)] Parasitology Reference and Research Laboratory, National Centre for Microbiology,

9 Majadahonda, Madrid, Spain

10 [2)] Departamento de Ciências e Tecnologia, Universidade Licungo, Quelimane, Zambézia,

11 Mozambique

12 [3)] Field Epidemiology Services, National Infection Service, Public Health England, London,

13 United Kingdom

14

15

16 [*] Corresponding author. D. Carmena, Parasitology Reference and Research Laboratory,

17 National Centre for Microbiology, Carlos III Health Institute, Ctra. Majadahonda-Pozuelo

18 Km 2, 28220 Majadahonda, Madrid, Spain

19 *Email address*: dacarmena@isciii.es (D. Carmena)

20 Tel.: +34 91 822 3641

21 Fax: +34 915097919

22

23 [§] ASM and SB contributed equally to this article.

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Abstract

Objectives: Chronic infections by enteric parasites including protist and helminthic species produces long-term sequelae on the health status of infected children. This study assesses potential associations linked with enteric parasite infections in symptomatic and asymptomatic children in Zambézia province, Mozambique.

Methods: In this prospective cross-sectional study, stool samples and epidemiological questionnaires on demographics and risk associations were collected from symptomatic children ($n = 286$) from clinical settings and asymptomatic ($n = 807$) children from 17 schools and creches aged 3–14 years. We detected enteric parasites by PCR-based methods. We calculated prevalence (adjusted for age, sex, house construction, drinking water, and latrine use) and odds ratios (OR) for risk associations with logistic regression, after adjusting for district, neighbourhood, and symptoms.

Results: Numbers and adjusted prevalences (95% confidence intervals in brackets) for the symptomatic and asymptomatic populations were *G. duodenalis* 120, 52% (22–82), 339, 42% (25–59); followed by *S. stercoralis* 52, 14% (9–20), 180, 20% (15–25). Risk associations for *G. duodenalis* included drinking untreated river/spring water, OR 2.91 (1.80–4.70); contact with ducks, OR 14.96 (2.93–76.31); dogs, OR 1.92 (1.04–3.52); cats, OR 1.73 (1.16–2.59), and a relative with diarrhoea, OR 2.59 (1.54–4.37). Risk associations for *S. stercoralis* included having no latrine, OR 2.41 (1.44–4.02); drinking well water, OR 1.82 (1.02–3.25), and increasing age, OR 1.11 (1.04–1.20).

Conclusions: We found a high prevalence of intestinal parasites regardless of the children's symptoms. Drinking well or river water, domestic animals, and latrine absence were contributing factors of human infections.

Introduction

The protozoa *Cryptosporidium* spp., *Giardia duodenalis*, and *Entamoeba histolytica*, and, to a lesser extent, the Stramenopile *Blastocystis* sp., are the commonest causes of parasitic diarrhoeal illness. *Cryptosporidium* infection is the second cause of diarrhoeal death in children ≤5 years in many countries including Mozambique [1,2]. *Giardia duodenalis* is the commonest protozoa causing intestinal disease globally (estimated 2.8 x 10^8 cases/year) [3], whereas invasive amoebic infection by *E. histolytica* is the fourth cause of mortality worldwide due to parasitic infection [4]. Although the pathogenic potential of *Blastocystis* sp. is unclear, evidence associates it with intestinal and extra-intestinal disorders [5]. The soil-transmitted helminth (STH) *Strongyloides stercoralis* infects up to 370 million people annually [6]. Asymptomatic chronic infections by these pathogens have been linked with growth faltering, malnutrition, stunting, chronic anaemia, and cognitive impairment [7-10].

Little is known on risk factors for intestinal parasites in Mozambique. Protozoan infections (0.5–37%) were reported in a community-based study in Beira [11], in infants and young children in Manhiça [2], and in HIV- and tuberculosis-infected individuals in Chowke [12]. Regarding STHs, S. *stercoralis* prevalence estimates include 1.1% in rural children with diarrhoea in Manhiça by microscopy [13] and 48% in Beira by PCR [11]. Risk factors for childhood enteric infections and death from diarrhoeal illness have been investigated in urban Maputo [14] and rural Manhiça [15,16], respectively.

This study assesses potential risk and/or protective associations with enteric parasite infections in symptomatic and asymptomatic children living in Zambézia province, Mozambique.

70 **Methods**

71 *Study population and collection of samples*

72 A prospective molecular epidemiological study was conducted with children aged 3–14 from
73 10 of the 22 districts of the Zambézia province, Mozambique, between October 2017 and
74 February 2019; cross-sectional for schools and an incidental cohort for clinics. Official
75 census information was obtained by district of residence for age, sex, and data regarding main
76 drinking water source, house material, and latrine availability [17].

77 In primary health clinics, children with gastrointestinal complaints (chronic or acute
78 diarrhoea, bloating, abdominal pain) were invited to participate in the survey. In school
79 settings (range: 35–2,111; mean: 651 schoolchildren) informative meetings were held for
80 interested families. Schoolchildren were excluded if they had diarrhoea in the last 7 days
81 before sample collection. Participating schoolchildren were given sampling kits (uniquely
82 labelled sterile polystyrene plastic flask with spatula) and stool samples were collected on
83 the following day by a member of our research team. An aliquot (2–3 g) of each faecal
84 specimen was transferred to REAL Minisystem devices (Durviz, Valencia, Spain) for stool
85 sample conservation and concentration. Preserved samples were maintained at room
86 temperature up to three months before processing.

87 *Questionnaire survey*

88 Individual standardized questionnaires (Supplementary Table S1) in Portuguese were
89 completed by a member of our research team in face-to-face interviews with each
90 participating child at sample collection. Questions included demographics, hand and
91 vegetable washing, presence of diarrhoea in the participant or family, domestic animals and

92 livestock, type of house material, and source of drinking water, use of water treatment-
93 chlorine or boiling, latrine use and rural/urban residence.

94 *DNA extraction*

95 Genomic DNA was extracted from ca 200 mg of concentrated faecal material using the
96 QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's
97 instructions. Extracted and purified DNA samples in molecular grade water (200 µL) were
98 kept at −20 °C until further analysis.

99 *Molecular detection of* Giardia duodenalis

100 Detection of *Giardia duodenalis* was carried out by a real-time PCR (qPCR) method
101 amplifying the small subunit rRNA (*ssu* rRNA) gene of the parasite [18]. Amplification
102 reactions were conducted in total volumes of 25 µL with 3 µL template DNA, 12.5 pmol of
103 the primer set Gd-80F/Gd-127R, 10 pmol of TaqMan™ probe, and 1× TaqMan™ Gene
104 Expression Master Mix (Applied Biosystems, California, USA).

105 *Molecular detection of* Entamoeba histolytica *and* E. dispar

106 Detection and differential diagnosis of *E. histolytica* and *E. dispar* was carried out by a qPCR
107 method targeting the *ssu* rRNA gene of the *E. histolytica*/*E. dispar* complex [19,20].
108 Amplification reactions (25 µL) consisted of 3 µL template DNA, 12.5 pmol of the primer
109 set Ehd-239F/Ehd-88R, 5 pmol of each TaqMan™ probe, and 1X TaqMan™ Gene
110 Expression Master Mix (Applied Biosystems).

111 *Molecular detection of* Strongyloides stercoralis

112 Detection of *S. stercoralis* was achieved by a qPCR method amplifying the *ssu* rRNA gene
113 of the nematode [21,22]. Amplification reactions were conducted (in duplicate) in total
114 volumes of 25 µL with 10 µL template DNA, 0.2 µM of the primer set F/R, 0.5 µL of 50×
115 Sybr Green (Invitrogen, San Diego, CA, USA) and 1× Quantimix EasyMaster Mix (Biotools
116 B&M Laboratories, Madrid, Spain).

117 *Molecular detection of* Cryptosporidium *spp.*

118 *Cryptosporidium* spp. was detected using a nested-PCR protocol targeting the *ssu* rRNA gene
119 [23]. Both PCR reactions were conducted in a total volume of 50 µL including 3 µL of DNA
120 sample and 0.3 µM of the primer pairs CR-P1/CR-P2 for the primary reaction and CR-
121 P3/CPB-DIAGR for the secondary reaction.

122 *Molecular detection of* Blastocystis *sp.*

123 Detection of *Blastocystis* sp. was conducted by a direct PCR targeting the *ssu* rRNA gene of
124 the parasite [24]. The PCR reaction contained a total volume of 25 µL including 5 µL of
125 template DNA and 0.5 µM of the primer set RD5/BhRDr.

126 Main PCR features for the molecular detection of *G. duodenalis*, *E. histolytica*, *E.*
127 *dispar*, *S. stercoralis*, *Cryptosporidium* spp., and *Blastocystis* sp. are shown in
128 Supplementary Table S2. All qPCR reactions were run on a Corbett Rotor-Gene 6000 qPCR
129 cycler (QIAGEN). All direct and nested PCR reactions were run on a 2720 thermocycler
130 (Applied Biosystems). Reaction mixtures included 2.5 units of MyTAQ DNA polymerase
131 (Bioline GmbH, Luckenwalde, Germany), and 5× MyTAQ Reaction Buffer containing 5 mM
132 dNTPs and 15 mM $MgCl_2$. PCR amplicons were visualised on 2% D5 agarose gels (Conda,
133 Madrid, Spain) and stained with Pronasafe nucleic acid staining solution (Conda).

134 *Statistical analyses*

135 We analysed the data using EpiData 4.2.0 (EpiData Association, Odense, Denmark) and Stata
136 software, versions 13 and 15 (STATA Corp., College Station, Texas, US). We calculated the
137 crude prevalence of each parasite and using census data, weighted for a) the district age/sex
138 population and b) district age/sex population, latrine use, water source, and house material
139 [17]. Age was coded into three categories (3–6, 7–10, 10–14) for weighting but used as a
140 continuous variable in the risk analysis; categorical variables were re-coded as binary. The
141 chi-square and/or the Fisher's exact test were used to compare infection with binary variables.
142 A probability (*P*) value <0.05 was considered evidence of statistical significance. We
143 examined for possible confounders (change of >20% in OR of other factors) and interactions,
144 particularly the effect of water treatment on water source by stratification. Where stratified
145 analysis showed effect modification, this was included as an interaction in the multivariable
146 model. To combine symptomatic and asymptomatic populations, we included “a priori” the
147 symptomatic variable in the multivariable models. Risk associations and then other parasites
148 with a *P*-value ≤ 0.2 from the univariable analysis were selected in the multivariable logistic
149 regression model, using Akaike's information criterion (AIC) and Bayesian information
150 criterion (BIC) to determine selection and evaluate the final model. Odds ratios (OR) and
151 their 95% confidence intervals (CI) were calculated using Wald test. To account for
152 clustering, we ran multilevel models with district and/or neighbourhood nested within district
153 as random effects.

154 *Ethics and regulatory issues*

155 This study was approved by the Ethics Committee of the Health Institute Carlos III (CEI
156 PI17_2017-v3) and the National Bioethics Committee for Health of the Republic of
157 Mozambique (52/CNBS/2017).

158 **Results**

159 *Prevalence of parasites*

160 There were 1093 children enrolled, aged 3–14 years, of which 286 were enrolled from six
161 primary health care centres and a hospital clinic and resided in 37 different neighbourhoods
162 in six districts (Supplementary Tables S3 and S5). The other 807 children were enrolled
163 from 17 schools and one creche and resided in 66 neighbourhoods (including the 37) in10
164 districts (Supplementary Tables S4 and S5). School children had a median age of 8 (range
165 4–14, IQR 5–10); clinic children 7 (range 3–14, IQR 5–10). There was a 50% split of males
166 and females.

167 We detected infection with at least one parasite in 663/1093 (61%) children, two or
168 more parasites in 194/1093 (18%) children, three or more parasites in 72/1093 (7%); four
169 parasites in 17/1093 (2%) and no child with all five parasites. Table 1 shows the crude
170 prevalence for each group; Table 2 the adjusted estimated prevalences. Numbers (adjusted
171 prevalences) for the symptomatic and asymptomatic populations were *G. duodenalis*
172 120(52%*),* 339(42%); followed by *S. stercoralis* 52(14%), 180(20%); *Blastocystis* sp.
173 11(1.6%), 143(17%); *E. dispar* 6(1.4%), 115(12%); with *Cryptosporidium* spp. 2(0.9%),
174 11(3.9%). No child was infected with *E. histolytica*. The asymptomatic group had
175 significantly higher prevalences for *Blastocystis* sp., *E. dispar* and co-infections. The
176 commonest symptoms in the symptomatic group with parasites (n=160), were diarrhoea 128,

177 (80%); weakness 81, (51%); decreased appetite 41, (26%); and vomiting 27, (17%)
178 (Supplementary Table S6). The significant associations (excluding *Giardia* co-infection,
179 comparing those symptomatic without any parasite) were *G. duodenalis* with diarrhoea:
180 101/120 cases (84%); OR 3.27 (95% CI 1.72–6.36 $P = 0.000$) and *Cryptosporidium* spp. with
181 abdominal pain: 2/2 cases (100%); OR 12.8 (95% CI 2.78–infinity $P = 0.0258$).

182 *Risk association analysis.*

183 As everyone answered "yes" to handwashing and washing vegetables, these questions were
184 omitted. (Supplementary Table S7). Water treatment by chlorine or boiling was combined
185 into one variable as 139 (13.8%) people had used chlorine, 5 (0.5%) people boiling and 7
186 (0.6%) people both. For *Cryptosporidium* spp., age was inversely related to infection,
187 however, there was inadequate power to run a multivariable model. Multivariable models for
188 other protozoa are shown in Table 3a.

189 For G. *duodenalis*, after controlling for district and neighbourhood, risk associations
190 included contact with ducks (only accounting for 5 cases), diarrhoea in a relative, contact
191 with dogs, contact with cats, and having a house material of other (usually rented
192 accommodation of zinc/straw roof and concrete blocks) compared to wood, adobe or
193 masonry. Drinking river/stream water as a primary or secondary source of water was
194 associated with *G. duodenalis,* but mitigated by treatment either by chlorine and/or boiling.
195 Water treatment also lowered the odds of *G. duodenalis* in people who did not drink
196 river/spring water as primary or secondary sources.

197 For *E. dispar*, after controlling for neighbourhood and symptoms, risk associations
198 were river/stream as a main source compared to tap water, contact with ducks, latrine
199 absence, *Blastocystis* sp. infection, and increasing age. Treatment of water had no effect.

200 For *Blastocystis* sp. carriage, after controlling for district, risk associations included
201 age, as a quadratic function, with an inverted U-shaped curve, peaking around age 9 years.
202 *Blastocystis* sp. was strongly associated with rural residence and less importantly with contact
203 with ducks, and latrine absence. House material of adobe was protective compared to wood,
204 masonry or other.

205 Risk associations for *S. stercoralis*, after adjustment for district, were latrine absence,
206 increasing age and use of well water; having a house material of adobe was associated with
207 a lower risk.

208 Assessing the risk of co-infection (Table 3b) shows that for coinfection with 2
209 organisms, the highest risk was *Blastocystis* sp. and *S. stercoralis;* but for 3 organisms this
210 was *Blastocystis* sp., *G. duodenalis* and *E dispar.*

211 **Discussion**

212 We found a high prevalence of intestinal parasites in paediatric populations in Zambézia
213 province (Mozambique) regardless of symptoms, with over half the children having at least
214 one parasite. Univariable and multivariable analyses revealed that infection/colonization by
215 enteroparasites followed pathogen-specific, age-related patterns. Drinking untreated water
216 and having contact with domestic animals were identified as risk associations for some of
217 them.

218 Previous studies in young children in Mozambique also found that most infections
219 occurred in reportedly asymptomatic children in Maputo [14], and that *G. duodenalis* was
220 more prevalent in asymptomatic than in symptomatic children in the Manhiça district [15].
221 These findings are compatible with an endemic scenario where persistent infections and re-
222 infections are common.

223 *Cryptosporidium* infections were more prevalent in young children, while increasing
224 age was a risk association for carrying *Blastocystis* sp. and *E. dispar*. Pathogenic
225 *Cryptosporidium* spp. and *G. duodenalis* are well-known to primarily affect young children
226 in sub-Saharan Africa including Mozambique [2,25]. Immature adaptive immune system in
227 infants may account for their high susceptibility to infection [26]. In contrast, the age-related
228 increased occurrence of *Blastocystis* sp. and *E. dispar* is indicative of persistent enteric
229 colonization, suggesting that both protists are mainly non-pathogenic commensals, hence
230 more prevalent in the asymptomatic group. Similar age-related patterns for *Blastocystis* sp.
231 carriage have been observed in healthy children in Spain [27] and in orphan children and
232 their caregivers in Thailand [28]. *Blastocystis* sp. was strongly associated with co-infection;
233 *E dispar* with three or more infections.

234 Drinking river/stream as a primary or secondary source of water was identified as a
235 risk association for *G. duodenalis* and *E. dispar* infection/carriage. The fact that water
236 chlorination/boiling reduced the odds of *G. duodenalis* (but not of *E. dispar*) in children
237 strongly suggests that waterborne transmission is an important factor in the epidemiology of
238 diarrhoea-causing enteroparasites in Mozambique [14-16]. Contact with dogs, cats, and
239 ducks were associated with increased risks of having giardiosis, whereas contact with ducks
240 increased the likelihood of carrying *Blastocystis* sp. and *E. dispar*. Domestic animals,
241 poultry, and livestock have all been demonstrated to be natural hosts of zoonotic *G.*
242 *duodenalis* genotypes in Côte d'Ivoire [29] and other African countries [30].

243 No DNA of *E. histolytica* was detected in any of the stool samples investigated,
244 confirming other PCR-based studies conducted in Ethiopia [31], Mozambique [12], and
245 Nigeria [32]. In contrast, the parasite has been reported at high prevalences when microscopy

246 was used [33,34], suggesting that most of the latter results should be attributed to the
247 morphologically identical but non-pathogenic *E. dispar* [32].

248 Risk associations for *S. stercoralis* included absence of latrines, having a house
249 material other than adobe, and increasing age. In the absence of adequate housing/sanitary
250 facilities, defecation in open spaces is an important source of environmental contamination
251 with the infective larvae of this STH. *Strongyloides stercoralis* has a characteristic
252 autoinfection cycle thus leading to chronicity in untreated cases. Patients with subclinical
253 infections and impaired immunity may accelerate autoinfection and trigger the
254 hyperinfection syndrome, explaining why infection rates accumulate with the age of infected
255 individuals.

256 Our study findings are improved by weighting for risk associations and demographics
257 to obtain a generalised prevalence for the 10 districts. We also corrected for random effects
258 from districts and/ or neighbourhood and symptoms in the risk analysis. Although, we cannot
259 imply causation, the risk associations may suggest avenues for intervention. Other limitations
260 were differences in sampling procedures in the asymptomatic/symptomatic paediatric
261 populations, lack of microscopy data to validate the qPCR results for *S. stercoralis*, and the
262 prolonged period between sampling and diagnosis which precluded us from informing
263 individuals and initiating appropriate treatment.

264 Our results highlight high asymptomatic carriage and thus the importance of
265 population interventions aimed at providing safe drinking water, improved sanitation and
266 hygiene to reduce the environmental contamination by faecally-orally transmitted pathogens.
267 We recommend molecular-based studies to ascertain the actual role of environmental and
268 animal reservoirs as sources of human infections in Mozambique.

Transparency declaration

Conflict of interest

The authors declare no conflicts of interest.

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Contribution

SB and DC conceived and designed the study protocol. ASM, PCK, BB, MHdM, AD, ED, and JMS carried out laboratory analyses. ASM, SB, KB, and DC analysed and interpreted the data. IF contributed reagents and materials. SB, and DC supervised laboratory and data analyses and wrote the draft of the manuscript. All authors read and approved the final manuscript.

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393 **Supplementary material**

394 **Supplementary Table S1.** Blank epidemiological questionnaire (in Portuguese) used in this
395 study.

396 **Supplementary Table S2.** Main features of the PCR methods used to amplify the small
397 subunit rRNA gene of *Giardia duodenalis*, *Entamoeba histolytica*, *Entamoeba dispar*,
398 *Strongyloides stercoralis*, *Cryptosporidium* spp., and *Blastocystis* sp. in this study.

399 **Supplementary Table S3.** Percentage of symptomatic children infected with each intestinal
400 parasite species for the seven clinical settings investigated in the Zambézia province,
401 Mozambique.

402 **Supplementary Table S4.** Percentage of asymptomatic schoolchildren infected with each
403 intestinal parasite species for the 18 schools investigated in the Zambézia province,
404 Mozambique.

405 **Supplementary Table S5.** Number and percentage of symptomatic and asymptomatic
406 children infected with each intestinal parasite species for the 10 districts investigated in the
407 Zambézia province, Mozambique.

408 **Supplementary Table S6.** Clinical manifestations by parasite species reported in
409 symptomatic children attended at clinical settings in the Zambézia province, Mozambique.

410 **Supplementary Table S7.** Descriptive and univariable analysis of the variables of interest
411 potentially associated with an increased exposure risk to the five intestinal parasites considered
412 in the present study, Zambézia province, Mozambique.