International Journal for Parasitology: Drugs and Drug Resistance 7 (2017) 262–271

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Reduced efficacy of albendazole against *Ascaris lumbricoides* in Rwandan schoolchildren

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ARTICLE INFO

Article history:
Received 28 March 2017
Received in revised form
17 June 2017
Accepted 21 June 2017
Available online 23 June 2017

Keywords:
Soil-transmitted helminth
Benzimidazole
Albendazole
Resistance
Ascaris
Deworming
Tubulin

ABSTRACT

Control of human soil-transmitted helminths (STHs) relies on preventive chemotherapy of school-children applying the benzimidazoles (BZ) albendazole or mebendazole. Anthelmintic resistance (AR) is a common problem in nematodes of veterinary importance but for human STHs, information on drug efficacy is limited and routine monitoring is rarely implemented. Herein, the efficacy of single dose albendazole (400 mg) was evaluated in 12 schools in the Huye district of Rwanda where *Ascaris* is the predominant STH. *Ascaris* eggs were detected by wet mount microscopy and the Mini-FLOTAC method to assess cure rate (CR) and faecal egg count reduction (FECR). Blood and faecal samples were analysed for co-infections with *Plasmodium* sp. and *Giardia duodenalis*, respectively. *Ascaris* positive samples collected before and after treatment were analysed for putatively BZ-resistance associated β-tubulin gene single nucleotide polymorphisms. The overall CR was 69.9% by Mini-FLOTAC and 88.6% by wet mount microscopy. The FECR was 75.4% and the 95% calculated confidence intervals were 50.4–87.8% using sample variance, 55.4–88.8% by bootstrapping, and 75.0–75.7% applying a Markov Chain Monte Carlo Bayesian approach. FECR varied widely between 0 and 96.8% for individual schools. No putative BZ-resistance associated polymorphisms were found in the four *Ascaris* β-tubulin isotype genes examined. Since FECRs <95% indicate reduced efficacy, these findings raise the suspicion of BZ resistance. In the absence of respective molecular evidence, heritable AR in the local *Ascaris* populations cannot be formally proven. However, since FECRs <95% indicate reduced efficacy, BZ resistance may be suspected which would be alarming and calls for further analyses and routine monitoring in preventive chemotherapy programs.

1. Introduction

Human soil-transmitted helminths (STHs) are highly prevalent in many developing countries with *Ascaris lumbricoides* alone infecting approximately 800 million people (Hotez et al., 2008). (Pre-)School children bear most of the disease burden, and in endemic regions, they should receive preventive anthelmintic chemotherapy (PAC) once or twice yearly, depending on STH prevalence. Recommended drugs are the benzimidazoles (BZ) albendazole (400 mg) or mebendazole (500 mg) given as a single oral dose (WHO, 2006).

The STH-related disease burden decreased almost 70% between 1990 and 2010 (Murray et al., 2012) but 600 million school-age children in 114 countries were still estimated to require PAC in 2011. In those African countries reporting to the WHO, the PAC coverage is only 23% (WHO, 2013) but treatment frequency and coverage are continuously rising and some countries such as Rwanda have achieved very high coverage (http://www.who.int/neglected_diseases/preventive_chemotherapy/sth/en/, last accessed 31.05.2017). Therefore, the potential selection of anthelmintic resistance (AR) by drug pressure is feared to eventually

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http://dx.doi.org/10.1016/j.ijpddr.2017.06.001

impair this intervention (Vercruysse et al., 2011a).

The cause of such fears lies in frequent and rapid selection of resistance against all major drug classes including multi-drug resistance in strongyle parasites of livestock (Kaplan, 2004; Wolstenholme et al., 2004). In humans, *Onchocerca volvulus* show reduced ivermectin efficacy in some areas where mass drug administration has been used over decades (Awadzi et al., 2004a, 2004b; Osei-Atweneboana et al., 2011). So far, confirmed AR in human STHs has not been reported (Vercruysse et al., 2011a). For human *Ascaris* infection, a meta-analysis performed a decade ago showed single-dose albendazole to achieve an average cure rate (CR) of 88% Keiser and Utzinger (2008). This figure was considerably higher (98%) in a more recent study involving seven trials conducted in South America, Africa and Asia (Vercruysse et al. (2011b).

However, the validity of CR as a parameter of efficacy is debated. CRs are considered to be highly variable and strongly depend on diagnostic sensitivity (Bennett and Guyatt, 2000; Vercruysse et al., 2011a). Very low faecal egg counts (FECs) may be missed by limitedly sensitive methods, which results in a false classification of cure. Since methods vary between studies, comparisons are difficult due to this bias. In contrast, faecal egg count reduction (FECR, = egg reduction rate) following treatment appears to be a much more stable parameter of efficacy and provides comparable data between different detection methods (Vercruysse et al., 2011a).

Diagnostic method and analytical approach are nevertheless also of importance for the assessment of FECR. Diagnostic tools differ in the presence of enrichment steps and in the amount of faeces examined (e.g. FLOTAC methods *vs.* wet mount microscopy), resulting in different analytical precision. Such differences in precision impact on the calculated 95% confidence intervals (CIs) and thus the precision of the FECR estimate but most statistical approaches do not take this into account (Torgerson et al., 2014; Levecke et al., 2015). Statistical methods to calculate 95% CIs further differ in dealing with paired data structure and overdispersion of FECs (high counts in a few individuals). A recently developed calculation method that considers the different sources of variation in a hierarchical Bayesian model uses a Markov Chain Monte Carlo method to estimate the FECR and its 95% CI (Paul et al., 2014; Torgerson et al., 2014; Wang et al., 2017).

BZs bind to tubulins of susceptible parasites preventing their incorporation into microtubules. This leads to a loss of microtubules, which finally kills the parasites (Whittaker et al., 2017). In the sheep strongyle *Haemonchus contortus*, BZ-resistance has been associated with a F200Y single nucleotide polymorphism (SNP) in the isotype-1 β-tubulin gene (Kwa et al., 1994, 1995). In one study, high-level resistance was additionally conferred by a loss of the isotype 2 β-tubulin gene (Kwa et al., 1993). Further isotype 1 β-tubulin gene SNPs (F167Y, E198A) have been implicated in BZ resistance in this parasite, but polymorphisms at codons 167 and 198 might play a more important role in other strongylid parasites (Drogemuller et al., 2004; Hodgkinson et al., 2008; Demeler et al., 2013a; AlGusbi et al., 2014; Redman et al., 2015). Molecular techniques may provide useful information on the emergence and spread of BZ-resistance.

In ascarids, five β-tubulin isotypes from *A. suum* and *A. lumbricoides* have been delineated (*Astbb-1* to *Astbb-4,* and *Altbb-1*) in previous publications (Demeler et al., 2013a). However, *Astbb3* is in fact only a shorter fragment of *Astbb-2*. Debate whether *A. suum* and *A. lumbricoides* are actually the same species is fuelled by evidence for cross-species transmission and the porcine origin of many *Ascaris* spp. found in humans (Leles et al., 2012; Betson et al., 2014; Nejsum et al., 2016). In fact, the *Altbb-1* gene can also be amplified among *Ascaris* obtained from African pigs (Krücken et al. unpubl.), and due to its close phylogenetic relationship, this isotype herein is named *tbb-1.2*.

Demeler et al. (2013a) showed that there are barely any one-to-one orthologs between strongylid and ascarid β-tubulin isotype genes. Therefore, it cannot be predicted which ascarid isotype is potentially involved in BZ resistance. An F200Y SNP in human *Ascaris tbb-1.2* has frequently been detected despite high BZ efficacy, suggesting that the SNP does not lead to BZ resistance in this species (Diawara et al., 2009, 2013).

The aim of the present study was to evaluate the efficacy of PAC applying a single-dose albendazole against *Ascaris* infections among Rwandan schoolchildren and to determine factors influencing efficacy. Different calculation methods to estimate efficacy were compared and molecular data on occurrence of BZ resistance associated β-tubulin SNPs are provided.

2. Material and methods

2.1. Study design and location

The study was conducted in October 2014 in the Huye district, Southern Province, Rwanda, and a sub-set of schools in October 2015. Study design and overall procedures have been reported previously (Sifft et al., 2016). Briefly, Huye district is located on the central plateau of Rwanda (altitude 1600–1800 m; average temperature 19 °C; annual rainfall 1200 mm). For each of the 14 sub-districts, one primary school of 500–1100 pupils was randomly chosen. Two schools later dropped out leaving 12 schools in the study. In every school, 150 children aged 6–10 years were randomly selected with the goal of recruiting 100 participants per school. Several days before examination, school meetings were held to explain purpose and procedures of the study to teachers and parents. Permissions were obtained from school directors, heads of local health centres and the district health office. Written informed consent and assent were obtained from the parents and the children, respectively. Ethical approval was granted by the Rwandan National Ethics Committee (242/RNEC/2014), and the Rwandan Ministry of Education (2543/12.00/2014) provided permission for study conduct.

2.2. Examination of children and interview data

All participating children were clinically examined, a brief medical history was obtained, and axillary temperature was measured. Age, sex, height, weight and mid-upper arm circumference were documented. Weight-for-age (WAZ), height-for-age (HAZ) and body-mass-index-for age (BAZ) were calculated using AnthroPlus software (WHO, 2009). Underweight, stunting and thinness were identified as WAZ, HAZ and BAZ scores, respectively, below -2 SD. Venous blood was collected into EDTA, and haemoglobin (Hb) concentration was measured using a HemoCue photometer (Ångelholm, Sweden). Anaemia was defined according to altitude-adjusted thresholds as Hb < 12 g/dL (Sullivan et al., 2008). On-site, children were tested for malaria parasites using immunochromatographic dipstick tests (SD BIOLINE Malaria Ag P. f/Pan, Standard Diagnostics Inc, Gyeonggi-do, Korea). Infected children received artemether-lumefantrine. Diagnosis of *Plasmodium* infection was ascertained by thick film microscopy and semi-nested PCR assays of all samples as described elsewhere (Sifft et al., 2016). In case of further illness upon clinical examination, treatment was provided in accordance with Rwandan treatment guidelines.

Trained interviewers conducted interviews with parents using structured questionnaires. Data collected included health and socio-economic status but in the present study only recent diarrhoea and vomiting (within preceding 2 weeks, stated by child or

parent) were regarded.

2.3. Deworming and faecal sample collection

Pre-treatment stool samples were collected alongside routine school-based deworming activities. Samples were obtained before observed, single-dose treatment with 400 mg albendazol (GlaxoSmithKline), and between 7 and 10 days afterwards.

2.4. Wet mount, mini FLOTAC examination and sample processing

Fresh faecal samples were screened on the day of stool collection for helminth eggs by direct wet mount microscopic examination (WHO, 1991). Preceding mini-FLOTAC examination, 5 g and 10 g aliquots of stool were weighed and Lugol's iodine solution was added to 25 ml or 50 ml total volume, respectively. Samples were stored at 4 °C overnight for disinfection. This procedure does not affect FLOTAC based results (Demeler et al., 2013b). Lugol-conserved samples (5 g) were centrifuged (180 × g for 3 min), the supernatant was discarded, and an additional wash step removed remaining iodine. These samples were examined using the standard Mini-FLOTAC technique (Barda et al., 2014; Maurelli et al., 2014) with saturated sodium chloride solution (density 1.2) to identify and quantify helminth ova. *Per* sample, ova were enumerated in a single chamber and the number of eggs was multiplied by 10 to calculate the number of eggs per gram stool (epg) (analytical sensitivity 10 epg). For STH positive samples, eggs were isolated from the 10 g reference samples by centrifugation (180 × g for 3 min), followed by washing of pellets, homogenisation with saturated sodium chloride solution and passage through a 200 μm sieve. Filtrates were centrifuged at 180 × g for 3 min and the supernatant was poured on a 22 μm sieve to collect eggs. Retained material was flushed into a centrifuge tube and concentrated using several centrifugation steps. Final volume was less than 100 μl, and these samples were stored at -80 °C. DNA was isolated from purified *Ascaris* eggs (NucleoSpin Soil kit, Macherey Nagel), eluted in 50 μl SE buffer and stored at -20 °C.

2.5. PCR amplification and sequence analysis

PCR primers (Table S1) were designed to be located within the introns flanking codons 167 or 198/200 in the *A. suum tbb-1*, *tbb-1.2*, *tbb-2* and *tbb-4* genes. For the *tbb-1.2* gene, published primers were slightly modified (Diawara et al., 2009). First round PCR reactions contained 0.2 mM dNTPs, 0.3 μM of each primer, 0.4 U Phusion Hot Start II High-Fidelity DNA polymerase (Thermos Scientific) in 20 μl 1 × HF buffer. The PCR run included initial denaturation at 98 °C for 30 s, 40 cycles of denaturation at 98 °C for 10 s, annealing at a target-specific temperature (Table S1) for 30 s and elongation at 72 °C for 30 s. PCR products were used as template in (semi-)nested PCR reactions performed under the same conditions. Amplicons were analysed on 1.0–1.5% agarose gels. PCR products were directly purified with DNA Clean & Concentrator™-5 or purified from agarose gels using Zymoclean™ Gel DNA Recovery Kit (Zymo Research). Sequencing was performed at LGC Genomics (Berlin, Germany). Sequencing primers used to determine sequences at the different β-tubulin codons and isotypes are listed in Table S1. Sequence chromatograms were visualised in BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and manually evaluated for the presence of SNPs in β-tubulin codons 167, 198 and 200 as well as for missense SNP within the amplicons.

2.6. Giardia duodenalis detection

Stool samples were screened for *G. duodenalis* by direct wet mount microscopy. In addition, 1 ml aliquots of fresh stool were stored at -80 °C, transported on dry ice, subjected to DNA extraction (QIAamp DNA sStool Mini Kit, Qiagen, Germany), and *G. duodenalis* was detected by real-time PCR assays as applied previously (Ignatius et al., 2012). Samples with Cq values > 36 were repeated. No sample had to be excluded because of evidence of faecal inhibitory factors (Cq value for the internal control PhHV-1 >36).

2.7. Statistical analyses

Analysis focuses on *Ascaris* only since hookworms (n = 24) and *T. trichiura* (n = 31) were too rare for meaningful conclusions (Vercruysse et al., 2011a). CR was calculated as the proportion of children positive for *Ascaris* at the pre-treatment survey who became negative 7–10 days post-treatment. The 95% CIs for prevalence and CRs were calculated as Wilson-Score intervals using the propCI function in the R package prevalence. Significant differences in proportions before and after treatment were calculated using the mid-p exact test implemented in the tab2by2. test function of the epitools R package. The Holm correction for multiple testing was applied using p. adjust in R.

The FECR and corresponding 95% CI were calculated on the group level as recommended by Vercruysse et al. (2011a) but applying three different methods. The first two methods use the mean egg count reduction as the estimate of the FECR calculated with the formula:

(1-(mean faecal egg count post treatment/mean faecal egg count pre-treatment)) × 100 = % FECR.

The first method calculates the approximate 95% CI from the sample variance as suggested by the World's Association for the Advancement of Veterinary Parasitology (Coles et al., 1992). This method does neither consider the paired study design nor the overdispersion of egg counts. The second method, a parametric bootstrap approach, assumes a negative binomial distribution of egg counts as suggested by Torgerson et al. (2005). A paired design was chosen and 10,000 bootstrap iterations were conducted. Both methods used the fecrtCI function implemented in the R package eggCounts 1.1–1 (Paul et al., 2014; Torgerson et al., 2014; Wang et al., 2017). In addition, the Bayesian method using hierarchical models to capture counting related Poisson variability and variability from overdispersion modelled as negative binomial distribution was used as implemented in the fecr_stan tool (eggCounts 1.1.-1 package). This method estimates the FECR as the mode of a posterior distribution generated using a MCMC approach and the 95% credible interval as a 95% highest posterior density interval (Paul et al., 2014; Torgerson et al., 2014; Wang, 2016). According to Vercruysse et al. (2011a), a FECR <95% indicates reduced efficacy and suggests drug resistance. In the following, FECRs with upper 95% confidence limits of <95% are classified as ***reduced efficacy***, FECR results including 95% in their 95% CI as ***suspected reduced efficacy*** and those with a lower 95% CI limit >95% as ***susceptible***. Reliable FECR estimates require that at least 30 individuals are positive before treatment or that the aggregation (many eggs in only a few individuals) in the data is low (overdispersion parameter κ > 0.01 in the negative binomial distribution) (Vercruysse et al., 2011a). The later parameter is automatically determined from the Bayesian approach and was used to judge the reliability of the estimates.

For the identification of parameters influencing FECR, FECRs were calculated for all pre-treatment *Ascaris* positive children individually (FECRi). If the post-treatment egg count was higher than pre-treatment, the FECRi was set to 0. Since this dataset is censored with values between 0 and 100, censored regression

models (censReg R package) were initially evaluated. However, since the vast majority of data points represented 100% or 0% FECRi, no conclusive regression models could be identified – a problem previously described by Vercruysse et al. (2011b). For age, pre-treatment egg counts, Hb and malaria parasite density, Spearman's correlation coefficients were calculated to determine potential effects on the FECRi. For analysis of FECRis with respect to categorical variables (*Plasmodium* infection, *Giardia* infection, diarrhoea, vomiting, fever, malnutrition), Mann-Whitney U tests or Kruskal-Wallis tests followed by Dunn's post hoc tests were conducted.

Since censored regression analyses consistently failed, a logistic regression analysis for presence of ***reduced efficacy*** (FECRi < 95%) was performed using the glm function in R. Children for whom no health parameters were recorded (n = 32) were excluded before the analysis since glm otherwise failed to converge. Variables were stepwise excluded using the drop1 function in R to optimize the Akaike information criterion. A likelihood ratio test was performed to compare the final model with the null model. The pseudo-R2 value according to Nagelkerke was calculated using the r2 function of the sjstats package.

3. Results

3.1. Treatment efficacy

3.1.1. Pre- and post-treatment prevalence of Ascaris sp.

Out of 1182 recruited children, paired stool samples (pre- and post-treatment) of 850 and 709 individuals were analysed by wet mount microscopy and mini-FLOTAC, respectively. Baseline characteristics and epgs before and after treatment for the children analysed using mini-FLOTAC are shown in Table S2 and S3, respectively. The overall pre-treatment prevalence of *Ascaris* was 33.3% (range for different schools 2.8–71.6%) (Table 1) using the Mini-FLOTAC method and 25.9% (range 0–47.7%) by wet mount microscopy. Following deworming, these figures declined to 13.4% and 5.6%, respectively. On a single school level, prevalence (mini-FLOTAC data) was reduced in all schools by treatment but differences were significant only for five schools. Prevalence remained above 15% in four schools.

3.1.2. Cure rates

Based on data obtained by the Mini-FLOTAC method, the overall CR was 69.9% (95% CI 63.4–75.4%) (Table 1 and Fig. 1A), with large variation between schools and a notably low figure in Kigoma (35.3%; Table 1). Only in three schools CRs >95% were achieved but in these, the number of pre-treatment positive children was very low. CRs based on wet mount microscopy (88.6%; 95% CI 83.8–92.2%) were significantly ($p = 0.002$) and overall almost 20% higher ($p = 0.002$) but showing a pattern between schools similar to mini-FLOTAC results.

3.1.3. Treatment efficacy based on egg count data

Overall and at the individual school level (except for Mbazi), mean epg decreased after treatment (Fig. 2 and Fig. S1). However, changes were not significant for six schools (including Mbazi). Three schools with very low numbers of pre-treatment positive children ($n < 5$) were excluded from school-level FECR analyses. Remarkably, 24 schoolchildren were *Ascaris* sp. negative before treatment but positive approximately one week later. Their median post-treatment epg was 370 (range 10–31120).

The FECRs and 95% CIs were obtained using (i) the approximated method (Coles et al. (1992) (Fig. 1B), (ii) the parametric bootstrapping method (Torgerson et al., 2005) (Fig. 1C) and (iii) the Bayesian/MCMC approach (Paul et al., 2014; Torgerson et al., 2014; Wang et al., 2017) (Fig. 1D). The three FECR estimates were identical (75.4%) but the 95% CIs differed considerably with the analytical approach: (i) using the approximated method 50.4–87.8%; (ii) based on the bootstrapping method 55.4–88.8%; and (iii) for the Bayesian method, 95% HPD 75.0–75.7%. Therefore, irrespective of statistical approach, the overall *Ascaris* study population was categorised as showing ***reduced BZ efficacy***. At the school-level, the same applied to the populations in Kigoma and Mbazi. By approximate and bootstrapping methods, all other schools were showing ***suspected reduced efficacy***. In contrast, the Bayesian method indicated ***reduced efficacy*** for Gatovu, Ngoma, Sovu, Rango, Sholi and Maraba but classified Karama as **susceptible**.

At least one criterion for reliable FECR estimates (Vercruysse et al., 2011a), was fulfilled for all schools analysed: (i) > 30 *Ascaris* sp. infected individuals were present in Sholi, Karama and Kigoma, and (ii) a low degree of aggregation ($\kappa > 0.01$) was met in all schools with most schools in the κ range 0.12–0.47. Only for Ngoma ($\kappa = 0.05$) and Gatovu ($\kappa = 0.07$) this value was lower but still above the postulated threshold.

3.1.4. Effects of independent variables on individual faecal egg count reductions

To identify factors that might influence the efficacy of albendazole, the FECRi were analysed. Spearman regression analyses revealed that age ($p = 0.095$), blood haemoglobin concentration ($p = 0.170$) or the *Ascaris* sp. epg before treatment ($p = 0.116$, only children with a positive epg before treatment included) had no significant effect on FECRis. In contrast, malaria parasite density was weakly but significantly correlated with a decreased FECRi ($p = 0.019$, $\rho = -0.444$). Table S4 summarises effects of co-infection with *T. trichiura*, hookworms, *Plasmodium* sp. and *G. duodenalis* as well as sex, presence of anaemia, fever, low WAZ, HAZ or BAZ, diarrhoea and vomiting on FECRi values. None of these variables except diagnosis of *Plasmodium* infection had significant effects. *Plasmodium* positive children showed significantly lower FECRis than those not infected ($p = 0.001$). The school had a significant effect on FECRis (Fig. S2A) with Kigoma showing significantly lower FECRis than Rango, Sholi, Sovu and Karama. Since the variables infection with *Plasmodium* sp. and school might be correlated, the prevalence of malaria parasites in children attending the different schools was also compared (Fig. S2B). This revealed that most comparisons were not significant except of Karama and Sovu showing a significantly lower prevalence than Kigoma and Sholi a higher prevalence than Karama. There was no clear pattern that high *Plasmodium* prevalence always resulted in low FECRis (Fig. S2).

Calculation of censored regression models failed due to the large number of 100% and 0% FECRi values. A logistic regression model for the presence of **reduced efficacy** was calculated considering all variables included in univariate analyses. The final model included the independent variables diarrhoea and school (Table S5) but only influence of the school was significant. Kigoma and Mbazi showed a significantly higher presence of **reduced efficacy** than the reference Karama ($p < 0.02$) (Table S5). An effect of *Plasmodium* infection on presence of reduced efficacy of albendazole against *Ascaris* sp. was not found in this multi-variate analysis.

3.2. Replication: preventive anthelmintic chemotherapy efficacy in 2015

In 2015, four schools were revisited and routine deworming efficacy monitored using Mini-FLOTAC for STH diagnosis. However, only few paired samples could be collected and STH prevalence (13.4%) (Table S6) and was lower than 33.3% observed in 2014 (Table 1). The overall CR was 87% as compared to 69.9% in 2014. Egg count data for these children are shown in Fig. S3. Due to limited

Table 1
Prevalence of and cure rates for *Ascaris* sp. in paired pre- and post-treatment samples of Rwandan children according to school.

School	Mini-FLOTAC							Wet mount microscopy			
	N[a]	n_{pre}[b]	Prevalence pre (%) (95% CI[c])	n_{post}[b]	Prevalence post (%) (95% CI[c])	p value (pre vs. post)	Cure rate[d] (%) (95% CI[c])	N[a]	n_{pre}[b]	n_{post}[b]	Cure rate[d] (%) (95% CI[c])
Ngoma	34	6	17.6 (8.3–33.5)	3	8.8 (3.0–23.0)	0.313	80 (37.6–96.4)	50	9	0	100 (76.9–100)
Mbazi	76	22	28.9 (20.0–40.0)	13	17.1 (10.3–27.1)	0.088	68.2 (47.3–83.6)	97	28	15	31.1 (17.9–50.7)
Rango	44	12	27.3 (16.3–41.8)	3	4.7 (1.3–15.5)	0.012	83.3 (55.2–95.3)	59	12	1	100 (81.6–100)
Mugogwe	85	4	4.7 (1.8–11.5)	2	2.4 (0–3.1)	0.446	100 (59.7–100)	91	3	2	100 (52.8–100)
Sholi	70	31	44.3 (33.2–55.9)	3	4.3 (1.5–11.9)	<0.001	90.6 (75.8–96.8)	89	31	1	96.8 (83.8–99.4)
Gathovu	64	8	12.5 (6.5–22.8)	3	4.7 (1.6–12.9)	0.130	75 (40.9–92.9)	58	6	2	83.3 (43.6–97.0)
Karama	67	48	71.6 (59.9–81.0)	16	23.9 (15.3–35.3)	<0.001	72.9 (59.0–83.4)	74	33	2	93.9 (80.4–98.3)
Githovu	36	1	2.8 (4.9–14.2)	0	0 (0–7.0)	0.500	100 (27.0–100)	39	0	0	na[e]
Tumba	14	1	7.1 (1.3–31.5)	0	0 (0–16.2)	0.500	100 (27.0–100)	24	2	0	100 (42.5–100)
Sovu	91	53	58.2 (48.0–67.8)	18	19.8 (12.9–29.1)	<0.001	88.7 (77.4–94.7)	86	41	4	96.5 (90.2–98.8)
Maraba	43	16	37.2 (24.2–52.1)	6	14.0 (6.6–27.3)	0.015	68.8 (44.0–85.8)	73	22	10	90.9 (72.2–97.5)
Kigoma	85	34	40.0 (30.2–50.6)	29	34.1 (24.9–44.7)	0.433	35.3 (21.5–52.1)	110	33	11	80.0 (64.1–90.0)
Total	709	236	33.3 (29.9–36.8)	95	13.4 (11.1–16.1)	<0.001	69.9 (63.8–75.4)	850	220	48	88.6 (83.8–92.2)

[a] N, Total number of children for which paired samples were analysed.
[b] n, number of infected children pre or post treatment.
[c] 95% CI, 95% confidence interval.
[d] Children that were positive only post treatment were excluded for calculation of cure rates.
[e] na, not available.

numbers, FECR values were not calculated.

3.3. β-tubulin genotypes

A total of 144 human *Ascaris* samples were available for molecular analysis; 98 samples (44 post-treatment, including 29 pre-/post treatment pairs) were from 2014 and 44 (13 post-treatment) from 2015. Codons 167, 198 and 200 from all four β-tubulin isotypes mentioned above were analysed for occurrence of SNP. Table 2 summarises data for available samples regarding their origin and the β-tubulin isotypes analysed successfully. Detailed data for all samples are provided in Tables S7 and S8 for 2014 and 2015, respectively. The observed genotype at codons 167 and 200 was always TTC encoding phenylalanine. In codon 198, differences between isotypes were found with GAG in isotypes 1 and 4 and GAA in isotypes 1.2 and 2. However, both codons encode glutamate. Therefore, no evidence for the changes F167Y, E198A or F200Y correlating with BZ resistance in strongylid nematodes was found.

4. Discussion

During the past years, efforts to reduce the burden of STH infections, particularly PAC in pre-school-age and schoolchildren, have increased considerably (WHO preventative chemotherapy database: http://www.who.int/neglected_diseases/preventive_chemotherapy/sth/en/, last accessed 31.05.2017). In Rwanda, pre-school-age children have reived PAC at least yearly. In consequence, there were ≥12 rounds of BZ-based PAC before this study in autumn 2014. The national coverage (proportion of the population requiring PAC that has been treated) ranged between 74.6% and 100% (median 96.2%). Systematic treatment of school-age children started in 2008. Since then, seven rounds of PAC were conducted before the present study was conducted – excluding a first PAC campaign with a negligible national coverage of 7,1% early in 2014. The remaining PAC programs targeting schoolchildren had a high national coverage (median 99.9%, range 82.7–100%). In 2015, after most of the data from the present study were obtained, school-age children were even treated three times with a national coverage of 98.6% in each round. In the Huye district, the situation is very similar to the nationwide numbers. For instance, pre-school-age and school-age coverage in 2015 were 98% and 87%, respectively (Rwanda Ministry of Health, 2016). Due to the high treatment frequencies and coverage, a risk of selection for resistant parasites is given and the present study was part of a program aiming to provide the tools for regular monitoring by the local health care institutions.

While efficacy of BZs against whipworms is generally poor, efficacy against hookworms and *Ascaris* sp. is considered good or even excellent (Keiser and Utzinger, 2008; Vercruysse et al., 2011b). Recently, 400 mg albendazole achieved an overall CR of 98.2% and a FECR of 99.5% in a study comprising seven sites on three continents (Vercruysse et al., 2011b). The authors concluded that a FECR below 95% should rise concern regarding reduced efficacy. The data from Huye, Rwanda, are clearly below this threshold. The same is true for the overall CR (69.9%). However, the low CR might be caused by the superior sensitivity of the Mini-FLOTAC method (analytical sensitivity of 10 epg) compared to the McMaster method (50 epg) used by Vercruysse et al. (2011b). In line with that, the CR based on wet mount microscopy (88.6%) was significantly higher than the figure derived from Mini-FLOTAC results but slightly lower than the 94% observed in this region in 2012 using the Kato-Katz technique (Staudacher et al., 2014). Nevertheless, even with the wet mount

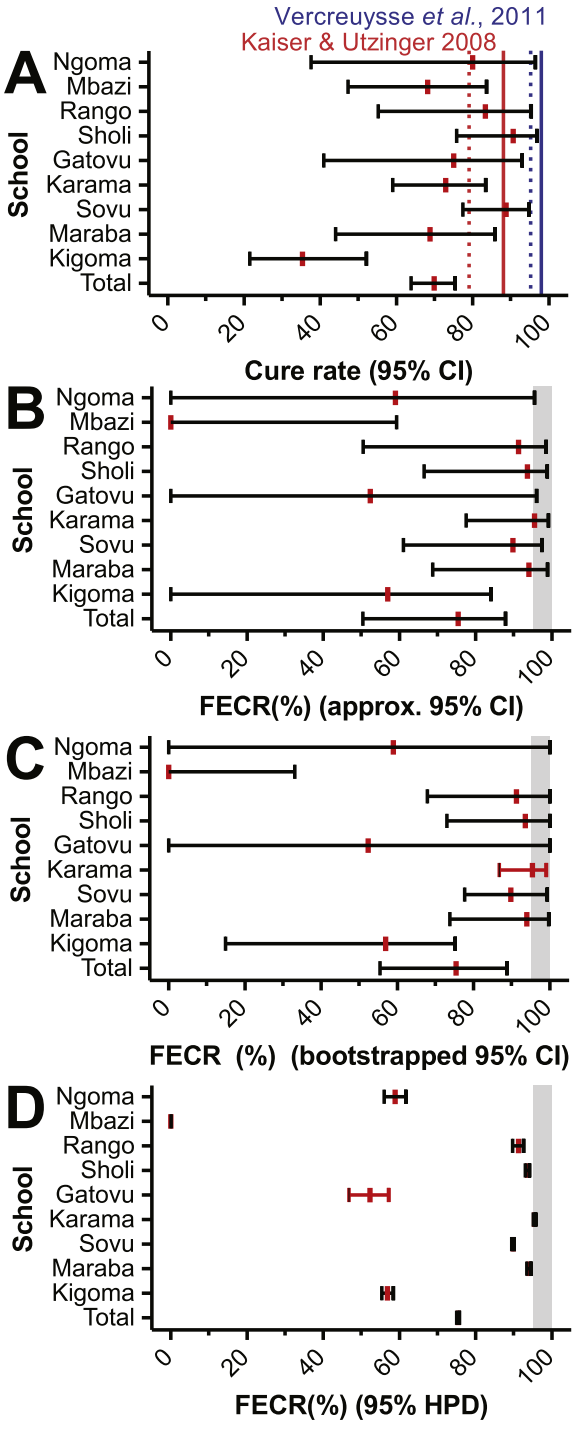

Fig. 1. Statistical analyses of treatment effects. Three schools for which only very few positive samples were found before treatment (between 1 and 4) are not shown here. (A) Cure rates for all samples from all 12 schools (Total) and for 9 individual schools (also presented in Fig. 1 or Fig. S1) are shown. Vertical lines indicate for comparison in red mean cure rate (continuous) and lower 95% CI (broken line) as determined in a meta-analysis by Keiser and Utzinger (2008) and in blue the mean (continuous) and lower range (broken line) of a recent study from 7 South American, African and Asian countries published by Vercruysse et al. (2011b). Feacal egg count reductions (FECR) were calculated as arithmetic means and 95% CI were calculated according to the approximate method postulated by Coles et al. (1992) (B) or using parametric bootstrapping with 10,000 iterations as described by Torgerson et al. (2005) (C). Alternatively, FECR and 95% credibile intervals were obtained as mode and highest posterior density intervals (95% HPD) after sampling from a Markow Chain Monte Carlo approach (D). In (B–D), the region between 95 and 100% FECR corresponding to full efficacy is highlighted by grey shading. Estimates of the FECR are shown in red and the 95% CI/HPD in black. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microscopy data, the overall CR was <90% and the upper 95% confidence limit was <95%, indicating reduced albendazole efficacy. In particular, the CRs in Kigoma and Mbazi were clearly below 90%, indicating that in these schools the treatment cannot be considered to be fully effective.

Data analysis on the school level further substantiates the suspicion that albendazole had reduced efficacy against ascarid infections of schoolchildren in the region. There was one school with an excellent CR above 90% and another school with a FECR above 95% while other schools showed low CRs and FECRs, in particular Kigoma and Mbazi. For the latter two, the data must be classified as reduced efficacy no matter which calculation method for FECR and 95% CIs/95%HPDs was used. *Ascaris* populations in circulation at other schools such as Rango, Sovu and Karama would be classified as showing a reduced efficacy using the Bayesian approach. This method considers all levels of variability and takes into account the high precision of the Mini-FLOTAC method which results in considerably narrower 95% HPDs.

The statistical methods used to calculate the FECR and its 95% CIs/HPD have an obvious influence on the interpretation of the data. While FECRs were basically identical, 95% CIs/HPDs – and thus the estimated degree of reduced efficacy – varied largely with the analytical approach. The statistical problems in determining anthelmintic treatment outcome parameters have been extensively discussed recently (Paul et al., 2014; Torgerson et al., 2014; Levecke et al., 2015; Wang et al., 2017). In particular, the design of the study (paired vs. unpaired), the degree of overdispersion of the data, the precision of the counting process and the counting-associated Poisson variation should be taken into account. None of these aspects is considered by methods calculating 95% CI from the sample variance. The bootstrapping method at least considers paired data structure and overdispersion. Only the Bayesian methods takes into account the precision of the counting method. In comparison with the Mini-FLOTAC method used in the present study, the lower detection limit of the standard McMaster protocol is five times higher. Levecke et al. (2015) have shown recently that the 95% CI is directly dependent on the actual number of eggs counted in total and not on the epg itself. Thus, a high counting effort should be rewarded by small 95% CIs while a strong extrapolation must be penalised by wide 95% CIs. One result of such considerations is the eggCounts package, which as applied in the present study, uses hierarchical models to account for different sources of variation such as overdispersion and the counting process. The resulting narrower confidence intervals following the Bayesian approach as compared to e.g. bootstrapping did not only allow to detect a comparatively higher number of populations with reduced efficacy, they also enabled to categorize the *Ascaris* population in one village (Karama) to be fully susceptible. The effect of the statistical analysis method on the classification of a populations phenotype as **susceptible**, **suspected reduced efficacy** or as **reduced efficacy** need to be kept in mind when comparing the present with previously reported data.

It must also be stated clearly that the model implemented in the eggCounts software package as it currently stands and was used here was evaluated using data from strongyle infections in small ruminants. Some of the respective assumptions might be violated in ascarid infections. For instance, there were a few cases in which no eggs were found before treatment but which were highly positive afterwards. When using the zero-inflated models implemented in eggCounts, these cases caused severe deviations of the estimate of the FECR from the other methods and the zero-inflated

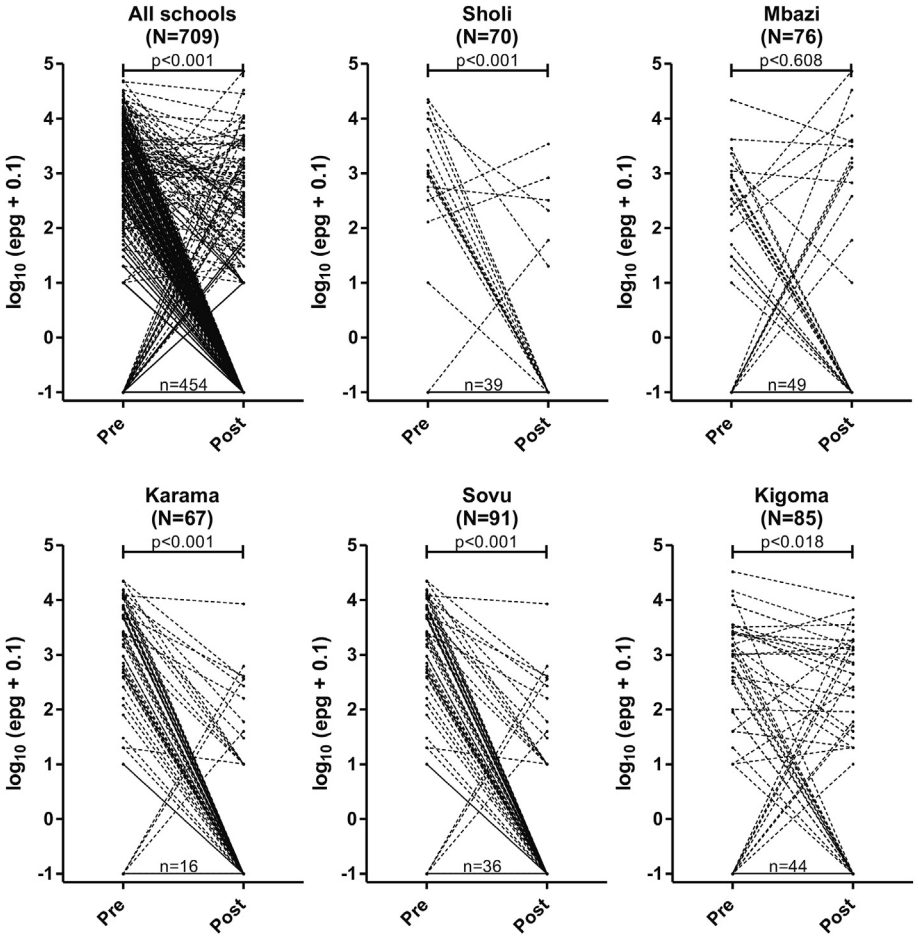

Fig. 2. Effects of treatment on eggs per gram faeces. Faecal egg counts are shown for all paired samples from 12 schools (All schools) or for selected schools (Sholi, Mbazi, Karama, Sovu, Kigoma). In order to improve the legibility of the diagrams, data for the remaining schools are shown in Fig. S1. N, number of paired samples; n, number of samples negative before and after treatment.

Table 2
Number of stool samples analysed per school and year for the presence of single nucleotide polymorphisms in different isotype β-tubulin genes.

School/year	Pre treatment				Post treatment			
	Isotype 1	Isotype 1.2	Isotype 2	Isotype 4	Isotype 1	Isotype 1.2	Isotype 2	Isotype 4
Ngoma/2015	3	5	4	4	2	2	2	2
Mbazi/2014	8	9	10	8	2	1	2	2
Mbazi/2015	9	10	11	11	2	1	1	0
Rango/2014	2	0	2	1	0	0	0	0
Sholi/2014	2	1	2	1	2	0	1	1
Gatovu/2014	1	1	0	0	1	1	1	1
Gatovu/2015	8	9	9	9	4	4	4	4
Karama/2014	8	2	8	3	6	7	10	10
Sovu/2014	12	2	11	3	10	12	11	10
Maraba/2014	3	0	2	0	3	3	3	3
Kigoma/2014	10	5	11	8	10	9	9	10
Kigoma/2015	4	3	2	3	6	6	6	6
Total	70	47	72	51	48	46	50	49

models were therefore not used in the final data analysis. Moreover, eggCounts uses a single value for the mean true eggs counts before and after treatment and models the reduction by introducing a reduction factor $(1 - \delta)$ with δ defined as the proportion of eggs remaining after treatment. This means that the same δ is expected for all individuals to model the true egg counts (not the observed ones which can vary, e.g., due to variability in the counting process). This assumption might be acceptable for the FECR in a group of animals coming from the same flock, grazing on the same pasture and most likely carrying the same spectrum of parasites. However, this assumption is most likely violated if data from many different schools are pooled. It is unlikely that data from a **susceptible** population such as from Karama and from a population with strongly **reduced efficacy** can be modelled with the same reduction factor. This problem is presumably less important when looking at the data which are coming from individual schools for

which a more homogenous population of *Ascaris* sp. parasites can be assumed.

It was aimed to replicate the findings in a second investigation in 2015. However, due to limited resources and administrative constraints, including repeated delays in the actual deworming date, only four schools could be included. The observed, overall CR of 87% is thus based on a small number of samples available in 2015, and the estimation of FECR was consequently omitted.

Among all variables tested to have an influence on the FECRi, only the school, *Plasmodium* infection and *Plasmodium* density had a significant effect on treatment efficacy in univariate analyses. However, the effects of *Plasmodium* were small. In contrast to the effect of the school, the *Plasmodium* effect was not confirmed in a multivariate analysis. Most likely, the results of the univariate analysis are therefore caused by confounding factors such as high *Plasmodium* infection prevalence in some schools with low FECRi.

Decreased anthelmintic efficacy suggests but does not prove presence of heritably resistant parasite populations. Nevertheless, the results presented here show that the local parasite population has experienced inefficient albendazole treatment and considerable proportions of the worm population survived treatment. Repeated BZ exposure of the local parasite population (including re-infections after deworming), particularly since the intensification of PCA in Rwanda in 2008 (Ruxin and Negin, 2012), has exerted considerable drug selection pressure. Surviving parasites were consequently exposed to sublethal albendazole concentrations, a constellation known to rapidly select for anthelmintic resistance (Vercruysse et al., 2011a; Knapp-Lawitzke et al., 2015). Several alternative reasons need to be considered. Incomplete treatment or vomiting of albendazole was excluded by directly observed treatment and repeated dosing if albendazole was vomited within 30 min. Impaired drug quality is unlikely considering that albendazole was produced by GlaxoSmithKline, provided by WHO, and distributed by the Rwandan health authorities. Excessively high temperatures as the most important component of inappropriate albendazole storage are rare in highland Rwanda. Unfortunately, no drug specimens were kept for the analysis of active ingredient. Nevertheless, the observation that high efficacy was achieved in one school argues against drug quality to be a major issue of concern. Faecal egg counts are known to vary in large ranges in ascarid nematodes anyhow and are considered to be dependent on factors such as diet fibre content, the daily amount (and consistency) of faeces passed and the worm age (Hall, 1981; Sinniah, 1982). Regarding the age, in an extreme case preadult, BZ resistant *Ascaris* sp. might survive treatment and have developed to highly fecundant worms a week later. This would be the simplest explanation for the high egg counts observed in a few post treatment samples despite negative pre-treatment samples.

For confirmation that reduced efficacy *in vivo* is actually due to drug resistance, comparison of susceptible and potentially resistant populations using *in vitro* assays is recommended (Vercruysse et al., 2011a). For the BZs, the egg hatch assay and the larval development assay are available for strongylid nematodes (Lacey et al., 1990; von Samson-Himmelstjerna et al., 2009a; Demeler et al., 2010). However, no such *in vitro* assays are available for *Ascaris* so far. This is due to the fact that *Ascaris* larvae do not hatch from their eggs in the environment and that their thick and highly impermeable shell prevents access of most drugs to the developing embryo. Development of such assays should be a priority as advocated by others too (Vercruysse et al., 2011a). An *in ovo* development assay determining effects of thiabendazole on embryonation in eggs, as recently described for *Parascaris* spp. (Tyden et al., 2016), is presumably a good starting point.

Molecular tests of parasite resistance have been applied for several strongylid species (von Samson-Himmelstjerna et al., 2009b; Martinez-Valladares et al., 2012; Demeler et al., 2013a; Ramünke et al., 2016). The large number of different β-tubulin paralogs in the genome of strongylid and ascarid nematodes leads to a complicated situation: most of the different isotypes in *Ascaris* sp. cannot be assigned one-to-one to orthologs in *H. contortus*. In the latter, isotype 1 has predominantly been implicated in BZ resistance but occasionally also isotype 2 (Kwa et al., 1993; Kotze et al., 2014; Ramünke et al., 2016). Phylogenetic analysis suggests that *Ascaris* sp. isotypes *tbb-1*, *tbb-2* and *tbb-1.2* might be the best candidates to look for resistance-conferring mutations (Demeler et al., 2013a). However, data from previous studies suggested that *tbb-1.2* is likely not involved since the F200Y exchange has frequently been detected in BZ susceptible worms (Diawara et al., 2009, 2013; Rashwan et al., 2017). Comparison with *T. trichiura*, which encodes only one β-tubulin gene in its genome, also makes the isotype *tbb-4* a candidate since the F200Y exchange increased in frequency in post-treatment samples (Diawara et al., 2009, 2013). In the present study, four β-tubulin isotype genes in the *A. suum* and *A. lumbricoides* genomes (Demeler et al., 2013a) have been analysed. Nevertheless, no SNPs correlating with BZ resistance in other nematode species were detected. One may conclude that the observed reduced efficacy of albendazole were not due to genetically resistant worms but caused by other factors as discussed above. However, there can be other genetic causes of BZ resistance, including other SNPs in the β-tubulin genes. Kotze et al. (2014) mentioned based on unpublished sequence data that at least nine β-tubulin isotype genes can be detected in *Ascaris*. Finally, BZ resistance in ascarids might be associated with non-drug target related resistance mechanisms. In *C. elegans* as well as in strongylid parasites, BZs can be metabolised by cytochrome P450 monooxygenases and also induce expression of P-glycoproteins (Kerboeuf et al., 2003; Laing et al., 2010; Jones et al., 2013, 2015). Inhibitors of P-glycoproteins and cytochrome P450 monooxygenases potentiate the effects of BZs in *C. oncophora* and *O. ostertagi* in *in vitro* assays (AlGusbi et al., 2014). Conceivably, such mechanisms might also be at play in ascarids.

5. Conclusions

In southern highland Rwanda, the efficacy of school-based deworming with albendazole against *Ascaris* infections proved to be highly variable and, overall, insufficient. In Rwanda, with its very high PCA coverage rate and thus drug selection pressure, these findings should be taken as a warning sign considering the role of surviving parasite in the emergence and spread of resistance (Vercruysse et al., 2011a). The β-tubulin genotypes do not provide an explanation for the inappropriate albendazole efficacy observed. This specifically emphasises the need for ongoing monitoring of the outcome of routine deworming.

Financial support

This work was supported by the German Federal Ministry of Education and Research [grant number 01DG13006A, MOPACUR].

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2017.06.001.

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