PLOS | NEGLECTED TROPICAL DISEASES

VIEWPOINTS

Role of DNA-detection–based tools for monitoring the soil-transmitted helminth treatment response in drug-efficacy trials

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Introduction

More than 1 billion people have been reported to be infected with at least one soil-transmitted helminth (STH) worldwide, according to the last published report of the World Health Organization (WHO) [1]. WHO guidelines for STH control mainly encompass periodic administration of benzimidazoles (albendazole or mebendazole) to at-risk people of the endemic areas [1]. However, extended use of benzimidazoles could entail a great selection pressure for parasitic-resistant strains. In veterinary medicine, anthelmintic resistance in gastrointestinal nematodes has been developed in response to their excessive use, and it is currently considered a serious threat to livestock health and welfare [2, 3]. In humans, the estimated efficacy of albendazole and mebendazole against *Trichuris trichiura* has been observed to significantly decrease over time [4]. This observed decrement in drug efficacy could be due to the development of anthelmintic resistance (among other reasons such as drug quality and administration, the increasing of drug-efficacy studies, improvements in sensitivity of diagnostic tools after treatment, etc) after years of mass drug-administration campaigns, which is one of the major concerns in STH control [5].

Monitoring anthelmintic efficacy trials have been traditionally done by microscopic approaches, although it is well known that microscopy's sensitivity may be insufficient in this context [6, 7]. We think that DNA-detection–based tools represent an accurate alternative to parasitological methods, and they should be evaluated and validated not only for monitoring worm burden before and after treatment but also for detecting genetic markers related to anthelmintic resistance.

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Monitoring infection intensity pre- and posttreatment

The cure rate (CR) and egg reduction rate (ERR) are both based on the microscopic detection of helminth eggs or larvae in stool samples and are traditionally used as indicators for monitoring drug efficacy. CR is calculated as the percentage of baseline-infected individuals that are diagnosed as negative posttreatment. ERR refers to the reduction in the number of eggs excreted posttreatment and is calculated using quantitative methods [6]. However, sensitivity, being microscopy's major limitation, especially in posttreatment-occurring low-intensity infections, may overestimate both CR and ERR. Besides, they are observer dependent, which leads to variations in egg counts and limits standardisation options [7].

Recently, quantitative detection of parasite-specific DNA in clinical samples by real-time polymerase chain reaction (qPCR) tests has demonstrated substantial improvement in diagnostic performance as compared to microscopy, including the capability to differentiate between hookworm species [8, 9]. However, despite its high sensitivity, qPCR has not been frequently used for monitoring STH-infection intensity in drug-efficacy trials. Mejia and colleagues used multiparallel qPCR to assess *Ascaris lumbricoides* burden pre- and postalbendazole administration in a cohort of 125 children, wherein all the participants detected positive by qPCR at baseline were below the threshold of detection after 21 days of treatment [10]. Of late, efficacy of a single dose of albendazole against *A*. *lumbricoides* and *Necator americanus* was assessed with a pentaplex qPCR in context of a controlled deworming trial [11]. However, in this study, the egg number in stool samples was not determined; as a result, the authors were not able to correlate ERR with the infection intensity reduction rate based on qPCR results [11].

Highly sensitive diagnostic methods might allow the detection of light infections posttreatment and consequently aid the identification of potential resistant strains, particularly with respect to drug-efficacy trials. However, more systematic studies are required to further explore the exact relationship between observed parasite intensity determined by faecal egg counts and the quantitative outcome of the qPCR [12].

Genetic markers associated with benzimidazole resistance

As discussed above, qPCR might aid in evaluating drug efficacy by accurately estimating infection intensity before and after treatment [10–11]; however, the detection of genetic resistance markers could also predict treatment failures. Benzimidazoles bind to the colchicine binding site of β-tubulin, thereby disrupting microtubule polymerization and leading to parasite death. Parasite microtubules have been reported to be involved in vital functions at cellular level, including mitosis, motility, and transport [13]. In veterinary medicine, benzimidazole resistance is mainly associated with a single nucleotide polymorphism (SNP) at codon 200 of isotype 1 of β-tubulin–encoding gene, which results in amino-acid substitution of phenylalanine to tyrosine (F200Y). A similar SNP at codon 167 (F167Y) or a glutamate to alanine change at codon 198 (E198A) has been associated as a drug-resistance marker [3] (Fig 1). However, few studies have investigated the presence, frequency, and association of these markers with benzimidazole resistance in human STH species.

The presence of putative benzimidazole-resistance SNPs has been studied by different molecular methods [14–21], which are presented in Table 1.

As observed, several molecular tools designed for detecting benzimidazole-resistant SNPs exist; however, technical and economic requirements could limit their application in low-income countries. SmartAmp technology has reported to be less expensive and highly efficient, and it has been also proposed as a quantitative technique [22] with potential applicability for mass screening in limited-resource settings. However, SmartAmp is still being validated, and

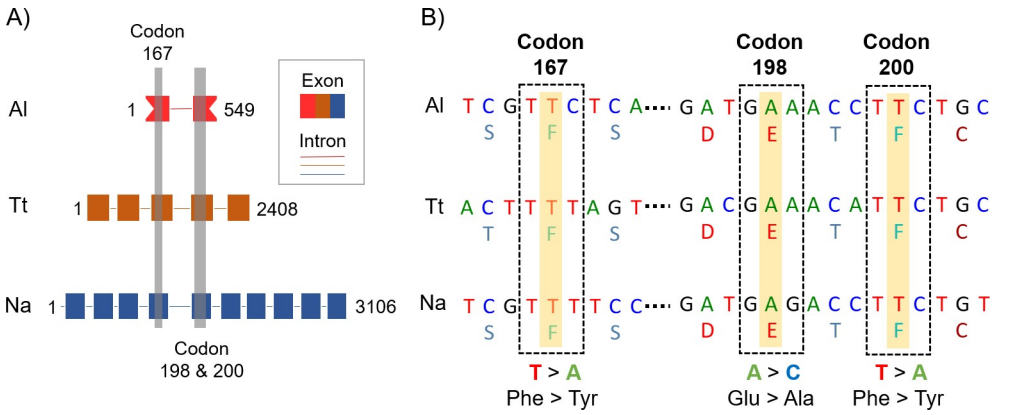

Fig 1. Putative benzimidazole-resistance SNP in human STHs. (A) Sequence information displayed in GenBank's flat file; the boxes represent exons, whereas the lines represent introns. (B) Detailed nucleotide sequence of the three putative resistance SNPs and the amino-acid transcript at codons 167, 198, and 200. Al, *Ascaris lumbricoides* β-tubulin 1 sequence, partial cds (GenBank: FJ501301); cds, coding sequence; Na, *Necator americanus* β-tubulin 1 sequence, complete cds (GenBank: EF392851); STH, soil-transmitted helminth; Tt, *Trichuris truchiura* β-tubulin 1 sequence, complete cds (GenBank: AF034219).

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currently only two studies describe this methodology for SNPs detection in STH (Table 2). The qualitative techniques that detect only the presence or absence of these SNPs possess a drawback: that the frequency of resistant alleles could not be measured in a pool of eggs. Conversely, pyrosequencing is more precise when compared with qualitative techniques, since it accurately determines multiple resistant allele frequencies in the same run. This technique has been widely used for detecting benzimidazole-resistant isolates in livestock nematodes [2], and therefore, we believe that pyrosequencing could be extremely helpful in understanding if there is a relationship between the presence of SNPs and their frequency with benzimidazole efficacy in STH, despite the high cost of pyrosequencing. It is expected that benzimidazole treatment could select resistant strains, and consequently, frequencies of resistant alleles would increase posttreatment.

With regard to these techniques' application, qPCR was first applied to detect the changes at codons 167 and 200 of *β-tubulin* gene of *N. americanus* [14, 21] and *Ancylostoma duodenale*, respectively [14]. More recently, restriction fragment length polymorphism (RFLP)-PCR has

Table 1. Characteristics of the molecular tools used for detecting putative benzimidazole-resistance SNPs in human STHs.

	Cost	Quantitative	Strengths	Weaknesses
qPCR	++	Yes	• Real-time detection • Widely used technique	• Expensive equipment
RFLP–PCR	+	No	• Simplicity • Widely used technique	• SNP detection limited by commercial endonucleases • Time-consuming procedure • Less accurate than the others
SmartAmp	+	No	• Isothermal amplification (no thermocycler) • Real-time detection • High amplification efficiency • Rapid and simple	• Need further validation
Pyrosequencing	+++	Yes	• High throughput • Multiple SNP detection • Accurate SNP frequency	• Highly expensive equipment • Not widely available

qPCR, real-time polymerase chain reaction; RFLP–PCR, restriction fragment length polymorphism-polymerase chain reaction; STH, soil-transmitted helminth

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Table 2. Summary of the results obtained by molecular tools in the detection of putative benzimidazole-resistance SNPs for human STHs.

	A. lumbricoides			*T. trichiura*			*N. americanus*			*A. duodenale*			
	F167Y	E198A	F200Y	F167Y	E198A	F200Y	F167Y	E198A	F200Y	F167Y	E198A	F200Y	
RFLP–PCR	×	×	-	-	-	-	-	✓	✓	-	-	-	[18]
qPCR	-	-	-	-	-	-	✓	×	×/✓	-	×	×	[14, 21]
SmartAmp	✓	×	×	-	×	✓	×	✓	×	-	-	-	[19, 20]
Pyrosequencing	✓	×	×	✓	✓	✓	×	×	✓	-	-	-	[15–17]

F167Y, E198A, and F200Y: putative benzimidazole-resistance SNPs at codon 167, 198, and 200, respectively.

✓: method applied and SNP detected.

×: method applied and SNP not detected.

-: method not applied for SNP detection.

qPCR, real-time polymerase chain reaction; RFLP–PCR, restriction fragment length polymorphism-polymerase chain reaction; STH, soil-transmitted helminth

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been used to study SNPs presence in *N. americanus* and *A. lumbricoides* populations in Brazil [18]. As an alternative, SmartAmp was developed for rapid and efficient genotyping of *β-tubulin* gene in *A. lumbricoides*, *N. americanus*, and *T. trichiura* [19–20]. Hence, pyrosequencing has been successfully used for determining the presence and frequency of three SNPs in all STH species except for *A. duodenale* [15–17]. Principal findings regarding genotypic benzimidazole resistance in STH are summarized in Table 2.

As previously described, all three SNPs (F167Y, E198A, and F200Y) have been detected in *T. trichiura* and *N. americanus*, whereas only F167Y has been observed in *A. lumbricoides*. However, most of these studies described only the presence or absence of SNPs and did not evaluate their association with the treatment response. However, so far only F200Y of *T. trichiura* has been noted to be associated with low response against albendazole [14, 15]. Diawara and colleagues measured the resistant allele frequency in *T. trichiura* by pyrosequencing in the stool samples collected from benzimidazole-naive populations [15] and albendazole-administered participants [16]. In nontreated areas, genotypic resistant profile (up to 63%) was noted, which explained the widespread suboptimal efficacy of benzimidazole against *T. trichiura* [15]. Moreover, a significant increase (from 3.1% to 55.3%) in F200Y frequency was observed post-treatment, which entailed the selection of resistant genotype [16]. The obtained results were in line with the findings observed in cases of veterinary helminths, wherein F200Y was reported as the most common SNP implicated in benzimidazole resistance [3]. However, this assertion must be cautiously interpreted, since the number of analyzed samples was low and the applied methodology was not harmonized across the study sites [16].

The case of *Strongyloides stercoralis* and ivermectin

Despite being recognized as an important intestinal helminthiasis to incorporate into deworming campaigns, strongyloidiasis has been commonly neglected in STH interventions [21]. The inefficiency of microscopy to detect *S. stercoralis* and the latter's unique feature of internal reproduction within the human host, makes worm burden less relevant, thereby rendering CR as a sole efficacy indicator [23]. Therefore, quantitative molecular methods such as qPCR would be even more important to accurately estimate the infection intensity pre- and post-anthelmintic treatment.

Apart from this, albendazole displays low efficacy against *S. stercoralis*, and ivermectin is the drug of choice [24]. Ivermectin has been extensively used in mass drug-administration campaigns for onchocerciasis and lymphatic filariasis [25] and, together with albendazole, is being evaluated for STH deworming campaigns due to higher efficacy of the combination,

particularly against *T*. *trichiura* [4]. Although, low response against ivermectin has been reported in veterinary helminths [2], we ignore the impact mass ivermectin administration (past and future) could entail in the development of anthelmintic resistance in *S*. *stercoralis* infections. Hence, in context of a potential widespread use of ivermectin against STH, we think that monitoring its efficacy and ivermectin-resistance emergence becomes of utmost importance, which has never been done so far.

Conclusions

Early detection of resistant strains is crucial for the successful performance of drug-efficacy trials with anthelmintics. Despite a wide range of different molecular tools, not enough systematic studies have been reported that could definitively associate low drug efficacy by ERR with the presence of anthelmintic-resistance genetic markers.

The authors of this viewpoint joined efforts in 2018 for the development of the STOP project (Stopping Transmission Of intestinal Parasites), funded by the European & Developing Countries Clinical Trials Partnership (EDCTP), with the main aim of moving forward with the control and elimination of STH infections. To our knowledge, there are two similar projects that are evaluating molecular tools for monitoring drug-efficacy trials in human STH: the DeWorm3 project, which will test the feasibility of interrupting STH transmission using biannual mass drug administration targeting all age groups and with large scale application of PCR for monitoring drug-administration campaigns [26], and the Starworms study, with the overall aim of recommending the best diagnostic methods for monitoring drug efficacy and molecular markers to assess anthelmintic-resistance emergence in STH-control programs [22]. In contrast, STOP is built around a multicenter, randomized, clinical trial for evaluating safety and efficacy of fixed dose of ivermectin and albendazole coformulation in 1,800 STH-infected children in Mozambique, Kenya, and Ethiopia. During the development of the trial, we will evaluate DNA-detection–based assays for accurately estimating the efficacy of the new coformulation. Besides, we would determine the usefulness of this new drug combination against potential STH-resistant strains in comparison with albendazole. We believe that the study of genetic resistance should be incorporated in future evaluation of anthelmintics, especially in drug-efficacy trials, for a better understanding of the potential impact of this problem in the drug assessment.

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