

Monitoring Anthelmintic Efficacy for Soil Transmitted Helminths (STH)

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Working group on Soil-transmitted helminthiasis.

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Abbreviations

ALB – Albendazole

AR – Anthelmintic Resistance

BZ - benzimidazoles

CR- Cure Rate

ERR - Egg Reduction Rate

EHT – Egg Hach assay

FEC – Faecal egg Counts

FECRT – Faecal Egg Count Reduction Test

GPELF - The Global Programme to Eliminate Lymphatic Filariasis

LEV - levamisole

IVM – Ivermectin

MBD - mebendazole

ML – Macrocyclic Lactones

PARASOL - Novel solutions for the sustainable control of nematodes in ruminants

SCI - Schistosomiasis Control Initiative

SNP - Single Nucleotide Polymorphism

STH – Soil-transmitted helminths

TST - Target Selective Treatments

BACKGROUND

1 - Prevalence and importance of Soil-Transmitted Helminths

The three major Soil-Transmitted Helminths (STH's), *Ascaris lumbricoides* (roundworm), *Necator americanus/Ancylostoma duodenale* (the hookworms) *Trichuris trichiura* (whipworm) are amongst the most prevalent parasites worldwide. Features common to all three include:

- Highest prevalence is observed in areas of rural poverty
- Morbidity is attributable to the chronic consequences of infection
- Intensity of infection is a key determinant of morbidity and transmission
- Inadequate sanitary conditions that affect the world's poorest people are key determinants of epidemiology
- Low mortality but high morbidity
- Affected populations frequently suffer poly-parasitism

It is estimated that there are more than one billion cases worldwide, of which 450 million have significant morbidity attributable to their infection, the majority of whom are children. An additional 44 million infected pregnant women suffer significant morbidity and contributory mortality due to hookworm-associated anemia. Approximately 135,000 deaths occur per year, mainly due to infections with the hookworms, or the roundworm *A. lumbricoides* (Awasthi et Bundy, 2007). When measured in disability-adjusted life years (DALYs) lost, the number of healthy years lost to premature death or disability, STH infections are as important as malaria or tuberculosis (Table 1).

Table 1 - Comparison of the STH Infections with other major diseases in developing countries (modified from Chan, 1997).

<u>Disease</u>	<u>DALYs lost annually</u>	<u>Deaths</u>
Hookworm infection	22.1 million	65,000
Ascariasis	10.5 million	60,000
Trichuriasis	6.4 million	10,000
Total STH Infections	39.0 million	135,000
Tuberculosis	34.7 million	2-3 million
<u>Malaria</u>	<u>46.5 million</u>	<u>112 million</u>

2 - Anthelmintics

The major means of controlling STH infections is by the periodic administration of one of the four anthelmintics recommended by the WHO: mebendazole (MBD), albendazole (ALB), levamisole & pyrantel. While ivermectin is not recommended for the treatment of human STH, except for strongyloidiasis, it has activity against ascariasis, hookworm and trichuriasis albeit less (virtually no detectable effect at doses normally used against *Necator americanus*) than the 4 drugs listed above. However, in the new perspective of preventive chemotherapy this drug requires attention as it is used for onchocerciasis and in "combination treatment" for lymphatic filariasis, and thus will have collateral effects in STH co-infection.

The benzimidazoles (BZ) – MBD and ALB - are the most frequently used anthelmintics to treat infections with STH's. Calculations by WHO have indicated that the cost for a treatment can be as low as 10 cent per child per year, including drug and delivery (Montresor et al., 2002). The clinical pharmacology of MBD and ALB in humans have been subject to extensive review (Dayan, 2003). A notable conclusion of this review is that the paucity of comprehensive data on the pharmacology of both drugs in humans. Significant deficiencies include incomplete data on:

- pharmacokinetics and metabolism during pregnancy and lactation
- potential drug interactions (nutrition, fasting....)
- genetic and phenotypic (diet etc) factors that might affect pharmacokinetics

3 - Indicators of efficacy

For anthelmintics used in veterinary medicine, the efficacy of an anthelmintic is determined (in standardized experimental conditions) during dose determination and confirmation studies (efficacy determined by reduction in worm counts) and field efficacy trials (efficacy determined by reduction of egg counts). For a claim against any worm species (immature or adult) a reduction of > 90% is requested (both for worm and egg counts). To monitor efficacy for the animal gastro-intestinal nematodes in field conditions the Egg Reduction Rate (ERR) is the standard used.

The two indicators that are used to determine the efficacy of an anthelmintic used in human medicine are the Cure Rate (CR) and the ERR. While in clinical

medicine the focus is on the Cure Rate, from a public health perspective - as in veterinary parasitology – the ERR is a more appropriate standard. Although, the ERR can be considered as the best (if not only) indicator, the conclusions (and a priori wrong) of many efficacy studies are based on CR's. Two important points to emphasize are:

1. Currently, for the human STH, large scale mass chemotherapy campaigns are taking place in various parts of Africa, Asia and South America, particularly targeting school children. Although WHO recommends a parasitological check every few years in large scale treatment programmes, these are being conducted infrequently without a coherent long-term strategy and without any standardized monitoring protocol.
2. In natural conditions the actual efficacy of an anthelmintic is whatever is observed at the time of treatment, however, this may be different from the efficacy under experimental conditions due to the influence by confounding factors (see 2, page 20).

4 - Anthelmintic efficacy in human helminthiasis: current situation

There are a vast number of studies investigating the CRs and ERRs of (BZ) anthelmintics against human STH, however, many of these are not directly comparable because several of these studies may have been confounded by methodological considerations (e.g. type of diagnostic tests, methods for determining the FEC/ERRs, treatment regimens, drug dosages, geographical location).

Table 2 shows the range of reported percentage CR of anthelmintics based on Janssens (1985) & Stepek et al. (2006) and Figure 1 shows the range of reported percentage CR and ERR of BZ based on Bennett and Guyatt (2000).

Table 2 – Percentage cure rates for the anthelmintics used to control STH

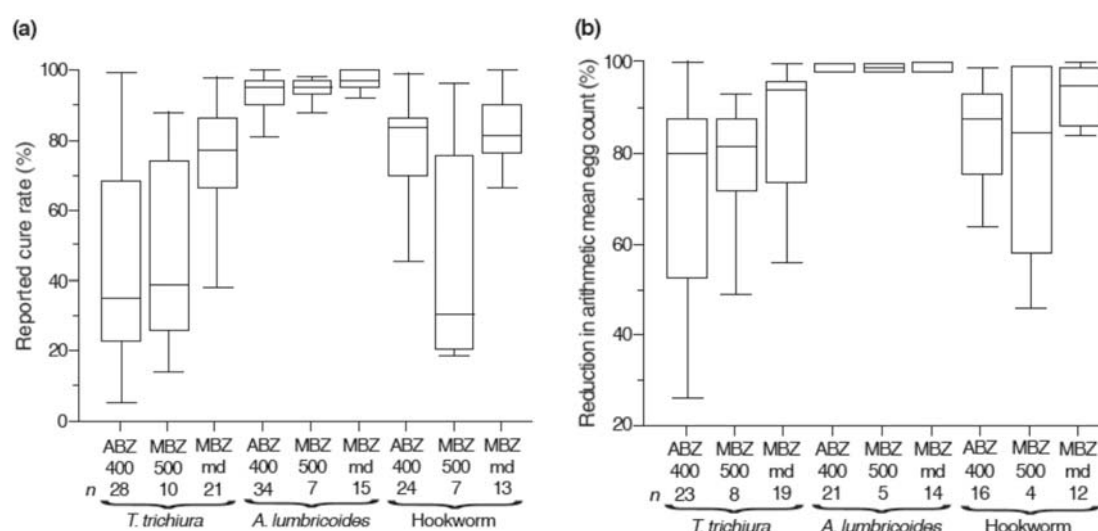
Anthelmintic	GI nematodes (% CR)¹		
	Hookworms	<i>A. lumbricoides</i>	<i>T. trichiura</i>
Mebendazole	9–60 ²	95–100	45–100
Albendazole	33–95	67–100	10–77
Pyrantel	37–88	81–100	0–56
Levamisole	66–100	86–100	16–18

Ivermectin	0 - 20	50–75	11–80
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¹ From Janssens, 1985; Stepek et al., 2006

² In areas of high transmission (prevalence >50%) according to an unpublished J&J meta-analysis conducted by Prof. Nilanthi deSilva

Figure 1 – Reported drug efficacy data as CR (a) and ERR (b) for 400 mg single-dose ALB, 500 mg single-dose MBD and multiple-dose (md) MBD (100 mg twice daily for three days) in treating *T. trichiura*, *A. lumbricoides* and hookworm infection.



Boxes represent the interquartile range of the data set, from the first quartile to the third, and the lines dividing the boxes represent the median. The whiskers indicate the range of the data, extending up to 1.5 interquartile ranges from the first and third quartiles, (therefore excluding extreme outliers). n = number of studies.

A major concern in the control of nematode infections is the low efficacy of single dose MBD for hookworm and whipworm infections, suggesting that this dosing regimen may be sub-optimal. The large variations are especially apparent for the CR (see Table 2 & fig 1^a), less for the ERR (see Fig 1^b). Assuming the use of high-quality drugs is maintained, this does not pose a problem in evaluating the efficacy of BZ for *Ascaris* infection because in all cases the efficacy is high, but it does present a problem in setting any benchmark for monitoring the emergence of drug resistance in hookworm and *Trichuris*.

The possible development of Anthelmintic Resistance (AR) to currently available anthelmintics is a subject of significant interest. In livestock resistance to all three of the major anthelmintic classes used (BZ, imidothiazoles/tetrahydropyrimidines & macrocyclic lactones) is widespread, and has been extensively studied (Wolstenholme et al, 2004).

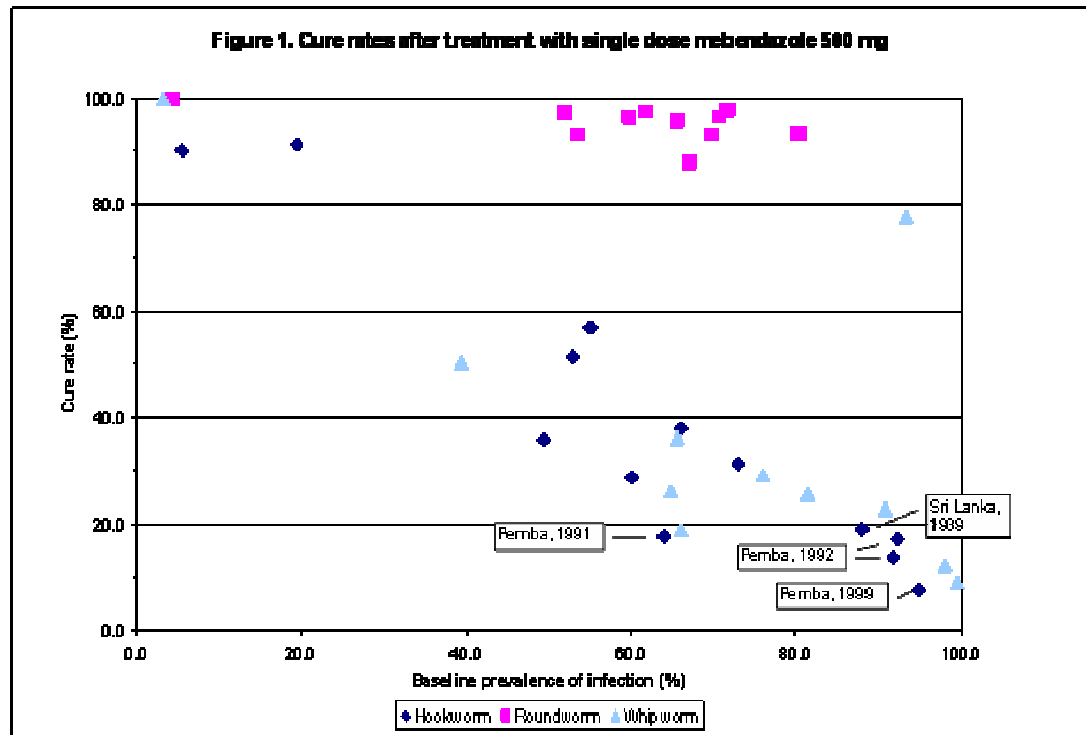
While it is possible that a similar situation observed for veterinary STH might

develop for human STH, currently there is no conclusive data demonstrating that resistance alleles have been selected in human STH, and that these alleles have spread in the parasite populations. Nevertheless, there are already worrying signs that anthelmintic efficacy may be declining, and unless the problem is taken seriously and appropriate measures implemented to prevent further decline in efficacy, in the next decade medical doctors may be facing similar problems to those currently being faced by veterinarians (Geerts and Gryseels, 2001).

Only one study has been published on AR in whipworms: the presence of the Phe200Tyr SNP in β -tubulin was observed in *Trichuris trichiura* (Diawara et al., 2007) and none for *Ascaris lumbricoides*. On AR in hookworms a (limited) numbers of studies were published:

- The study of De Clercq et al. (1997) in Mali on *N. americanus* was suggestive that AR against MBD developed (CR of 22.9%, ERR 6.5%, ED₅₀ of 0.117 instead of 0.069). However, when Sacko et al (1999) compared the efficacy of pyrantel ABL and MBL in the same localities they fell short of providing conclusive evidence of AR against MBL.
- Reynoldson et al. (1997) suggested that the locally (Australia) *A. duodenale* is resistant to pyrantel (results based on only 15 individuals).
- On Pemba Island, Zanzibar, the efficacy of MBD against hookworms in school children appeared to have fallen over a period of 5 years, during which time the children were regularly treated with MBD (ERR fell from 82.4% to 52.1%). This suggested the possibility of emergence of MBD-resistant hookworms on Pemba Island (Albonico et al., 2003).
- Recently, a single dose MBD was found to have disappointing efficacy (ERR of 31%) against hookworms infections in Vietnam (Flohr et al., 2007), although repeated dosing and single dose ALB treatment achieved higher efficacy (75% and 88%, respectively).
- Figure 2 shows a meta-analysis conducted by Prof. Nilanthi de Silva from the University of Kelanyia for the efficacy of MBD against the three major STH (personnel communication). It shows high rates of drug failure (low efficacy) against hookworm, particularly in Pemba (Albonico et al., 2003). While the biologic basis for this has not been extensively investigated it is possible that it represents anthelmintic resistance (AR).

Fig 2 – Cure rates after treatment with a single dose of MBD



To conclude, these studies cited above provide circumstantial - but not conclusive - evidence of reduced efficacy in hookworms. There has been, however, some debate about whether these reports on reduced efficacy in hookworms represent genuine cases of AR, based on the selection of resistance alleles in the parasites. It is important to consider following points:

1. To confirm these data there is a need to deal with potential confounding factors e.g.:
 - low number of studies, many significantly underpowered
 - no standardised protocols and diagnostic techniques
 - no agreed threshold to define AR
 - and many others
2. From veterinary experience, observable levels of resistance (= no effect on morbidity after treatment) may appear *suddenly* after an extended period of drug pressure during which the resistant phenotypes and genotypes gradually

increase, but are rarely detected clinically

3. It is difficult to know what the frequency of resistance would need to be before it could be detected, especially for human STH where dosing is often inadequate. The FECRT used in veterinary medicine, is not likely to detect resistance until at least 25% of the worm population is already resistant. However, some of the *in vitro* assays have been shown to detect 5-10% resistant worms when looking at measurements other than the LD₅₀.

5 - Anthelmintic resistance: genetics

Knowledge of the genetics of AR in helminths is incomplete. Nevertheless a number of important principles derived from the study of worms of livestock are likely to be relevant for human helminths. It is accepted that the rate at which AR develops in a given helminth population depends on many factors, among them the frequency of resistance alleles in the initial untreated population. Usually this frequency is estimated at a very low level. However, in untreated *Haemonchus contortus* populations, the initial frequencies of RFLP polymorphs associated with resistance to benzimidazoles (BZ) at the isotype 1 and 2 β -tubulin loci were 46 and 12%, respectively; surprisingly high figures (Beech et al., 1994). Similarly high (10 - 20%) frequencies of polymorphs associated with IVM resistance were reported in unexposed *H. contortus* (Anderson et al., 1998). However, these were not actual alleles, but RFLP polymorphs which may have contained more than a single allele, so that the frequency of a resistance causing Single Nucleotide Polymorphism (SNP) may have been less than this figure.

In this respect, Elard et al. (1999) determined the actual frequency of the 200Tyr codon in β -tubulin, in five different populations (each with between 38 and 101 individual parasites) of BZ susceptible *Trichostrongylus axei* and found for the total population of 281 diploid organisms genotyped, that the overall frequency of alleles containing the 200Tyr codon was 2.7%. However, the range of frequencies of the resistance-associated codon 200 was between 0% and 12.0% in the five different susceptible populations. The fast development of resistance to IVM and BZ in helminths of livestock may be explained, in part, by initial frequencies of resistance alleles being moderately high in some populations of nematodes. Data on the prevalence of putative AR alleles in STH of humans is only now being generated (see e.g. Schwenkenbecher et al., 2007) and needs to be investigated as a priority.

The number of genes involved in resistance and their dominance or recessiveness are other factors with an important impact on the rate at which AR spreads. Although contradictory reports have been published on *H. contortus* (the best studied helminth of veterinary importance), resistance to BZ may be polygenic with at least two, possibly three, genes involved, with the alleles being recessive (Prichard, 2001). However, polymorphism in beta-tubulin isotype 1 seems to be most important for BZ resistance in *H. contortus* (Mottier and Prichard, 2008). Levamisole resistance seems to be caused by one gene or gene cluster, the alleles of which are autosomal recessive (Anderson et al., 1998), and IVM resistance appears to be polygenic (Prichard, 2007b). Although there is ongoing debate about the number of genes involved in resistance to these different anthelmintics, of major importance is that there is a general agreement that reversion to susceptibility is rare once AR has developed in helminths of livestock, even when other drugs with completely different mechanisms of action are used for prolonged periods. This is an observation of major importance and is supported by a significant body of field and experimental data (reviewed by Conder and Campbell, 1995).

Although the findings in veterinary helminths may not be extrapolated directly to humans, the chance that similar phenomena might occur in STH of medical importance should not be overlooked.

6 - Factors contributing to the development of drug resistance

6.1. Treatment frequency

This is an important determinant of the speed of selection of AR: the greater the drug pressure, the faster the selection of resistant nematode strains. Treatment frequencies of 5 or more a year (up to 10/year) are not uncommon in livestock (Dorny et al., 1994); in humans the frequency of treatments is limited to 1 to 3 per year for *Trichuris/Ascaris*/hookworms (Warren et al., 1993; Renganathan et al., 1995). However, even at these lower treatment frequencies, selection of AR has been repeatedly reported in sheep and goat nematodes (Coles., 1995; Boudsocq et al., 1999). This is especially the case when the same drug has been used over prolonged periods, as is the case with BZ's in the control of STH, these lower treatment frequencies might be able to select for resistance. This has been clearly shown in nematodes of livestock, where farmers tend to use a single drug until it fails (Reinemeyer et al., 1992).

6.2. Refugia

The phenomenon of *refugia*, i.e. the proportion of the parasite population that is not exposed to drugs and thus escapes selection for resistance, is a very important factor, whose impact on the development of AR is too often overlooked (Van Wyk, 2001). The size of refugia will be mainly determined by (1) the fraction of the population treated (i.e. mass treatments versus selective or targeted selective treatments) and (2) the proportion of the worm population present in the environment where it is not subject to drug action (e.g. in the soil). This is in turn influenced by a range of factors including e.g. climate and longevity of the infective stage. For most of the STH of human importance there is a poor understanding of these factors, which are important parameters for the construction of relevant mathematical models.

In the past, strategic or prophylactic mass treatments of livestock have been the rule. This practice is certainly responsible for many of the AR problems veterinarians are now facing, an example of which is the “drench-and move” system, whereby all animals in a flock are treated before they are moved to clean pastures containing few or no worms in refugia, a strong selector for AR. Only recently veterinary parasitologists realized that a balance has to be found between treatment efficacy and delaying the development of AR. Mathematical modelling has shown that it is possible to delay the development of AR by not treating part (e.g. 20%) of the herd or flock. Recently (2006), the European Union has provided 2.9 Million Euro for an international research project to investigate and develop sustainable, low-input methods for internal parasite control in ruminants (Target Selective Treatments = TST). The project known as PARASOL (Parasite Solutions), an abbreviation for “Novel solutions for the sustainable control of nematodes in ruminants”. This project has been funded for a period of three years and involves 12 academic partners and 5 business ventures from 12 EU countries as well as Africa (<http://www.parasol-project.org>). The PARASOL-Project aims to reduce the need for drugs by developing TST only for animals showing clinical evidence of parasitic disease or reduced productivity. Animals with low worm burdens do not show symptoms and therefore do not require treatment. In addition, these strategies will reduce the risks of residues in food and in the environment, and provide a parasite population *in refugia* to limit the development of anthelmintic resistance.

In control programmes of human helminths, treatment is often directed at specific target groups such as school-aged children (they often remain in school for 5

years and therefore every child receive a limited number of treatments) and other population groups at greatest risk of morbidity. This effectively provides refugia, especially for hookworms as the adults tend to harbour greater worm burdens than children, and thus targeting school children likely reduces the selection pressure. This effect is further magnified because compliance is often less than 80% (Chitsulo et al., 2000). On the other hand:

- mass treatment with ALB is now a standard component of the The Global Programme to Eliminate Lymphatic Filariasis (GPELF)
- ALB is being distributed to all communities as part of the the Schistosomiasis Control Initiative (SCI) activities.
- in human parasitology the aim is to have high coverage of those we wish to target.

The size of refugia is also largely determined by factors such as the timing of the treatment and the climate immediately prior to treatment as both will influence the selection pressure. The generation of parasites, which develops after treatment in dry environments, may completely consist of a high proportion of resistant worms because climatic conditions will kill previously deposited eggs and larvae. However, in wetter environments, pre-parasitic stages of susceptible worms might survive on pasture and dilute the resistant genes in the next worm generation. However, the relevance of the timing of treatment on the refugia for the human STH has not been studied yet.

It needs to be stressed that we do not have the necessary information on the biology of human STH to enable mathematical modeling of sufficient accuracy to be of assistance in informing policy with respect to strategies for control. It is necessary first to collect relevant data in order to derive realistic estimates of the parameters that will then allow modelers to develop accurate and realistic mathematical models. For example we do not have any readily available information on the duration of viability of infective hookworm larvae in the soil. Those aspects of a parasite's life-cycle that will have the greatest influence over the rate of spread of drug resistance will vary between species and according to the genetics of drug resistance (e.g. whether resistance is a recessive trait). However, basic biology such as the longevity of adult worms is likely to be highly influential but remains poorly understood. It is important to know not only the average value but also its variability within a particular population to allow more accurate modeling. Also, recommending

treatment at certain times when a high proportion of parasites are in refugia in the environment would delay the spread of resistance but on the other hand would increase the rate of reinfection and reduce the effects of treatment, so a compromise had to be achieved.

6.3. Underdosing

Underdosing may constitute an important risk factor for the development of AR. As was shown in the models developed by Smith et al. (1999), the impact depends on the initial (before exposure to a given anthelmintic) and the resultant (after treatment) frequency of resistance alleles in the helminth population. Depending on their ability to kill all or part of the susceptible homozygote, heterozygote and/or homozygote resistant helminths, specific dose regimens can be identified that by underdosing promote resistance and others where resistance is impeded. Assuming that resistance is determined by a single major gene comprising two alleles at a single autosomal locus and low initial frequency of the allele for resistance, the most dangerous dose is the one that kills all susceptible homozygotes but none of the heterozygous or homozygous resistant genotypes. In contrast, when the initial frequency of the allele for resistance is high, the dose, which promotes resistance most strongly, is that which kills all susceptible homozygotes and all heterozygotes, but none of the resistant homozygotes (Smith et al., 1999).

Sub-optimal (subcurative) regimens are the rule in human treatment when anthelmintic are administered in single doses at dose rates that do not produce virtually 100% efficacy, a practice that is widely advocated and implemented in public health helminth control programmes. While the operational objectives are laudible: to ensure compliance of the target population, where the objective is only morbidity control (Warren et al., 1993) this may have unwanted consequences when applied widely and over a significant period. Taking into account these restrictions, the current approach might select for resistance under certain conditions. As can be seen in Table 2, the efficacy of most anthelmintics, at the dose rates used, is only moderate for the human STH (compared to veterinary STH with efficacies of > 98%). It is apparent that currently recommended dose regimens are underdosing parasites in humans. However, data beyond the scope of this paper indicate that increasing the size of the single dose of BZ will not always result in increased efficacy because the nature of their antiparasitic action depends on prolongation of contact time (Vercruysse, 2005); instead repeated dosing is necessary, an approach

that is difficult to implement in Public health programs.

Thus currently recommended regimens could be a contributing factor to the development of AR in STH. On the other hand, subcurative dose rates may exert less selection pressure for AR development than would dose rates and repetitive treatments which initially produced virtually 100% efficacy. These alternative possibilities need to be investigated experimentally and considered in models of possible AR development. The subject of anthelmintic dose rates and the pharmacodynamics and pharmacokinetics of anthelmintics in humans will be the subject of another report.

OBJECTIVES OF THE POSITION PAPER

During a WHO-World Bank meeting (Washington, Nov 2007) it was decided that monitoring the efficacy of anthelmintics should be a key-issue and a Working Group was established to write a position paper on the subject.

The overall objective of the present position paper is to make recommendations for “Monitoring of drug efficacy in large scale treatment programmes for human helminthiasis”, with the STH’s being specifically considered in this report.

The specific objectives were to make recommendations for:

1. The coprological and associated molecular methods to be used to monitor the efficacy of anthelmintics used in control programmes
2. The confounding factors to consider that may affect anthelmintic efficacy
3. The studies to be designed to monitor efficacy of anthelmintics (FECRT)
4. The statistical analysis to be used for the FECRT
5. The methods to be considered to evaluate AR for human STH, including the research needed on improved tools for determining the presence of AR
6. The algorithm and referral process needed for monitoring AR in endemic areas

These recommendations should allow to proceed with the monitoring of drug efficacy in large-scale treatment programmes for human infections with STH and to identify the extent of AR.

METHODOLOGY

1 - Coprological methods to monitor the efficacy of anthelmintics

1.1. Standard method to evaluate efficacy

The current method of choice to determine the efficacy of an anthelmintic, in veterinary medicine, is to compare infection levels before and after treatment. Infection levels can be measured either by worm counts (method of choice) or Faecal Egg Counts (FEC). It is evident that for human STH only FEC can be used as a measure of infection levels.

Whichever method is used to quantify the FEC, the uncertainty around this method of estimating parasite intensity should be acknowledged. Several reports have indicated a distinct density dependence effect on FEC in canine hookworms (Krupp, 1961; Kopp et al., 2008). From Strongylid infections in ruminants it is also known that the first signs of immunity development are reductions in worm length and worm fecundity, even before reductions of worm establishment are observed. Thus, in older animals low FEC are not always suggestive for light worm burdens and it may well be that this is also occurring for hookworms.

For a good interpretation of FEC it is thus essential that information is available on the e.g. helminth species, age, gender, nutritional status, previous contacts with STH etc.

1.2. Available techniques to detect nematode eggs in faeces

In veterinary parasitology quantitative techniques to determine FEC are commonly utilized, the most widespread is the McMaster method. In human medicine, only few techniques are commonly employed to determine FEC. While the Kato Katz technique is most widely utilized, this technique is especially appropriate to detect eggs of *Schistosoma mansoni*, but less so for the detection of eggs of STH, and particularly for quantification of egg burden of hookworm due to lysis of fragile hookworm eggs during processing.

Of major importance is that consensus should be reached on the most appropriate (sensitive and practical) technique to be used to detect and quantify the eggs of STH in medical helminthology. Considerations should include:

- The technique (s?) should be inexpensive and easy to perform in field conditions. However, it may be better to aim for a two-step mechanism: (1) a field applicable test - a sort of standardized pre-screening that may detect

preliminary evidence that AR should be investigated, and (2) for subsequent, more thorough laboratory investigation, using more sophisticated confirmatory techniques

- Quantitative tests should be recommended (= able to calculate ERR) in preference to qualitative tests (= only CR can be determined)
- Each recommended technique should be properly evaluated (e.g. sensitivity, robustness and practicability) under different conditions.

Primarily the FLOTAC and McMaster techniques are designed to be quantitative. The other coprological techniques are qualitative, however, they also can be used to determine (approx ?) FEC.

The McMaster Technique (most common technique used in veterinary parasitology) has been used for a hookworm study (Kotze et al, 2005) and has the advantage of not needing any apparatus other than the McMaster counting chamber. The FLOTAC technique, has been described recently both for human and veterinary medicine (Cringoli et al., 2006) and its application to counting hook worm eggs described (Utzinger et al. 2007). A recent study compared several methods for detecting STH (Goodman et al, 2007) and of the methods used alone, Wisconsin flotation and simple gravity sedimentation each provided the highest sensitivity for detecting eggs of STH.

A list of 5 techniques (+SOP) to detect eggs in faeces are show in Annexe 1.

- ✓ FLOTAC method
- ✓ Midi Parasep[®] SF Solvent Free Faecal Parasite Concentrator
- ✓ McMaster Method
- ✓ Kato Katz technique on double samples (SOP still to be completed)
- ✓ Formol-ether Concentration Technique (SOP still to be completed)

1.3 Ring testing

There is evidence for considerable variation of results obtained in different laboratories using the same coproscopical method on the same test material. Recently, Bogoch et al. (2006) reported that when the rates of detection of STH in sodium acetate-acetic acid-formalin (SAF) solution-preserved specimens were compared, large discrepancies between European and African centres were observed.

Since the evaluation of the efficacy of anthelmintic treatments within control programmes is mainly based on the use of such basic diagnostic tools, it is important

to eliminate factors contributing to data variation as much as possible. This can be done by evaluating the reproducibility of the respective diagnostic procedure followed by a process aiming at the identification of sources for variation (e.g. laboratory specific consumables, often neglected minor differences in test performance, storage of sample prior to testing). The aim of such a ring test procedure is to standardize the respective protocol as much as necessary to achieve the highest level of reproducibility possible under practical conditions. At the same time this will allow a thorough description of the level of variation associated with the different standardized methods. The first step in any ring testing approach will be the formulation of an initial test protocol or standard operating procedure (SOP). Uniform samples, distributed by one laboratory to all other laboratories at the same day under conditions preventing changes of the test stages (e.g. anaerobic storage of STH eggs), will then be tested in a number of laboratories. In this way samples with few, medium and high numbers of parasite stages should be tested to assess the reproducibility with respect to sensitivity and quantification. This has to be done specifically for different parasite species, or at least for different groups of parasites, i.e. hookworms and ascarids.

Recommended procedure

1. A consensus group, consisting of (veterinary and human) parasitological laboratories should be identified and established to agree and distribute information on the FEC methods to be assessed.
2. The different coprological tests should be compared.¹
3. A ring test (= Quality Assurance Program) is recommended to enable confidence in the approach and comparability between different laboratories in outcomes of any coprological (and later other) test.
4. The SOP (+ necessary material) should be made available to laboratories involved in FECRT studies.

¹ Note JV – We are comparing currently the sensitivity of Flotac, McMaster, Telemann (= Ritchie, sedimentation/flotation technique) and ParaSep techniques on 100 stool samples of monkeys. Results should be available in February.

2 - Confounding factors affecting anthelmintic efficacy

IT IS VERY IMPORTANT TO MAKE A DISTINCTION BETWEEN “TO EVALUATE EFFICACY” FROM “TO DETERMINE THE PRESENCE OF AR”. MANY (CONFOUNDING) FACTORS MAY DETERMINE THE EFFICACY OF AN ANTHELMINTIC, THEY SHOULD FIRST BE ELIMINATED BEFORE AR CAN BE ASSUMED.

Confounding factors are e.g.

- a) Conditions such as diarrhoea and some foods which increase the rate of passage of substances through the gastrointestinal tract may reduce the period over which an anthelmintic may be absorbed and act on the parasites, reducing efficacy.
- b) Greater availability, and therefore improved anthelmintic activity, is possible through temporary (12 hours) fasting (e.g. during Ramadan)
- c) The infection levels:
 - i. high infection levels may interfere with the pharmacokinetics (= reduced bioavailability) of an anthelmintic;
 - ii. high number of immature stages are present when infection levels are high and these stages may be less sensitive to anthelmintics (e.g. praziquantel for schistosomiasis);
 - iii. different pre-treatment intensity of infection may give different CR and ERR post treatment. Indeed, individual studies that have segregated the drug efficacy data by intensity class have demonstrated a reduction in CR and ERR with high intensities of *T. trichiura* and hookworm infections (Bennett and Guyatt, 2000). This factor should be taken into account in the statistical analysis.
- d) The quality of the anthelmintic (intrinsic quality, bioavailability and/or degradation during storage/transport)
 - i. Generic anthelmintics may be of high quality, unfortunately sometimes generic anthelmintics may be of low quality, affecting efficacy
 - ii. For intestinal species, particle size is very important (fine particular size is far superior to coarse), and this is likely to be affected by

passage along the intestine (Kelly et al., 1975; Wesche, Barnish, 1994)

- iii. For any anthelmintic showing low efficacy a batch of the anthelmintic should be tested to determine the quality of the drug by analysis of the active ingredients, purity, particle size etc. However, in Developing Countries only few specialised laboratories may have the capacity of undertaking such analyses²; another approach may be to determine the efficacy of an anthelmintic (BZ) used for medical purpose in natural infected cattle (easy and cheap to perform) i.e. if the FECRT is also low for the cattle STH, it is likely that the drug is of low quality³ rather than AR is the cause (AR in cattle is still rare).
- e) Physiological variations, such as inter-individual variability, age- related changes in drug distribution
- f) Drug interactions due to concomitant therapy (anti-inflammatory or antibiotic drugs)
- g) Co-morbidities (e.g. gastrointestinal diseases, malnutrition and immunodeficiency) may impair anthelmintic efficacy as many drugs require a competent immune system to achieve optimal efficacy
- h) Anthelmintics may reduce worm fecundity, a factor which is also worm-density dependent. As the AR is determined on FEC and not on the expulsion of adult worms this is an important factor.

Recommended procedure

- 1) The different confounding factors which may affect anthelmintic efficacy should be reviewed e.g. quality of anthelmintics, intensity of infections... and guidelines need to be established how to identify these factors.
- 2) Regions with different epidemiological situations (e.g. intensity of infections) and where worm control programmes are infrequently applied should be identified.
- 3) Studies should be initiated in these regions to determine the confounding

² The FAO & IFAH will sign in January 2008 a memorandum of agreement with as objectives to establish standards and protocols for quality control of trypanocidal drugs (and later anthelmintics), to establish good laboratory practices for chemical analysis, to transfer the methodology and technology to West and East Africa.

³ less than 30% of the anthelmintics used in veterinary medicine in Africa are of optimal quality (unpublished reports)

factors that may affect anthelmintic efficacy.

3 - The Faecal Egg Count Reduction Test (FECRT)

3.1. Introduction

At present, the FECRT is the only available test in the field to monitor the efficacy of an anthelmintic. As already stated (see 2) it is important to first exclude the confounding factors, which may affect drug efficacy, before considering AR. It is also important to consider that, at least for veterinary STH, the FECRT is able to detect AR only when at least 25% of the helminth population carries resistance genes (Martin et al., 1989). Finally, while the FECRT has the advantage that it can be used with any available anthelmintic for STH, thresholds to decide if AR is suspected still need to be worked out for the human STH.

The FECRT is based on the parasitological assessment of ERR, and should detect a reduction in egg counts following treatment. The FECRT is a standard test used in veterinary parasitology (Coles et al., 1992) and can provide data to assess drug efficacy against nematodes of humans. AR is considered to be present in livestock helminths if the ERR is less than 95% and if the lower 95% confidence interval is below 90% (Coles et al.; 1992). However, these values are based on expected ERR in drug-susceptible parasites of greater than 99%. These values would not be applicable to human STH where expected ERR cover a wide range, often falling well below 90%. For human STH, recommended procedures are described in a report from WHO Informal Consultation (WHO, 1999). An ERR below 70% in the case of *A. lumbricoides* or below 50% for *T. trichiura* or for the hookworms when BZs are used⁴, should trigger further, more detailed examinations. Furthermore, if in chemotherapy-based helminth control programs the difference of ERR between baseline survey and subsequent monitoring surveys shows a significant decline, then the suspicion of drug resistance should be investigated by further tests (WHO, 1999).

The interpretation of the FECRT (especially for human hookworms) may be further complicated by the several reports that have indicated a distinct density dependence effect on faecal egg count in canine hookworms (Krupp, 1961). Recently, Kopp et al (2008) showed that, in some cases, fecal egg counts were a

⁴ The proposed cut-off values of 70% in the case of *Ascaris lumbricoides* or 50% for *Trichuris trichiura* or for the hookworms are, considering the expected efficacy levels (table 1), too low. Compared to efficacy levels to the closely related animal helminths drug efficacies in drug naive populations should be higher than 80%.

poor indicator of drug efficacy in canine hookworms: while significant reductions in worm counts were observed following drug treatment, the FEC actually increased (fecundity increased in the small residual worm population).

Finally, it may be considered during FECRT studies to collect data on clinical signs (before and after treatment) and if possible to identify morbidity markers (weight, diarrhoea score, others?). However, in subtle and chronic infections like STH this may well be difficult. Particularly because we are working on positive but otherwise “healthy” children to test drug efficacy and 2-3 weeks are too a short time to test any clinical improvement. Changes in weight or iron status needs 6-12 months to become manifest, if ever, and need a large sample size, as well as a placebo group.

3.2. Study designs for FECRT

Before high-quality FECRT studies to monitor efficacy of anthelmintics can be designed (and guidelines proposed) it will be necessary to have baseline data on many relevant issues for which, until now, we don’t have the necessary information.

3.2.1. Inclusion criteria for subjects

Theoretically, only those subjects shown to be positive for worm eggs should be included in efficacy studies as ethically, it may be considered inappropriate to treat people without worms. However, on the other hand it can be argued that:

- when being done by way for prophylaxis it is acceptable
- it is routine for many of the control programmes to treat everyone in endemic areas regardless of their worm burden as the cost of diagnostics is high relatively to the treatments
- the proven safety of the anthelmintics in non-infected people
- the definition of “negative” depend on the sensitivity of the diagnostic test and since often control programmes rely on insensitive tests it is likely that the so called negatives are positives.

The minimum pre-treatment egg counts for each species of STH for patients to be included in the calculation of results still need to be established. This will also depend on the method (e.g. sensitivity) used for detection.

Important aspects to consider are:

- Subjects should be allocated to treatment groups on the basis of random allocation within sex and age stratified cohorts, to ensure that as far as possible both sexes and each age class is fairly distributed among all treatments. This should NOT be done on the basis of egg counts.

- Pregnant women should be excluded from trials, or possibly included in the placebo treated group. It is desirable also to conduct a pregnancy test in the field on all mature females prior to allocation to groups.
- On the treatment day, subjects should be observed to take the treatment in the presence of those running the trial, ensuring that the drug has been swallowed.
- On completion of the survey, all subjects originally administered ineffective or subtherapeutic treatment should be given the most effective drug.
- Equivalent medical care should be offered freely to all who participated or who did not but were part of the community where the trial was based.

In practice group sizes are then self-selected, because they met the above listed inclusion criteria (including (?) infected). On ethical grounds it is not then possible to exclude people from treatment if they meet the inclusion criteria.

3.2.2 Assessment of drug efficacy

Given the large differences in drug efficacies that have been seen within the same species in different geographical locations it is important to understand the variability in normal drug response (i.e. before the start of mass chemotherapy) so that atypical responses can be identified. Therefore, it is important to have baseline data on drug efficacy before the beginning of the chemotherapy-based intervention.

If AR is defined as a heritable change in a population of worms that enables them to survive drug treatments that are generally effective against the same species and stage of infection at the same dose rate (Prichard, 1980; Coles & Kinoti, 1997) then it will be necessary to establish a change in drug efficacy over time. This will require longitudinal cohort studies over a number of treatments, with FEC conducted immediately before and after each round of treatment. However, If a drug efficacy trial is carried out on a sample of the population that will be subject to periodic treatment, it may be not necessary to repeat efficacy monitoring after each treatment round, especially if treatments are done two or three times/year.

The duration and design of these longitudinal studies should be guided by the ethics of the situation and members of the community should be given anthelmintics with a different mechanisms of resistance (if the mechanisms of resistance are not known, different mode of action could be a surrogate for different mechanisms of resistance) if suboptimal responses are reported over time.

3.2.3 Study designs

Three study designs can be proposed:

- i. The basic trial format is to include only a treatment group and compare the FEC before and after treatment.
- ii. Where possible a placebo treated group should be included (e.g. treated with a vitamin supplement rather than the anthelmintic); on ethical grounds, this group should be treated with the most effective drug available when the trial has been completed. If a placebo is used a blind randomised trial is recommended and a placebo with similar shape and taste of the treatment should be used.
- iii. Where possible two drugs, operating through different modes of action (e.g. benzimidazole vs. pyrantel group) should be used for treatment.

3.2.4 Interval of sampling post-treatment

For the STH's the interval between treatment and re-sampling should be not less than 7 days, and no greater than 21 days. The timing of post-treatment examination that has been used in several recent efficacy trial for STH and recommended by WHO was three weeks. It was considered to be a time that (1) excludes new infection after treatment as is it as long as the minimum pre-patent period and (2) ensures that all live and dead eggs from worms present at the time of treatment have had time to be expelled. However, an interval of 3 weeks may be too long and ideally a period of 10-14 days should separate treatment from post-treatment survey; longer intervals may result in an apparent lower efficacy due to the maturation of immature stages (= suggesting lower efficacy for BZ).

Annexe 2 gives a proposal for the SOP for the FECRT. It will be critical to establish SOPs regarding the size of such trials, the frequency of parasitologic examinations so that the results of such trials are sufficient to detect changes in drug efficacy. It is clear that to obtain robust data it is necessary to use robust statistical approaches such as bootstrapping.

3.3 - Statistical analysis (FECRT)

3.3.1. Introduction

It is very important that any statistical analyses of the results from the FECRT studies are standardized. Where possible (large group size) data should be analyzed by general linear models that can cope with negative binomially distributed data, or data should be transformed appropriately to meet the requirements of parametric analysis (but in this case great care should be taken in comparing groups by means, whether arithmetic or geometric). Analysis of changes in prevalence rates (i.e. cure

rates) is weak and not really very informative, so analysis of quantitative FEC should be given priority.

Where group sizes turn out to be too small for parametric analysis, non-parametric tests (Kruskall-Wallis, Mann-Whitney U test, Meddis tests etc.) can give some idea about whether groups responded differently to treatment.

3.3.2. Number of individuals

The number of individuals to include in a trial is a problem of some concern and it is very difficult to give any clear recommendation that will satisfy all concerned. It is important to aim to do trials that are sufficiently powered to give us the information we need. Most previous trials were done with a too few numbers of subjects. The golden rule is to include as many as are available and can be practically assessed in an individual trial, given the resources available, however:

- Investigators should expect a drop off rate in compliance, that is some of those given the treatment may not provide post-treatment stools for analysis for a variety of reasons.
- To increase the number of subjects will always remain a key-issue as many unexpected factors may impede attendance during samplings.
- To determine FEC is a time consuming procedure and how many tests, which can be conducted by a field team, depends on the size of the team and resources available.

The last WHO Informal Consultation (1999) on this subject recommends 200 individuals per group, However, practicalities and local circumstances dictate who is available and how many people are willing to participate.

Therefore, we can only give rough guidelines here, because the ideal group sizes depend on local conditions, not least the prevalence of infection and the degree of aggregation of worms in the most susceptible sectors of the community. Ideally group sizes should be selected on the basis of power calculations, but this requires prior knowledge of prevalence and abundance of infection in the subject community where the trial is to be based. In practice, surveys are usually conducted on communities, which have come to the attention of the investigators as failing to respond to treatment or for some other reason. Having conducted FEC on the community, on ethical grounds investigators are then obliged to treat everybody whether group sizes are adequate or not.

Given the fact that groups of children or adults are usually less homogeneous

than herds or flocks of animals and taking into account the unavoidable drop-out rate, the number of people in the study groups should be higher (preferably 50 or more) than the number of animals (15) advised by the WAAVP. Another factor is that typically efficacies of 50 – 90% are seen for anthelmintics against human STH and this is lower than normal efficacies seen in livestock studies (> 96%). With the lower efficacies variances are normally much larger than when efficacies of 95 – 100% are expected and the greater variances mean that group sizes should be substantially higher than in animals studies in order to maximise the chances of detecting a significant effect of treatment.

3.3.3. *Basic approaches for data-analysis.*

Firstly investigators should look for bias in allocation to groups before treatment. Thus a statistical analysis should be conducted on the FEC at the first survey (pre-treatment) of those who successfully completed the trial with the expectation that some of those allocated to groups will fail to turn up for treatment or fail to provide a post-treatment stool sample. Note that often individuals provide the first stool sample then miss out on treatment, but still provide a second stool sample, so these, depending on number, may be useful in establishing an untreated group.

Secondly, we can look for differences between treatment groups at the second, post-treatment survey.

Thirdly, we can calculate for each individual the change in faecal egg counts between surveys and base the analysis on the quantitative change in FEC (i.e. FEC_1 minus FEC_2) or employ paired sample tests for this purpose.

For large surveys, as undertaken in veterinary medicine (> 1000 animals), and when high prevalences is expected it may be recommended to compare only the group (geometric or arithmetic) mean of the FEC before and after treatment.

3.3.4. *Calculation of efficacy*

Drug efficacies can vary enormously depending on the manner in which summary statistics of drug trials are calculated (Sacko et al., 1999). Therefore, it is important to standardize the methods used for assessing and interpreting drug efficacy, or to agree to analyse data from all trials in every instance.

Annexe 3a describes the different formulae used to calculate efficacy. Given the enormous differences in FEC between communities, and variation in degrees of aggregation of FEC, and the lack of thresholds, it is still too early to determine definitively which of the currently available methods is best for human studies. All

the calculations are relatively simple and easily computed with widely available software, and therefore until more studies report on outcomes of treatment all the calculations should be carried out during the analysis of each trial and published in full. In time this may enable a consensus view, perhaps following a meta-analysis of several trials, on which approach is most meaningful and relevant for the detection of AR in human clinical trials. Furthermore, it would be of considerable value to perform a simulation study, comparing the different analyses. In simulations studies the end points are known, and as a consequence, the simulated data can be evaluated by each different method of analysis and the accuracy of each method can be assessed in terms of Type I and Type II error rates. Such an analysis was recently performed on egg reduction data in horses (Vidyashankar et al., 2007) (Annexe 3b)

It may also be worth considering the option by Montresor et al (2007) (Annexe 3c) in which the analysis of reduction of proportion of class of intensity of infection is proposed. The use of the "category of intensity of infection" allow to describe in a very simple and direct way the epidemiological changes occurring in a STH infected population when a pharmacological intervention is implemented. The main objective of a STH control intervention is to reduce morbidity, the reduction of the number of individuals harbouring large number of parasites (the individual in the category of infection of heavy intensity) seems to be the best indicator of the reduction in morbidity in the population studied. This way is more representative of the benefit of the drug in reducing heavy infections and it does control for different intensities of infections at baseline which may affect drug efficacy.

Recommended procedure

1. A consensus group, consisting of WHO and (veterinary/human) parasitological laboratories should be established to develop guidelines for FECR studies
2. Regions (and corresponding laboratories) should be identified where FECRT can be assessed
3. A priority should be to identify regions where control programmes are commonly and occasionally applied⁵

⁵ In India (Vellore) I identified a region where people are treated three times a year with ALB (n = 75,000) and adjacent regions where people are only occasionally treated

4. The key-issue will be to reach a consensus on the threshold for AR for a specific drug. This should be based on a meta-analysis of randomised efficacy trials for any specific anthelmintic. In addition to a given threshold, a declining efficacy over time needs also to be evaluated.

4 - Other methods of choice to evaluate AR for human STH

4.1 Biological test available to detect AR

4.1.1. Egg Hatch Test (EHT) - BZ

The EHT is one of the standardized tests to detect drug resistance recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP). The EHT test assesses the ability of thiabendazole at given drug concentrations to inhibit the embryonation and hatching of freshly collected nematode eggs, expressed as the dose required to inhibit 50% of the eggs (ED₅₀). In nematodes of animals, ED₅₀ values in excess of 0.1 mg/ml of thiabendazole are considered to be indicative of BZ resistance (Coles et al., 1992), although this may well be too high and the discriminating dose (LD₉₉) should be 0.1 mg/ml (Coles, personnel communication). Annexe 4 gives the SOP for the EHT

Given the biological characteristics of human helminth eggs, the EHT is the most appropriate test for use with human hookworms, since their eggs hatch rapidly. The EHT is not useful for *Ascaris* and *Trichuris* which develop to the infective stage within the egg in the external environment but do not hatch externally to the host. The WHO (1999) has promoted the evaluation and standardization of the EHT in human helminthology.

- The first to use the EHT were De Clercq et al. (1997) who determined the ED₅₀ for a sensitive (kept in laboratory animals) and a suspected resistant (Mali) strain of *N. americanus*:
 - ✓ sensitive strain ED₅₀ : 0.069 µg/ml TBZ
 - ✓ Mali strain ED₅₀: 0.117 µg/ml TBZ
 - A series of studies were carried out in 1998-2000 on Pemba Island, Zanzibar, to evaluate, adapt and refine the EHT as a test to detect BZ resistance in human hookworms (Albonico et al., 2005).
 - Similar studies were carried out in Papua-New Guinea (Kotze et al., 2005)
-

and showed that the EHT is a reproducible test to perform in the field and that it proved effective in quantifying drug sensitivity in drug-susceptible hookworms.

However, the ability of EHT to indicate drug resistance in larval or adult hookworms remains to be determined, and more studies are needed to further standardise and validate the technique, and to define a threshold for resistance for human hookworms. Although that the EHT is fairly standardised in veterinary medicine, yet evidence of variability between laboratories exists and several factors need to be standardised/agreed upon in order to minimise variation (von Samson Himmelstjerna, 2007, submitted).

4.1.2. Larval development tests – BZ, LEV & ML's

For the larval development assay it is assumed although not proven that its sensitivity is slightly better than for EHT and BZ drugs, with resistance being detected when 10% of the worm population is resistant (Dobson et al., 1996). Studies are needed to determine that the test can be used to detect resistance in human STH.

With both the EHT and LDT, the sensitivity may be improved by using discriminating doses (=LD₉₉). This approach is routinely used in field testing of insecticides as organisms surviving the LD₉₉ are by definition resistant. However this would require the robust establishment of values using known susceptible isolates (such as *Necator americanus* maintained in laboratory hamsters and *Ancylostoma duodenale* in dogs) and must be reproducible between laboratories.

4.1.3. Motility and other assays (PYR / LEV, ML's)

A motility assay has been used to measure the response to ivermectin in human hookworm larvae (Kotze et al., 2005). However, its ability to detect resistance is also unknown. Recently, a motility assay and a novel assay (the larval arrested morphology assay, LAMA) showed some ability to discriminate between strains of the canine hookworm, *A. caninum*, showing different levels of resistance to pyrantel (Kopp et al., 2008). However, the sensitivity of the assays is unclear. As described above (4.1.2) further studies are required to determine the suitability of these assays for resistance testing with human hookworms.

4.2. Molecular Markers for Benzimidazole Resistance in STH

Several PCR protocols have been published for the analysis of BZ-resistance associated beta-tubulin single SNPs in parasitic nematodes from livestock. Conventional PCR methods were found suitable for testing single worm/larval DNA

samples (Elard et al., 1999; Kwa et al., 1994; von Samson-Himmelstjerna et al., 2002; Silvestre and Humbert, 2000). Recently real-time PCR was developed for the use of pooled larvae DNA (Alvarez-Sanchez et al., 2005; Walsh et al., 2007). One of these procedures was applied to a set of eleven *Haemonchus contortus* isolates and it was shown that the beta-tubulin isotype 1 codon 200 allele SNP frequencies corresponded closely with the respective BZ-phenotypes (i.e. EHT data). Furthermore, in partially selected isolates with susceptible phenotype status, the molecular method detected a loss of susceptible alleles (Walsh et al.; submitted). It was recorded that the data obtained for the beta-tubulin isotype 1 codon 200 by the two molecular tools (i.e. real-time PCR and pyrosequencing) were almost identical. These findings show that molecular tools for the assessment of BZ-resistance status are nowadays a practical options for routine use in the field. However, this requires a good understanding of the genetic characteristics of resistance in the respective target species. Because different levels of resistance were associated with beta-tubulin polymorphisms within, and between, sheep trichostrongyle and horse nematodes, it is necessary to understand resistance associated SNPs on a species-specific level. Closely related model parasites from veterinary hosts may be helpful in this regard providing approximate values that may be expected, but by no means absolute in this respect. The potential for developing molecular markers for the BZ anthelmintics has been recently reviewed (Prichard, 2007a).

Both real-time PCR and pyrosequencing assays were developed for both codon Phe200Tyr and Phe167Tyr in individual microfilaria of the human filarial nematode, *Wuchereria bancrofti* (Schwab et al., 2005). Recently, pyrosequencing assays (see below) have been developed for the Phe200Tyr SNP in *A. lumbricoides*, *T. trichiura* and *N. americanus* (Diawara and Prichard, unpublished). Of interest was the finding of Tyr200 in *T. trichiura* samples (Diawara et al., 2007). Other studies of interest were published by Albonico et al. (2004) on the molecular sequencing of the β -tubulin gene of human hookworms as part of an investigation of possible BZ resistance on Pemba Island and by Schwenkenbecher et al. (2007) using real time PCR as a tool for monitoring sequences associated with drug resistance in hookworms (assays were developed for β -tubulin isotype 1 of *N. americanus* and *A. duodenale*).

However it must be remembered that changes in drug transport (P-

glycoprotein) may also contribute to BZ resistance (Kerboeuf et al., 2002; Blackhall et al., 2008).

The quantitative *de novo* sequencing of short target sequences, by pyrosequencing, offers the opportunity to assess the allele frequencies of single or multiple resistance-associated DNA polymorphisms at high throughput and low cost. This procedure can be considered as highly suitable for routine testing of known sequence polymorphisms, similar to the real-time PCR. In addition, pyrosequencing offers the opportunity to test multiple polymorphisms in one analysis and can be used on pooled samples, e.g. nematode eggs recovered from stool samples, to obtain proportions of alternative SNPs (Annexe 5). Methods for using real time PCR for SNP analysis in nematodes can be found in Schwab et al. (2005) and Schwenkenbecher et al. (2007).

Annexe 6 shows the Pyrosequencing Assay Protocol for β -tubulin (isotype 1) Phe200Tyr SNPs in nematode parasites of humans (after Diawara et al., 2007).

4.3 The research needs for the improvement of the tools available for determining the presence of AR

A fundamental prerequisite for the meaningful use of molecular tools for the diagnosis of AR in human helminths is knowledge of the molecular markers associated with resistance. Because no confirmed resistant isolates of STHs have been reported from people so far, it should be our first goal to produce data on polymorphism in genes, which have been associated with anthelmintic resistance in veterinary nematodes, in phenotypically resistant and susceptible populations of human STHs. Ideally these should be derived from confirmed field cases and from different geographical regions. Since this can not be achieved as long as resistance in human STHs is not confirmed, alternative strategies should be pursued:

1. One approach is to measure the genetic polymorphism in genes, that have been associated with anthelmintic resistance in veterinary nematodes (e.g. β -tubulin in BZ resistance), in human STHs, and at the same time assess response to anthelmintic treatment as FECR in the same populations.
2. Another approach is to undertake longitudinal studies in which the genetic polymorphism is assessed along with response to treatment, as FECR, with increasing rounds of anthelmintic treatment.
3. A third possibility is to use human STHs that have been adapted for passage through laboratory animals (such as *N. americanus* in hamsters and *A.*

duodenale in dogs) for experimental selection of AR and at the same time, to analyse putative molecular markers in these drug selected populations.

4. A fourth possibility would be to use closely related model species, for example canine hookworm or *A. suum*, for experimental selection of AR in their natural animal hosts and again analyse the putative molecular markers in these drug selected populations.

In the case of the two approaches using animal hosts as models for the human STHs or closely related animal STHs, a relationship between the anthelmintic response phenotype and the molecular markers would need to be confirmed in populations of human STHs obtained from people.

Recommended procedure

- 1) A consensus group should be established to develop biological and molecular test to detect AR
- 2) Strains of STH, suspected to be resistant, should be identified and tested
- 3) Strains of STH, suspected to be resistant, should be archived for future development and improvement of molecular tests for AR

5 - Algorithm and referral process for monitoring AR in endemic areas (M. Albonico)

For comparison purposes, and to provide unselected parasite material for baseline genetic analysis, anthelmintic response data and parasite material should ideally be collected before widespread chemotherapeutic intervention occurs. Baseline data should be collected according to the recommended protocol and it is important that similar procedures are adopted during subsequent ongoing monitoring for AR. Quality testing of the methods needs to be carried out with internal and external quality control procedures in order to validate the results. The following procedures may be proposed when preventive chemotherapeutic intervention for STHs is planned.

District level: Baseline information on FEC pre and 2 weeks post treatment with the drug that will be utilised in mass deworming campaigns should be collected before the beginning of the mass chemotherapy intervention. In area where mass treatment is carried out, after 4 treatment rounds (after 2 years if the intervention is carried

every six months), it is recommended that the FECRT be repeated. If no sign of reduced efficacy are detected the AR monitoring should be ongoing every two years. In cases where treatment failure is suspected in specific foci an *ad hoc* monitoring survey using the FECRT should be carried out on demand, and if possible, parasite material (eggs, larvae or adult STHs) should be collected for DNA analysis in a reference laboratory.

National level: Baseline information on EHA with thiabendazole on hookworm eggs should be collected before the beginning of the chemotherapy-based intervention.

From areas where mass treatment is carried out, after 4 treatment rounds (after 2 years if the intervention is carried every six months) it is recommended to repeat the monitoring survey for hookworm AR by EHA .

If no signs of reduced efficacy are detected, the AR monitoring should be ongoing every two years. In the case in which treatment failure is suspected in specific foci an *ad hoc* monitoring survey by EHA should be carried out on demand.

Reference laboratories. Isolates (adult worms, pool of larvae or eggs) for molecular analysis should be collected (see Annexe 5) as baseline and reference material in endemic countries before any chemotherapy-based intervention for STH control. Even if standard methodologies for analysis of genetic polymorphism are still under development, it is good practice to store such reference material for future molecular analysis at reference laboratories. Samples of nematode eggs, larvae or adult worms (e.g. for 5 – 10 % of the study population) should be collected routinely every two years from areas where mass treatment campaigns are carried out. If treatment failure is suspected in specific foci, an *ad hoc* collection of eggs, larvae or adult worm samples should be obtained for monitoring purposes. The isolates should be sent to a Reference laboratory for molecular testing.

OVERALL RECOMMENDATIONS

Before making any overall recommendations we need to realize that the most difficult, but perhaps the most important, will be to pick up the first warnings of impending resistance before it becomes widespread (= to detect AR at an early stage). To monitor efficacy of anthelmintics (with emphasis on good use, quality of drugs, etc.) or to detect resistance once when it has become a serious problems is easier.

The decision the working the group has to make is to decide how to proceed in the future, using the present position paper as a guideline. We should thus:

1. Find out what the best (most sensitive) coprological methods are. Methods should be evaluated and advantages/disadvantages should be compared. Once we have decided on a "state of the art" test - define a SOP and perform ring test (quality assurance) with diagnostic laboratories willing to collaborate
2. Design a monitoring system (FECRT) to evaluate anthelmintic efficacies including e.g. sampling procedures, minimum number of individuals to be sampled, minimum egg counts to be included, data analysis
3. Work out a standard checklist of potential confounding factors (and how to identify them) to separate them from potential drug resistance
4. Find out and reach consensus on what threshold levels for potential drug resistance are or could be - using the FECR test with the method(s) worked out under heading 2.
5. Initiate FECRT studies in regions where there is either no widespread chemotherapeutic intervention and or where control programmes are ongoing.
6. If signs of reduced efficacy are detected possible confounding factors should be first excluded. If AR is suspected parasite (eggs, larvae) should be collected and submitted to reference laboratories (to be identified) for further analysis
7. Work out methods to confirm drug resistance in the lab - biological methods for the time being and - hopefully - molecular methods in the (near) future. But before being able to move on this, we must have some potentially resistant parasite strains at hand (see 6)

Data obtained from the FECRT studies will lead to:

- ✓ Validation of the statistical methods proposed.

- ✓ Agreed guidelines for the thresholds of efficacy for the common STH
- ✓ Establishment of standard operating procedures for surveillance tools and guidelines for monitoring systems
- ✓ Determination of the thresholds for the FECRT for the STHs in regions where it may be expected that AR is likely not to occur (limited use of anthelmintics) and in regions where anthelmintics are frequently used
- ✓ Identification of the full range of factors involved in the development of AR or of confounding factors affecting efficacy (e.g. quality of the anthelmintics)

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Annexe 1 – Quantitative coprological techniques to detect eggs in faeces - SOP

1.1 – Flotac technique

A novel multivalent faecal egg count method, the FLOTAC technique, has been described recently both for human and veterinary medicine (Cringoli, 2006) and its application to counting hook worm eggs described (Utzinger et al. 2007). This method is facilitated by the FLOTAC apparatus (Fig. 1), which has been designed to carry out flotation in a centrifuge, followed by a transversal cut (i.e. translation) of the apical portion of the floating suspension. Manual centrifugation is also possible. The FLOTAC technique allows quantification of eggs and/or larvae of nematodes and trematodes as well as cysts and oocysts of intestinal protozoa in up to 1 g of faeces or even more. It should therefore improve the sensitivity of the “classical” diagnostic tools in human parasitology, such as the Kato-Katz technique, the ether-based concentration method, the sedimentation technique, etc.

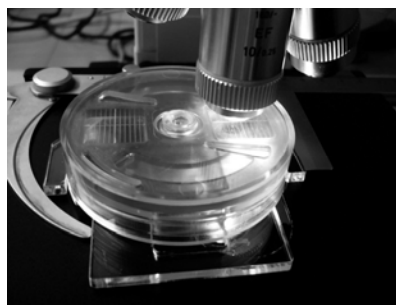


Fig. 1. The FLOTAC apparatus under the microscope.

The FLOTAC apparatus is a cylindrical-shaped device composed of three principal physical components: the body (Fig. 2a), the translation disc (Fig. 2b), and the reading disc (Fig. 2c), and of other auxiliary physical components. The FLOTAC apparatus holds two sample flotation chambers, 5 ml each, for a total volume of 10 ml (Fig. 2d), with two ruled grids (Fig. 2e). Each grid (18×18 mm) contains 12 equidistant ruled lines that are transparent, and hence permit the counting of parasitic elements under them. Where the dilution of faeces is 1 in 10, the reading of 2 grids (total volume is 10 ml = 1.0 g of faeces) permits an analytic sensitivity of 1 EPG/LPG/OPG; the reading of 1 grid (total volume is 5 ml = 0.5 g of faeces) permits an analytic sensitivity of 2 EPG/LPG/OPG.

Regarding the human field, in a recent study, Utzinger et al. (2007) compared the diagnostic performance of three methods (Kato-Katz, ether concentration and FLOTAC techniques) for hookworm diagnosis on 102 schoolchildren in Côte d'Ivoire. The observed hookworm prevalence as assessed by the FLOTAC, Kato-Katz and ether concentration techniques were 65.7%, 51.0% and 28.4%, respectively. Considering the combined results as the diagnostic ‘gold’ standard, the FLOTAC technique had a sensitivity of 88.2% compared with 68.4% for the Kato-Katz and

38.2% for the ether concentration techniques. The FLOTAC technique thus showed promise as an important new tool for individual hookworm diagnosis and for rigorous monitoring of soil-transmitted helminth control programmes.

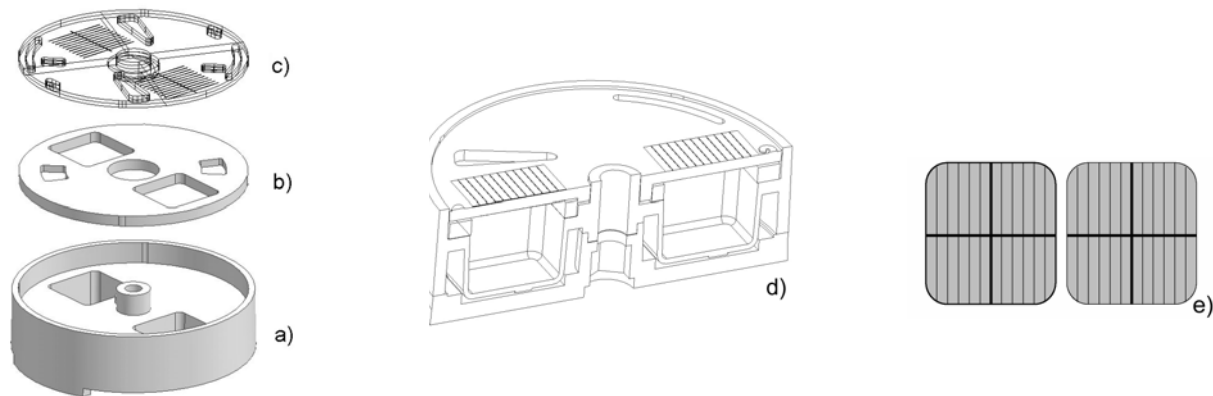


Fig. 2. The three principal components of the FLOTAC apparatus: the body (a), the translation disc (b), and the reading disc (c); the two sample flotation chambers (d); the ruled grids (e).

In addition, it is important to underline that the FLOTAC technique is multivalent. Hence, the technique can be used for the simultaneous diagnosis of different helminths as well as intestinal protozoa. This issue is of relevance because multiple-species parasitic infections are the rule rather than the exception in the developing world and hence a combination of different diagnostic approaches is recommended.

FLOTAC technique – Standard Operating Procedure

- 1) Take a part of faecal suspension conserved in SAF or formalin (minimum 1 g of faeces)
- 2) Add tap water (dilution ratio = 1: 10)
- 3) Homogenize
- 4) Pour the suspension through a wire mesh screen with an aperture of 350 μm
- 5) Place 10 ml of the filtered suspension in a tube = 1 g of faeces
- 6) Centrifuge at 2.000 rpm x 1 min
- 7) Discard the supernatant
- 8) Fill the tube with the flotation solution up to 10 ml
- 9) Mix the suspension in the tube
- 10) Fill the two chambers of the FLOTAC apparatus using a pipette
- 11) Centrifuge the FLOTAC at 1.000 x 5 minutes
- 12) Translate the FLOTAC and count the parasitic elements under the microscope
- 13) No multiplication factor is needed

FLOTAC technique – Equipment needed

- 1) Cups or beakers
- 2) Wire mesh screens
- 3) Tubes (15 ml)
- 4) Flotation solutions
- 5) FLOTAC apparatus
- 6) Manual or electric centrifuge
- 7) Optical microscope

Manual centrifuge – Standard Operating Procedure

- Place 4 FLOTAC apparatus over the 4-place rotor of the manual centrifuge
- Rotate the handle 60 times/minute for 5 minutes

Manual centrifuge – Equipment needed

Hettich Hand Centrifuge (model 1011) ideal for field research or any situations where electricity is limited. This Centrifuge can be easily clamped to a bench or table – independently of an external power supply. This Centrifuge is available with 4-place rotor for 15 ml conical tubes.

In the FLOTAC equipment, a special adaptor for the centrifugation of 4 FLOTAC apparatus suitable for this centrifuge will be provided.

References

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1.2 - Midi Parasep[®] SF Solvent Free Faecal Parasite Concentrator

The object of the test is to identify nematode eggs (+ *Schistosoma*) in human faecal samples with minimum exposure to samples. The method has only so far been used qualitatively. Its performance for quantitative nematode and schistosome egg counts is under investigation. The optimum way for counting eggs from the concentrator is under investigation

Equipment & material

Balance: accurate to 0.1g

Bench centrifuge: capable of taking 50 ml tubes

McMaster slide

Microscope: dissecting or compound

10% formaldehyde solution: technical grade

Triton X-100.

Saturated sodium chloride: technical grade

Procedure

1 Add 8 mls of 10% formaldehyde to tube and 1 drop of surfactant (Triton X-100). Screw on top, label and weigh.

2 Add about 400 mg human faecal sample to the tube. Reweigh tube.

3 Mix thoroughly then replace stopper with filter thimble and conical tube.

4 Invert tube and centrifuge for 3 minutes at 200xg. Carefully unscrew to avoid forming an aerosol, and pour off supernatant.]

5 Egg counting.

a) add 1 ml of saturated sodium chloride, mix then pipette under a McMaster slide [requires testing to see about clarity of the suspension in the slide – might need to add >1ml. Could be 1ml of saturated sodium chloride plus stain, if relevant. e.g. stored hook worm eggs/*Schistosoma* eggs?]

b) Suck up sample using FE5 series Workstation. Count sample in 2 cells (total volume 80µl)

Data collection and storage

a) McMaster. Using total of 1 ml and McMaster slide. Record numbers and species of eggs, hookworms, *Ascaris*, *Trichuris*. Divide number by weight of faeces e.g. 0.4 if 400 mg. Multiply this count by 3.33 to give total epg.

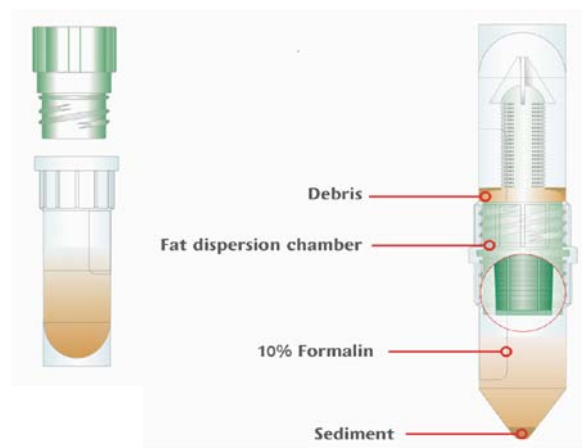
b) FE5 series Workstation. Record numbers and species of eggs, hookworms, *Ascaris*, *Trichuris*. Divide number by weight of faeces e.g. 0.4 if 400 mg. Multiply this count by 12.5 to give total epg. Recent studies have shown that where known numbers of nematode eggs are present the counting procedure is quantitative (unpublished).

Problems

The use of the system for quantitative eggs counts is being examined. Currently used widely for qualitatively for diagnosis where a positive sample means treatment. The veterinary version has been re-engineered to give same results as improved modified McMaster..

Diagram - Midi Separator

a) sample collection b) after centrifugation

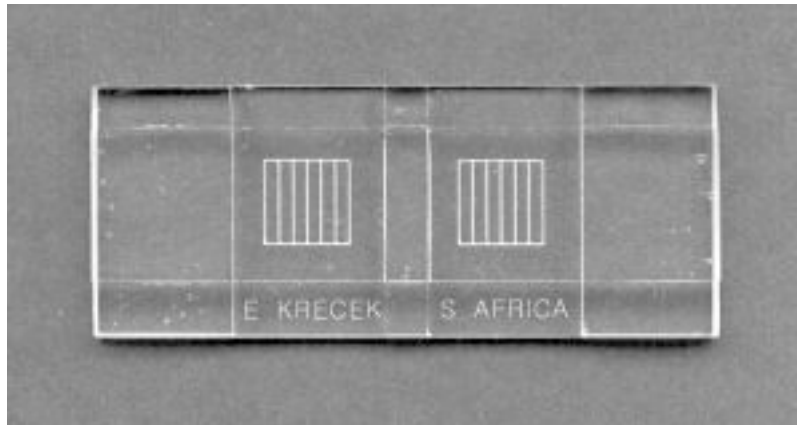


1.3 McMaster technique

The McMaster technique uses a counting chamber which enables a known volume of faecal suspension (2 x 0.15 ml) to be examined microscopically. Thus, if a known weight of faeces and a known volume of flotation fluid are used to prepare the suspension, then the number of eggs per gram of faeces (FEC) can be calculated.

The quantities are chosen so that the faecal egg-count can be easily derived by multiplying the number of eggs under the marked areas by a simple conversion factor.

The McMaster chamber has two compartments, each with a grid etched onto the upper surface. When filled with a suspension of faeces in flotation fluid, much of the debris will sink while eggs float to the surface, where they can easily be seen and those under the grid counted.



Equipment List

- * Two beakers or plastic containers
- * Balance
- * Tea strainer, cheesecloth or dental napkin
- * Measuring cylinder
- * Stirring device (fork, spatula, tongue depressor)
- * Pasteur pipettes and rubber teats
- * Flotation fluid (choice of solution dependant on species expected to be present and availability of reagents)
- * McMaster counting chamber
- * Compound microscope

Procedure

1. Weigh 4 grams of faeces in a plastic beaker (100 ml).
2. Add 60 ml of a saturated salt + sucrose solution (NaCl 333 g, sucrose 200 g in 1L of aq dest).
3. Homogenise and pour the faecal suspension 3 times through a tea strainer to withhold the large debris.
4. Homogenise the filtrate by pouring it 10 times from one beaker to another and fill up one side of a regular McMaster counting chamber by using a pasteur pipette. Repeat for the other side.
5. Allow the counting chamber to stand for 2 minutes, place under a light microscope and examine using a 100x magnification. All the eggs under the two separate grids are counted (this represents a volume of 2x 0.15 ml)
6. The number of eggs per gram of faeces is obtained by multiplying the total number of eggs or oocysts under the two grids by 50. The sensitivity of the test is 50.

1.4 Kato Katz technique on double samples

Materials and reagents

1. Applicator sticks, wooden.
2. Screen, stainless steel, nylon or plastic 60-105 mesh.
3. Template, stainless steel, plastic, or cardboard . Templates of different sizes have been produced in different countries. A hole of 9 mm on a 1 mm thick template will deliver 50 mg of faeces; a hole of 6 mm on a 1.5 mm thick template, 41.7 mg; and a hole of 6.5 mm on a 0.5 mm thick template, 20 mg. The templates should be standardized in the country and the same size of

templates should always be used to ensure repeatability and comparability of prevalence and intensity data.

4. Spatula, plastic.
5. Microscope slides (75 x 25 mm).
6. Hydrophilic cellophane, 40-50 mm thick, strips 25 x 30 or 25 x 35 mm in size.
7. Flat-bottom jar with lid.
8. Forceps
9. Toilet paper or absorbent tissue.
10. Newspaper.
11. Glycerol-malachite green or glycerol-methylene blue solution (1 ml of 3% aqueous malachite green or 3% methylene blue is added to 100 ml of glycerol and 100 ml of distilled water and mixed well). This solution is poured onto the cellophane strips in a jar and left for at least 24 h prior to use.

Procedure

1. Place a small mound of faecal material on newspaper or scrap paper and press the small screen on top so that some of the faeces are sieved through the screen and accumulate on top.
2. Scrape the flat-sided spatula across the upper surface of the screen to collect the sieved faeces.
3. Place template with hole on the centre of a microscope slide and add faeces from the spatula so that the hole is completely filled. Using the side of the spatula pass over the template to remove excess faeces from the edge of the hole (the spatula and screen may be discarded or, if carefully washed, may be reused).
4. Remove the template carefully so that the cylinder of faeces is left on the slide.
5. Cover the faecal material with the pre-soaked cellophane strip. The strip must be very wet if the faeces are dry and less so if the faeces are soft (if excess glycerol solution is present on upper surface of cellophane wipe with toilet paper). In dry climates excess glycerol will retard but not prevent drying.
6. Invert the microscope slide and firmly press the faecal sample against the hydrophilic cellophane strip on another microscope slide or on a smooth hard surface such as a piece of tile or a flat stone. The faecal material will be spread evenly between the microscope slide and the cellophane strip. It should be possible to read newspaper print through the smear after clarification.
7. Carefully remove slide by gently sliding it sideways to avoid separating the cellophane strip or lifting it off. Place the slide on the bench with the cellophane upwards. Water evaporates while glycerol clears the faeces.
8. For all except hookworm eggs, keep slide for one or more hours at ambient temperature to clear the faecal material prior to examination under the microscope. To speed up clearing and examination, the slide can be placed in a 40°C incubator or kept in direct sunlight for several minutes.
9. *Ascaris* and *Trichuris* eggs will remain visible and recognizable for many months in these preparations. Hookworm eggs clear rapidly and will no longer be visible after 30-60 minutes. Schistosome eggs may be recognizable for up to several months but it is preferable in a schistosomiasis endemic area to examine the slide preparations within 24 hours.
10. The smear should be examined in a systematic manner and the number of

eggs of each species reported. Later multiply by the appropriate number to give the number of eggs per gram of faeces (by 20 if using a 50 mg template; by 50 for a 20 mg template; and by 24 for a 41.7 mg template). With high egg counts, to maintain a rigorous approach while reducing reading time, the Stoll quantitative dilution technique with 0.1 mol/litre NaOH may be recommended (see *Basic laboratory methods in medical parasitology*, WHO, 1991)..

1.5 Formol-ether Concentration Technique.

SOP not available

Annexe 2 – FECRT Draft SOP

1. Explain the objectives of the trial to the people of the communities that have been selected for treatment and obtain agreement of village/community elders or heads of families.
2. Ensure that all local ethical rules are adhered to (preferably of an international standard), and that informed consent has been obtained from all subjects to be examined and/or treated, and if they are young children from their parents or guardians.
3. Record as much detail about each subject participating in the trial as is culturally and ethically acceptable (e.g. sex, age, racial group, family in village, household, occupation, religion etc.). This is usually done at registration for the trial.
4. It is most important to register whether each subject has been treated recently with any medicine (including local ethno-medical herbs/foods), but particularly anthelmintics.
5. At this meeting dispense suitable containers (small buckets, plastic bags etc.) for each subject, pre-numbered with a unique reference. For added security to ensure that no confusion is generated subsequently) these contained should also have the name and household number of the subject (although for cultural/ethical reasons this may not always be possible). Note that there may be no selection at this stage, all willing participants should be invited to join.
6. Survey those who agree to participate first for presence/absence of helminths and record quantitative data on eggs per gram of faeces of each subject for each species detected in the community.
 - a. Collect individual faecal samples (3-5 g). This is usually done overnight, with people bringing their samples in the morning, or leaving them in a prearranged location in the village (e.g. a hut/house).
 - b. Count using a standardised technique (still to be decided) as soon as possible after collection.
 - c. Only store at 4°C for 24 hours if using samples for culturing (to differentiate *Ancylostoma*, *Necator* and *Oesophagostomum*).
7. Arrange into treatment groups using random numbers, but do so within sex and age matched cohorts to ensure that both sexes and all age groups are present in each treatment group.
8. At the very least two treatment groups should be included, one treated with the drug the other no treatment. If numbers permit increase the number of treatment groups by using drugs that have different mods of efficacy.
9. Placebo groups may be treated with vitamins or inactive components that are formulated to resemble exactly the formulation of the active drugs.
10. In randomised, double-blind, placebo-controlled trials (the gold standard of clinical trials), the drugs should be coded, with each drug, including placebo, being available under a range of codes (not just one code for each drug). Those administering the treatment in the field should have no a priori knowledge of which code corresponds to which drug. Those analysing the data should not be involved in the field work other than to initially allocate treatment codes to subjects by random means within single sex, age stratified cohorts. The treatment codes should only be broken once the statistical analysis has been completed.

11. Select individuals who were positive for helminth eggs at survey 1 (it is accepted that for local, diplomatic, other reasons some uninfected individuals may need to be included)
12. Choose schoolchildren 8 to 14 of age if *Ascaris* and *Trichuris* are the helminths of concern. Hookworm (*Necator*) infections are heaviest in adults so all members of the community will need to be included, perhaps excluding only the youngest age groups. If *Ancylostoma* is present the youngest age groups must be included since this species can be transmitted in milk and infections can be heavy in neonates.
13. Use a minimum of 20 subjects per group if possible, although larger sample sizes such as 50 or even 100 increase the power of subsequent analysis.
14. Individually weigh children (or other technique to estimate weight) and give manufacturers recommend dose orally. Confirm that each individual has swallowed the drugs in your presence. **DO NOT DISPENSE THE DRUGS ON THE PROMISE THAT THEY WILL BE TAKEN LATER**
15. Collect a 2nd faecal sample between one and two weeks after treatment. For each subject record the dates accurately so that any variation in the period from treatment to second faecal analysis can be taken into account in evaluation of the results.
16. During the collection of the second sample enquire whether the subjects have taken any other forms of treatment since the administration of the anthelmintics being assessed
17. All the data should be stored as hard copies in suitable ledgers or on cards, and transferred to computer databases or spreadsheets as soon as possible. Once completed, all data should be triple checked for consistency with field notes and hard copies. It should then be backed up to avoid the risk of loss.
18. Once this is complete, the analysis and evaluation can commence.
19. Analysis is best done by individuals who were not involved in the field work and who have no knowledge of the identity of the coded treatment groups. The code identity should only be broken once the analysis has been completed.
20. Interpretation of results: still to be decided

Annexe 3 – calculation of drug efficacy

Annexe 3a – Procedures described by Sacko et al (1999) to calculate efficacy

Procedure 1 - The cure rate, which represents the number and percentage of individuals who were positive for hookworm eggs at the pre-intervention survey but showed no hookworm eggs at the post-intervention survey.

Procedure 2 represents the mean of the change in EPG at the individual subject levels between the pre- and postintervention surveys, thus for each subset of data

$$\left\{ \sum_{i=1}^{i=n} [(T1_i - T2_i)] \right\} / n \text{ where } i = i \text{ th subject.}$$

Procedure 3 represents the mean percentage change in EPG at the individual subject levels between the pre- and post-intervention surveys, thus for each subset of data

$$\left\{ \sum_{i=1}^{i=n} [(T1_i - T2_i) / T1_i] \times 100 \right\} / n.$$

Procedure 4 is based on percentage change in mean EPG for each subset, thus $\{T1 - T2\} / T1 \times 100\%$.

Procedure 5 (COLES et al., 1992) is based on arithmetic means and calculated as $1 - \{T2/C2\} \times 100$.

Procedure 6 (PRESIDENTE, 1985) is based on geometric means and calculated as $(1 - \{T2/T1 \times C1/C2\}) \times 100$.

Procedure 7 (DASH et al., 1988) is based on arithmetic means and calculated as $(1 - \{T2/T1 \times C1/C2\}) \times 100$.

$T1$ = EPG at pre-intervention survey, $T2$ = FEC at postintervention survey, $C1$ = FEC in placebo group at preintervention survey, $C2$ = FEC in placebo group at postintervention survey.

Sacko, M., De Clercq, D., Behnke, J.M., Gilbert, F.S., Dorny, P., Vercruysse, J., 1999, Comparison of the efficacy of mebendazole, albendazole and pyrantel in treatment of human hookworm infections in the southern region of Mali, West Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93, 195-203.

Note - A formula proposed by prof P. G. Smith statistician of the LSHTM was published in Albonico et al. (1994) A randomised controlled trial comparing Mebendazole 500 mg and Albendazole 400 mg against *Ascaris*, *Trichuris* and the hookworms. *Transactions of the Royal Society of Tropical Medicine*. 88, 585-589)

Procedure 8

$$\text{ERR: } 100[1 - \exp(-D)]\%,$$

where D is the mean difference for a particular treatment. The difference between pre and post intervention is calculated for each individual and D is the mean (geometric) of the differences.

Annexe 3b – Calculation of drug efficacy (Vidyashankar et al., 2007)

Vidyashankar et al. (2007) showed recently that data transformation led to a spurious increase in power that resulted in higher Type I error rates (decreased accuracy). Also – they found that taking variance into account was important, something that is not done with the procedures typically used and listed in Annexe 3a. As described in the model, modelling and bootstrapping appear to offer the best approach. The impression is that mathematicians/statisticians/modellers can disagree on the best modelling method, but ultimately, it probably matters little when compared to not using a modelling method. Meaning that any of the models with bootstrapping that are chosen will be far superior to simple arithmetic calculations that are usually done. Some good real data sets together with simulated data sets that are produced based on analysis of the real data need to be analyzed using these models to determine which is/are the best approach(s).

Model

Fecal egg count data exhibits variability due to subjects, time, geography, dose, parasites, and the treatment. In the work on fecal egg count reduction in horses, Vidyashankar, Kaplan, and Chen (2007) have recently shown that by not accounting for variability and trend, a scientist may either over estimate or under estimate resistance by a substantial percentage.

Making matters even more difficult, most of the problems that confound the ability to make an accurate inference on resistance in veterinary parasites are magnified in humans. Human subjects in a community are more heterogeneous than are animals on a farm, isolates of human parasites are likely to be much more heterogeneous than are isolates of veterinary parasites, and observed levels of worm egg reduction following treatment of drug-susceptible parasites in humans is much more variable. Unlike in the veterinary situation where anthelmintic drugs administered to animals harbouring drug-sensitive worms consistently yield greater than 99% percent reduction in egg counts following treatment, in humans, egg reductions frequently yield a wide variability in response (see Table 2). Consequently, significant statistical problems arise in making accurate clinical inferences on levels of observed egg reduction. These issues make it very difficult to assign a precise definition of resistance in terms of percent reduction. Likewise, it is very difficult to determine appropriate “cut-off” values for establishing a diagnosis of resistance or suspected resistance in human STH. Because of these important differences in the nature of the data between human and veterinary gastrointestinal helminths, a “cut-off” approach as is used in veterinary medicine is likely not the most appropriate method to use in humans.

We therefore suggest the following methodology to evaluate egg reduction and resistance in human STH. Using historical data and the detailed data concerning egg counts we will develop appropriate confidence intervals for expected percent worm egg reductions for a range of drugs, drug dose levels and worm species combinations. These confidence intervals will be model based and take into account various sources of variability and trends. A practical outcome of these analyses will be the development of a Table of Confidence Intervals that can be made available to field workers.

A second level of analysis will be to use the historical data to analyze which of the 7 procedures listed above yield accurate results in terms of Type I and Type II error

rates. One or more of these procedures can then be selected as a standard and used by field workers to calculate the relative change in worm egg counts; namely the percent egg reduction amongst homogeneous subjects. The definition of homogeneous subjects will be made available in the table. The field scientist can then use simple arithmetic calculations (as listed in procedures 1-7) together with the Table of Confidence Intervals made available to him/her, to classify the drug (for a specified drug, at a specified dose level, and to a specified worm species) into one of three categories: likely resistant, suspected resistant, and fully efficacious.

The table of confidence intervals should be updated every year and validity of the model should be evaluated using the cumulative data that is being collected. In addition, as *in vitro* and molecular tests for resistance improve in sensitivity and accuracy, results of these tests can be correlated back to the worm egg count reduction data to further improve the precision of the Confidence Interval Table.

Annexe 3c – Proposed method to evaluate the change in EPG in a population after pharmacological treatment (A. Montresor, WHO Hanoi)

In helminthology, the mean number of eggs per gram of faeces (EPG) is a commonly used indicator for estimating the severity of the infection in a population and the changes that occur after pharmacological treatment.

Mean EPG can be calculated as

- Geometric Mean
- Arithmetic Mean

In my opinion Geometric /Arithmetic Means are not a valid indicator in helminthology because:

1- The calculation of **Geometric Mean** includes a logarithmic transformation that normalizes the variance of the data.⁶ However, the mathematical procedures applied to normalize the variance, reduce the influence of high counts on the mean and therefore result in a problematic interpretation of the results.

2- **Arithmetic Mean** is not distorted by logarithmic transformations and potentially a good indicator. However, Arithmetic Mean does not assure homogeneity of the variance between groups compared; therefore parametric tests can not be used for the statistical interpretation of the results.

In my opinion an useful indication of the changes that occur in the helminth egg counts after pharmacological treatment is given by the Class of intensity (defined by WHO for each of the STH)

The advantages of this indicator are:

- The indicator is **simple to calculate** (very basic mathematical principles are involved in calculation)
- The indicator is **simple to interpret**: the reduction of the number of individuals harbouring large number of parasites is normally the main aim of any the control programme; analyzing the results of a pharmacological intervention by class of intensity give clear indication of the number of heavily infected before and after intervention

Some problem in interpreting the results of interventions using classes of intensity could arise if:

⁶ The homogeneity of the variance among the groups that are compared is one of the essential conditions for the application of χ^2 test to evaluate the significance

- Large EPG changes occur within the same class (e.g. for hookworms: EPG count >10 000 that are reduced by 50% would not result in a change of category for the individual.⁷) are underestimated by the method
- Small EPG changes occur over the threshold (e.g. from 4 100 EPG to 3 900 EPG¹) are overestimated using the method.

Therefore, I suggest that a proper evaluation the change in EPG in a population after pharmacological treatment should be carried out using a combination of two methods:

- **Arithmetic mean** to evaluate, from the public health point of view, the reduction of EPG in the community (and we can establish a level that we consider reasonable: e.g. a reduction of over 50% is certainly clinically valid from a public health perspective).
- An evaluation of the changes in the **class of intensities** (to be able to apply a proper statistical test and evaluate the statistical significance of the reduction).

References

Montresor, A. 2007. Arithmetic or geometric means of eggs per gram are not appropriate indicators to estimate the impact of control measures in helminth infections. *T. Roy. Soc. Trop. Med. H.* 101, 773-776.

⁷ The threshold between moderate and high intensity is 4 000 EPG

Annexe 4 – Egg Hatch Test (after Samson-Himmelstjerna et al. in prep.) (including anaerobic storage of eggs)

The object of the test is to determine whether nematodes eggs are resistant to benzimidazole anthelmintics. To demonstrate the reproducibility wherever possible four replicates per sample should be performed. There are no hazards involved other than that caused by handling of animal faeces. Gloves should be worn.

Eggs with an LD50 greater than 0.1 mg/l thiabendazole are designated as being resistant to benzimidazoles.

Equipment

Screw top bottle, plastic, 100ml

Shaker bottle with about 45 8mm glass balls

Sieve, 200mm, 0.15mm mesh

Collecting bowl

Centrifuge tubes, plastic or glass

Bench centrifuge

Pasteur pipette

Microscope slide

24 well plastic plate

Micropipette, 0-20µl and 1000µl

Macropipette, 1-5 ml

Magnetic stirrer

Microscope, compound or dissecting

Saturated sodium, chloride technical grade

Solutions of thiabendazole - see SOP Gram's iodine.

Dissolve 1g iodine and 2g potassium iodide in 100 ml double distilled water.

CAVE: Do not use tap water for preparation of eggs. Only double distilled or deionised water.

Procedure

1. Anaerobic storage of eggs. To a 100 ml screw top bottle add about 85 ml tap water, 20 8mm glass beads and about 10 g faeces. Screw on the top and shake vigorously for about 1 min to disperse the faecal material. Store at about 20°C for up to 7 days. Do not refrigerate.
2. Homogenize faeces in up to about 200 ml water until all faecal pellets have been broken up. Pour through the 0.15mm aperture sieve and collect the filtrate in the bowl. Pour the filtrate into the centrifuge tubes and centrifuge for 2 min at about 300xg (approximately 1500 rpm on the bench centrifuge). Pour off or gently suck off the supernatant. This step is eventually repeated 2 or 3 times to obtain a reasonable amount of sediment.
3. Transfer the sediment by mixing with saturated salt to a 50 ml centrifuge tube. Agitate the tubes to loosen the sediment then add saturated sodium chloride solution until a meniscus is formed. Add a cover slip (e.g. 32 x 32 mm) and re-centrifuge for 2 min at about 130xg (approximately 1000 rpm on the bench centrifuge). Alternatively, centrifugation can be performed without adding cover slip.
4. Pluck off the cover slip vertically and wash off eggs with double distilled water into a conical 15 ml glass centrifuge tube. Alternatively aspirate a small volume of

- flotation medium from the top of the 50 ml tube. Fill with double distilled water and centrifuge (about 2 min at 300xg).
5. Remove the water resuspend the eggs and count the numbers of eggs by removing aliquots with a micropipette. Adjust to give about 100 eggs per ml. Alternatively count eggs, add the appropriate volume of eggs to give from 150-200 per well and then add the double distilled water to give a final volume of 2 ml
 6. Prepare a stirred suspension of eggs. Eggs must be less than 3 hrs old or stored anaerobically and processed in less than 3 hours. IMPORTANT: Note the percentage of embryonated eggs by examining the state of development of 100 eggs.
 7. First add 10µl of thiabendazole solution to experimental wells to give final concentrations of 0.025, 0.075, 0.1, 0.2, 0.3, 0.5 mg/l (µg/ml) and control. Then add 1990 µl egg suspension. Seal plates with tape to avoid drying out. Incubate for 48 hours at 25°C. Note down the exact time when the incubation started.
 8. Add one drop of Gram's iodine to each well. Count at least 100 of the remaining eggs (dead and embryonated) and hatched larvae. Note down the exact time when the incubation was finished and also note when the counting was done.
 9. If possible count all stages (unembryonated and embryonated egg as well as larvae) per well. Preferably, inverted microscopes should be used.

Results

Calculate the ED₅₀ with programmable calculator allowing for unhatched numbers of eggs in control. Eggs with an ED₅₀ > 0.1 mg/l are indicative of resistance. Record percentage of eggs hatching in 0.2 and 0.3 mg/l. Hatching in these concentrations with an ED₅₀ < 0.1 mg/l may indicate resistance in a minor species of nematode.

Problems

Occasionally eggs do not embryonate and hatch in control wells. The reasons are not certain. Repeat the test. 'Tailing', i.e. small numbers of eggs hatching in high concentrations of thiabendazole indicate resistance in a minor species of nematode. Further research into this is required.

Reference

Coles, G.C., Bauer, C., Borgsteede, F.H., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J., 1992, World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary parasitology* 44, 35-44.

Preparation of thiabendazole (DMSO) solutions for EHT

The object is to make up solutions of thiabendazole for the egg hatch test for the detection of benzimidazole resistance. Avoid skin contact with dimethyl sulphoxide (DMSO). Use balance accurate to 0.1 mg.

Equipment

Balance, accurate to 0.1 mg.
Micropipettes, 0-20 µl and 100-1000 µl
Macropipette, 1-5 ml
Dimethyl sulphoxide (DMSO)
Thiabendazole (Sigma)
Screw top glass vials, 10 ml size

Procedure

DMSO stock solution: Weigh out 50 mg of thiabendazole and dissolve in 5 ml DMSO. It is easier to weigh the quantity put in the tube and adjust volume of DMSO accordingly. Stock solution A= 100 mg/10 ml.

Dilutions.

Final working concentrations 0.05, 0.1, 0.2 , 0.3 and 0.5 µg/ml in the wells of the 24 well plate.

Calculations

10 µl of solution is added to 2ml water. Therefore 10 µl of stocks must contain from 0.1 to 1 µg of thiabendazole = from 0.1 to 1 mg per 10 ml.

i) Dilute stock solution A: Add 1 ml stock solution A to 9 ml DMSO/water. Thiabendazole now 10 mg/10 ml = Solution B.

ii) Make tubes as follows:

Final working concentration

in well µg/ml	Volume B in µl	Volume DMSO/water in ml
0.05	100	9.9
0.1	200	9.8
0.2	400	9.6
0.3	600	9.4
0.5	1000	9

For test add 10 µl of solution to 2 mls water containing eggs. Mix by shaking plate. Refrigerate solution when not in use (DMSO will solidify).

Alternative assay technique: agar-based method (modified from Kotze et al, 2005)

This technique involves the exposure of eggs to various concentrations of thiabendazole impregnated in agar in assay plates prepared under standard conditions. These plates would be distributed for use in the field. The plates consist of serial dilutions of thiabendazole in 2 % agar in the wells of 96-well assay plates. Eggs may be prepared as described above (points 5.1 to 5.6) and then placed onto the wells of the assay plates. The plates are then placed into plastic bags, and incubated for 48 hrs at 25°C.

The assay is then assessed as described above (points 5.8 and 5.9).

Annexe 5 – Semi-quantitative isolation of nematode eggs from stool samples for DNA analysis

Store stool sample in sealed (cliplock) plastic bag with as much air excluded from the sample as possible (anaerobic storage). Keep sample cool (refrigerate) to slow hatching of eggs to larvae. Process stool samples as soon as possible.

1. Add approximately 2 grams of stool to a labelled and tared screw cap container
2. Reweigh to obtain quantity of stool sample. Record weight against sample number
3. Add ISS (see below) at a ratio of ~1:10 (stool:ISS)
4. Homogenize stools by shaking until uniformly suspended. It may be necessary to break up the stool sample with a spatula
5. Filter through cotton gauze to remove course particles
6. Distribute sample in 50 ml conical tubes, filling tubes to ~45mL with ISS solution
7. Centrifuge tubes for 2 min at 1500 - 2300 rpm
8. Discard supernatant
9. Suspended sediment in BW solution to ~ 40 ml, vortex
10. Centrifuged again at 1500 - 2300 rpm for 2 min.
11. Discarded supernatant once again
12. Overlay with 40 ml NaNO₃ saturated solution (if NaNO₃ is not available saturated NaCl could be used. However, NaNO₃ is more dense than NaCl) to top of tube and mix by inversion
13. Centrifuge for 10 minutes at 1000 RPM
14. *Take ~10 ml from top of tubes and pour onto top of filtration apparatus with 75µ mesh
15. *Gently wash top mesh sieve with clean water until all small particle have passed through top mesh
16. *Collect flow through and repeat with 25µ mesh in an identical fashion
17. *Gently repeat wash of 25µ mesh sieve with clean water
18. *Turn filtration apparatus on side and wash eggs off mesh into collection container(s)
19. Spin 1000 RPM for 5 minutes
20. Remove supernatant
21. Resuspend pellet in ~ 1.5 ml 70% ethanol (for DNA extraction) [or ~ 1.5 ml clean water if viable eggs required for in vitro assays], in a 1.5 ml labelled eppendorf (N.B. the alcohol may remove markings on the tube. Attach a tape to the tube with sample number to reduce possibility sample number will be erased. Keep record of sample number placement in rack/box)
22. Suspend eggs and take 20 µl onto slide, mix with a drop of lugols iodine, cover with a coverslip and observe eggs with a light microscope to determine nematode species present
23. Store eppendorf tubes with eggs in alcohol under refrigeration, but not frozen.

* If 25µ mesh and 75µ mesh filters are not available, mix 10 ml from top of tube with ~30 ml clean water, cap tube, mix by inversion. Centrifuge again for 5 minutes at 1500 – 2300 rpm. Discard supernatant. Resuspend pellet in ~30 ml water. Mix by inversion, centrifuge again. Repeat resuspension of pellet and centrifugation 1 – 3 times, until suspension almost clear. Proceed as in 19. above, to storage.

Subject/Sample information

Record:

1. Sample number (this should relate to subject identity, but subject name can be kept confidential)
2. Subject age
3. Location site
4. Date of stool collection
5. Date of last anthelmintic treatment received by subject
6. Number of anthelmintic treatments subject received, if known
7. Infection status if known (1 = nematode infection present; 0 = no eggs detected in stool sample, for each species of nematode – *Ascaris lumbricoides*, *Trichuris trichiura*, hookworms). See below for egg morphology.

Materials

Stock solution of Brij-35:

30% w/v (30 g of Brij-35 in 100 mL of distilled water) (This is how it is supplied by Sigma)

10X Isotonic Saline (10XIIS)

90 gm NaCl (or table salt) in 1 litre water

Isotonic Saline Solution (ISS)

100 ml 10x isotonic saline to 900 ml water

10X Brij-35 Working solution (10XBW)

Add 5 mL of Brij-35 stock solution to 1,000 mL 10X isotonic Saline

Brij-35 Working solution (BW)

Add 100 mL of 10X Brij-35 Working solution to 900 mL water

Sodium Nitrate solution

Dissolve 315 gm NaNO₃ in 950 ml dH₂O

Add dH₂O/sodium nitrate until 10ml of solution weighs 12 g

Sedimentation technique with Brij-35 for qualitative examination (Diagnosis)**Procedure**

1. Add ~20 g (ml) of stool to 50 ml conical screw cap tube
2. Homogenize stools with ISS at a ratio of ~1:10 (stool:ISS) by vortexing until uniformly suspended
3. Centrifuge sample for 2 min at 1500 rpm
4. Discard supernatant
5. Suspended sediment in BW to ~ 40 ml, vortex and centrifuged again at 1500 rpm for 2 min.
6. Discard supernatant once again
7. Suspend sediment with ~5 mL BW, depending on the amount of sediment obtained.
8. Add one drop of the suspension to a glass slide with a drop of lugols iodine
9. Cover with a coverslip and inspect with a light microscope.

Qualitative examination (Diagnosis)

1. Place ~1ml of stool suspension (from 7 above) in a 10 ml conical tube
2. Overlay with NaNO₃ to top of tube and mix by inversion
3. Centrifuge for 10 minutes at 1000 RPM

For immediate examination

Take a drop from the top onto slide, mix with a drop of lugols iodine, cover with a coverslip and inspect with a light microscope

For later examination

Take ~1ml from top of tube and place in a fresh 10 ml conical tube

Add water to 10 ml, mix by inversion

Centrifuge for 10 minutes at 1000 RPM

Discard supernatant

When ready for examination, place a drop of the sediment onto slide, mix with a drop of lugols iodine, cover with a coverslip and inspect with a light microscope

Quantitative count protocol (if required)

1. Collect faecal samples in plastic containers as usual.
2. Take ~2-3 ml sample into green parafix containers. These contain preservative and can therefore be used to check for parasites at a later date.
3. Take ~1 ml by sodium nitrate flotation as usual for rapid diagnosis of samples containing nematode eggs (most likely will have only hookworm)
4. If positive, place a yellow cap conical tube on the scales and record the weight of the empty tube (tare). (Otherwise tare the scales to weigh the contents)
5. Add ~1 ml into the yellow cap conical tube and record the precise weight of the sample.
6. Add sodium nitrate to tube until volume is ~10 ml.
7. Shake vigorously to get a uniform suspension.
8. Using a 1 ml pipettor immediately pipette sufficient volume of faecal solution into the 3 chambers of the McMaster slide (WHITLOCK worm egg counting Chamber). *This step is critical, the suspension must not be allowed to settle before the pipette is used to withdraw an aliquot for loading the slide (the minute the suspension stops being agitated, eggs will float toward the surface and the count will not be accurate). Hold the pipette horizontal while loading the counting chamber (otherwise, especially if not used to loading the chamber, eggs may float to the top of the pipette solution while you are loading the slide).* [If the pipette blocks, it may be necessary to insert a straining step through a sieve or gauze cloth.]
9. Nematode eggs will immediately float to the underside of the top glass plate of the counting chamber.

10. Place chamber on microscope; focus on the lines on the counting chamber (the eggs will be in the same field of vision).
11. Scan slide and count eggs

To determine the number of eggs per gram (epg) of faeces;

Multiply the number of eggs of each type counted in the scanned area of the slide by:

$$\frac{\text{Volume [weight in grams] of faeces sample} + \text{Volume Flotation solution}}{\text{Volume [weight in grams] of faeces sample} \times \text{Measurement volume used}}$$

(x eggs counted/volume of counting chamber scanned, e.g. 0.9mL) X (10mL/weight of faecal sample) [This formula is based on using a 0.3 ml counting chamber. This should be verified and the formula adjusted if the counting chamber has a different volume.]

Eg.: For 1g faeces in 9 ml of flotation solution (1g:10ml), place 1g faeces in 9 ml of Sodium Nitrate flotation solution and measure or count in all three chambers of the Whitlock McMaster slide (0.9ml).

$$\text{epg} = \# \text{eggs} \times (1+9) / (1 \times 0.9) = \# \text{eggs} \times 11.1$$

(in other words, in this instance, multiply the number of eggs counted by 11.1 to give the epg.)

Proportions of *Ascaris*, *Trichuris* and hookworm eggs can be estimated from their individual counts in a sample.

Annexe 6 - Pyrosequencing Assay Protocol for β -tubulin (isotype 1) Phe200Tyr SNPs in nematode parasites of humans (after Diawara et al., 2007)

gDNA extraction

DNA from individual adult worms or pools of eggs was extracted using the NucleoSpin[®] Extraction Kit (Qiagen) according to the manufacturer's protocol.

PCR amplification

A small fragment of DNA (~200 bp) surrounded the codons of interest (e.g. codon 200) is amplified using DNA from an adult worm and/or a pool of eggs. The reaction should contain 5 μ l 10 \times Hi-Fi PCR buffer, 2 μ l [50mM] MgSO₄, 1 U platinum Taq Hi-Fi (Qiagen), 1 μ l of each sense and antisense primers# [20 μ M], 1 μ l dNTP [10 μ M], 1 μ l of template DNA and sterile H₂O up to 50 μ l. The antisense primer is biotinylated at its 5' end (Invitrogen). The thermal cycling conditions should include an initial denaturation at 95°C for 3 min, followed by 50 cycles of 94°C for 45s, 55°C for 45s, and 68°C for 1 min and a final extension at 72°C for 6 min.

Capture of PCR product on beads

Biotinylated PCR products are immobilized on streptavidin-coated Sepharose[™] beads (Amersham Biosciences). A 96-well PCR plate was filled with 10 μ l of PCR product, 40 μ l of binding buffer ((Pyrosequencing[™]), 3 μ l of beads and sterile H₂O up to 80 μ l in each individual well. Using a shaker, the plate is incubated for 15 min at room temperature.

During the immobilization time, in a new 96-well PCR plate 2 μ l sequencing primer# and 38 μ l annealing buffer (Biotage[™]) are mixed.

Strand separation

The three troughs delivered with the Vacuum Prep Tool (Biotage[™] AB) are filled with approximately 180 ml of high purity water, 70% ethanol and denaturation solution (0.2M NaOH)*, respectively. The fourth trough is filled with 120 ml of washing buffer (10mM Tris acetate adjusted to pH 7.6 with 4M acetic acid)[†].

The probes of the vacuum are washed for 30 sec in high purity water, then the beads containing the immobilized template are sucked up by the Vacuum Prep Tool and the template denaturised by washing for 5 sec in 70% ethanol, then in the denaturation solution and finally in the washing buffer. The vacuum is turn off and the beads released in the plate containing the sequencing primers# (Invitrogen).

Primer annealing

The plate is heated at 80°C for 2 min using a PTC-100 programmable thermocycler (MJ Research, Inc), then left at room temperature for 10 min.

Pyrosequencing

Program setup on PSQ 96 MA Pyrosequencer[™] instrument from Biotage[™] AB:

Select "Simplex entries" (to look for only one position). Create a new entry (e.g. "Trichuris position 200"). Enter the sequence to analyse (including the SNP)#. Enter

the pyrosequencing primer sequence#. Choose the parameter “0002”. Activate the program for the microplate rows that you plan to use, and then the amount of each nucleotide (dATP α S, dCTP, dGTP, dTTP, substrate (S) mixture (luciferin, adenosine 5' phodphosulfate) (Biotage™) and enzyme (E) mixture (DNA polymerase, ATP-sulfurase, apyrase) (Biotage™) that need to be loaded in the PSQ™ 96 reagent Cartridge is calculated. The cartridge and the plate are placed into the PSQ 96 MA Pyrosequencer™ instrument from Biotage™ AB. Peak heights are measured and analyzed to determine genotypes by the AQ module in the PSQ™96 Single Nucleotide position Software (Biotage™ AB)

Primers

Species	SNP	1 st stage amplification primers		Sequence to be analyzed (including SNP)	Pyrosequencing primer
		Sense (5'-3')	Antisense (5'-3')		
<i>A. lumbricoides</i>	Phe200Tyr	AGGTTTCTGATG TGGTGTTGGA	TATGTGGGATTT GTAAGCTTCAG	GAGAACACCGATGAA ACCT	GAGAACACCG ATGAAACCT
<i>T. trichiura</i>	Phe200Tyr	AGGTTTCAGATA CAGTTGTAG	CAAATGATTTAA GTCTCCG	GAGAACACGGACGA AACAT	GAGAACACGG ACGAAACAT

Denaturation solution: 0.2 M NaOH

Chemical	Chemical formula	Molecular weight (g/mol)	Final concentration	Grams needed for 1 litre
Sodium hydroxide	NaOH	40.0	0.2 M	8.00

Dissolve chemicals in 950 ml MiliQ water. Fill up to 1L with MiliQ water.

Washing buffer: 10 mM Tris-acetate pH 7.6

Chemical	Chemical formula	Molecular weight (g/mol)	Final concentration	Grams needed for 1 litre
Tris	C ₄ H ₁₁ NO ₃	121.14	10 mM	1.21

Dissolve chemicals in 900 ml MiliQ™ water. Adjust pH to 7.6 with 4M acetic acid at +22°C +/- 1°C. Fill up to 1L with MiliQ water.

Note - Schwenkenbecher J et al., analysed the assay for β tubuline 1 of *Necator americanus* and *Ancylostoma duodenale*. However, Pyrosequencing is a preferred method if we are looking to establish SOPs (Ray Kaplan, personnel communication). This real time PCR method seemed to work well in this study, but as yet is still unproven to be a good test because all tests were negative and it is hard to prove a negative. Until we can test it with known resistant genotypes we cannot properly validate it. In contrast the Pyrosequencing is robust because it tells you exactly what is there and give precise comparative data.

References

Diawara A., Drake, L.J., Suswillo, R.R., Bundy, D.A., Prichard, R.K. (2007) Development of DNA assay in soil-transmitted nematodes of humans for the detection of single nucleotide polymorphisms (SNPs) associated with benzimidazole resistance. *Ann. Meeting Amer. Soc. Trop. Med. Hyg.*, Nov. 4-8, Philadelphia, PA, USA.

Schwenkenbecher J et al., Real time PCR as a tool for monitoring sequences associated with drug resistance in hookworms. *Molecular and Biochemical Parasitology*, 2007 Dec, 156(2), 167-74