

**DRUG RESISTANCE IN HUMAN HELMINTHS:
CURRENT SITUATION AND LESSONS FROM LIVESTOCK**

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Abstract

In this review the available reports on drug resistance in human helminths, particularly hookworms and schistosomes, are critically analysed. The experiences with helminths of livestock are then reviewed, in particular the factors contributing to the development of anthelmintic resistance, the mechanisms and genetics of resistance to various anthelmintic classes, and available methods for detection. These experiences appear to be worryingly similar with and relevant to the potential development of drug resistance in human helminths. Recommendations to reduce its risks are developed.

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1. Introduction

In recent years, several reports of apparent failures in the treatment of human schistosomes and nematodes have been published (33,81,116,132). Although the interpretation and the implications of these studies are still being debated, they have led to an increased awareness of the potential problem of anthelmintic resistance (AR) in the treatment and control of human helminths.

In view of the short but worrying history of AR in livestock, such concerns are not superfluous. At present, AR is the most important disease problem of the sheep farming industry in Australia, South Africa and possibly South America (140,146,147). Twenty years ago, however, many scientists considered drug resistance in helminths of livestock as an unimportant phenomenon. High prevalences of AR, often exceeding 50 %, have now been reported in all parts of the world for gastro-intestinal helminths of sheep, goats and horses kept in industrial livestock systems. Surprisingly, up to now very little problems with AR have been noticed in cattle helminths (58). Table 1 summarises the helminth species and the anthelmintic classes most frequently involved.

Even multiple drug resistance is not uncommon in helminths of veterinary importance. In parts of Paraguay (95) and South Africa (140), resistance is present against all available

broad-spectrum anthelmintics and farmers have started to give up sheep farming because of insurmountable problems with AR (138).

For purposes of discussion, anthelmintic resistance (AR) is defined as a heritable reduction in the sensitivity of a parasite population to the action of a drug. The reduction is expressed as the decrease of the frequency of individual parasites affected by exposure to the drug, compared to the frequency observed in the same population upon initial or prior to exposure (31). Although not unequivocal but generally considered as the most adequate, this definition encompasses two biologically distinct, but not always distinguishable processes: 1) existing drug-tolerant parasite lines may become more frequent, particularly under drug pressure; 2) previously susceptible parasites may undergo genetic mutations, possibly induced by drug exposure, and be selected under drug pressure .

The term tolerance refers to the innate unresponsiveness of a parasite to a drug, independent of prior exposure to that drug or to others belonging to the same class.

In advancing the cause for the wide-spread use of drugs to control human helminths, Cerami and Warren (20) believed that "helminths are less likely to develop resistance or would do so more slowly" as compared to other infectious agents because they multiply at a slower rate. This assumption has certainly not appeared valid in the case of livestock helminths, justifying caution in human helminths as well. AR may not be a medical problem yet, but for all we know the few reports so far may represent only the tip of an iceberg. Veterinary experiences have shown that the problems becomes apparent only when it is too late and reversion to susceptibility is no longer possible (31). Individual treatment failures may often remain unnoticed, as most helminth infections lead only to subclinical disease. Epidemiologically, there have been few efforts so far to examine or monitor the problem. The development of drug resistance, and AR in particular, usually follows a sigmoidal pattern: a long period of incubation with only a few, scattered cases is followed by a sudden explosion of the problem (145). Once AR becomes apparent, it may very quickly become a major problem in both clinical and preventive medicine.

For more than a decade veterinary researchers have drawn the attention of the medical community to the risk of AR development in human helminths, such as schistosomes and hookworms (26,28,62,128). Drawing from the lessons and errors in their own field, they urged medical workers to use anthelmintics more carefully in order to avoid or at least to

delay the development of AR. Nevertheless, the wide-spread drug use for the control of schistosomiasis, onchocerciasis and geohelminths has been increasingly advocated by scientists and international organisations, with drug companies willing to offer assistance (1,17,113,150).

In light of these issues we will critically review the available evidence of resistance to drugs of human helminths at present, and discuss the prospects for the future taking into account the veterinary experiences.

2. Reports on drug resistance in human helminths: a critical analysis

Early reports on possible resistance to santonin in *Ascaris lumbricoides* (86) and diethylcarbamazine (DEC) in *Onchocerca volvulus* (143) were not well documented and cannot be assessed as to accuracy and relevance.

We will concentrate here on the more recent and better documented reports on drug resistance of human nematodes (hookworms) and trematodes (schistosomes). AR of human cestodes has not yet been reported. Also, livestock cestodes do not seem to develop drug resistance easily; only a single report of drug resistance in tapeworms of sheep (*Moniezia expansa*) has been published (144).

2.1. Drug resistance in nematodes.

2.1.1. Use of anthelmintics

The main drugs used to treat human nematodes nowadays are mebendazole, albendazole, pyrantel pamoate and levamisole for intestinal nematodes, ivermectin for onchocerciasis, and DEC alone or DEC/albendazole and ivermectin/albendazole combination treatments for filariasis (1,35,153). Depending on local epidemiology, availability and cost, these drugs have been widely available in most health care systems for the curative treatment of clinical cases for many years.

In addition the use of anthelmintics is now being strongly advocated in a preventive, population-based way as well (1,17,113,150,154). It is estimated that some 1.3 to 2.0 billion people in the world suffer from helminth infections. Although direct mortality is low, intestinal helminth infections are believed to contribute to "general morbidity". Both intestinal helminths and schistosomiasis have been associated with anemia, stunted growth, poor nutritional status, reduced physical and intellectual abilities (17,18, 150); onchocerciasis has been associated with severe itching, skin diseases, poor health and even reduced chances for marriage. By providing single dose anthelmintics on a regular basis to entire populations or high risk groups (such as school children and pregnant women) it is hoped to reduce both morbidity and

transmission. It has even been proposed to combine albendazole, ivermectin and praziquantel at a low dose in a single tablet and to distribute it to virtually all school-aged children in the developing world (148,149). The proponents of these strategies recognise the risk of emergence of drug resistance, but usually judge it to be insignificant.

As mentioned, the veterinary experiences dictate otherwise. The recently published reports on drug resistance in human helminths must thus be taken seriously, yet examined critically.

2.1.2. Problems of defining drug resistance in hookworms

It should first of all be noted that complete cure of hookworm infection (and most other helminths for that matter) is usually not achieved with any drug. Depending on the dosage and the coprological method applied (with lack of standardization and control methods being a noteworthy problem), cure rates as low as 61% (400 mg) and 67 % (800 mg) for albendazole, 0% (single dose) and 23% (repeated dose) for levamisole, 30 % (single) and 37 % (repeated) for pyrantel pamoate, 27 % for thiabendazole, 19% (single) and 45% (repeated) for mebendazole have been reported (35,88).

Thus, at least some hookworm populations show some degree of (innate) tolerance to at least one of the drugs currently in use. The different susceptibility between the two species *Ancylostoma duodenale* and *Necator americanus* is well established. Most probably, also within the species the degree of tolerance varies regionally, even locally.

Secondly, results of field trials critically depend on the coprological methods used. The number of hookworm eggs per gram (EPG) measured by the Kato-Katz method, commonly used for schistosomes, is unreliable if not strictly standardised. This method consists of measuring 25 to 50 mg of sieved stools in a punched template after which the sample is allowed to clear with glycerin. As hookworm eggs tend to dissolve quickly and uncontrollably, the slides must be examined within 30 to 60 minutes (99,110). In the field, however, Kato slides are often difficult to read, unless the thick faecal smear has been allowed to clear for at least several hours, particularly

when the faeces are hard or dark, or when quantities over 25 mg are examined such as in the commonly applied Kato-Katz technique (83,106,109,131). In order to quantify hookworm eggs correctly, and certainly to compare the number of EPG between individuals or groups or in time, the method must be strictly followed. Qualitative methods, such as ZnSO₄ flotation or Ridley's formol-ether concentration, allow only semi-quantitations at best. The most sensitive method, stool cultures, is laborious and also only semi-quantitative. It is noteworthy, however, that the few therapeutic trials in which this method was applied have resulted in considerably lower cure rates than reported with other methods, and this for most of the drug regimens in use (88). Finally, even correctly measured egg counts or EPGs must be interpreted cautiously, as they are only an indirect measure of worm counts (the actual outcome indicator of transmission and treatment), and subject to inter- and intra-individual variations (38,74).

Contrary to veterinary helminthology, in which methods and cut-off values to define AR are well established and standardized (27), there are no such guidelines in human helminthology. *In vitro* methods for the biological confirmation of AR have not been developed or validated for human nematodes.

Also the local endemic situation and the timing of the follow-up are of paramount importance in tests for the detection of AR, and this in varying ways for different species and drugs. In endemic situations, people and particularly children who were cured are reinfected quickly and may reach the pre-treatment level of infection within a few months. Moreover, they may carry prepatent infections which are affected by some drugs but not by others such as mebendazole, which is hardly absorbed.

Therapeutic trials for human helminths demand rigorous statistical methods, as the worms are overdispersed (i.e. a large number of the worms being present in a small proportion of the hosts) within a population, due to physiological, immunological, ecological and behavioural factors. Study and control populations must therefore be large enough, randomly selected, and upon analysis any cluster bias must be excluded. A few "wormy" people in one or other group may lead to fatal flaws in the analysis of

the results (3,18). Clearly, lack of validated methodology and reference data, many confounding factors and the complex statistics complicate the interpretation of low drug efficacy.

2.1.3. Reports of drug resistance in hookworms

Two recent publications have invoked AR as the probable cause of failure of anthelmintic treatment of human hookworms. Both are community-based studies in field conditions, not clinical observations. De Clercq et al. (33) described a failure of mebendazole to treat *N. americanus* in Mali, whereas Reynoldson et al. (116) reported poor efficacy of pyrantel pamoate against *A. duodenale* in North West Australia. The salient features of both reports are summarised in table 2.

The authors mentioned other possible causes of reduced drug sensitivity of the hookworms such as a genetic change in the susceptibility of the local strain of hookworms (i.e. not through selection pressure by the drug) or host factors (such as local diets) which might have altered the pharmacodynamic properties of the drug.

However, some features which were present in one or both localities are suggestive of possible drug resistance. Since regions in Mali and Australia are remote, relatively isolated areas with probably a rather limited influx of infected foreigners, local helminth populations may have been isolated with little dilution or replenishment by (susceptible) helminths from elsewhere. Under these circumstances, AR would develop more rapidly, because the lack of influx of susceptible genotypes (2).

The possible development of resistance to mebendazole in human hookworms (Mali study) would not altogether be surprising, as benzimidazoles are known to be relatively good selectors of AR (8,118). In helminths of livestock, resistance to the benzimidazoles has appeared quickly and spread easily (31). On the other hand, the drug pressure in the Mali community was not especially noteworthy, as far as data are available (no history of previous mass treatments).

Pyrantel/morantel resistance in livestock helminths mainly developed as cross-resistance due to wide-spread use of levamisole (125). In the Australian study (116), there might be a plausible case for intense pyrantel pressure having led to specific resistance: it had been used for passive case detection as well as active community treatment for decades. Albendazole,

which had not previously been used in this population before, worked perfectly, thereby validating also the methodology.

The hypothesis of drug resistance in the Australian situation was inspired by clinical suspicion of resistance in an area where pyrantel pamoate had been used for a considerable length of time in the community. The reported efficacy of pyrantel pamoate (CR 13%, ERR - 46%) at the given (relatively low and single) dose and for the particular species is below those documented elsewhere, although cure rates as low as 19% have been described (35). The reported ERR is based on Kato slides from a small number of subjects, and may therefore be biased. The study did not include an untreated control group, a necessity for the correct interpretation in light of egg output variations or statistical bias due to aggregation. The follow-up period of 7 days was relatively short and no *in vitro* confirmation was attempted.

In conclusion, the situation and the data are suggestive, but fall short from providing conclusive evidence.

In the Mali study drug resistance was discovered within the context of a research project on schistosomiasis. Since there was no history of intense treatment or clinical suspicion of drug resistance, the local situation was not different from any other endemic area in Africa. Single dose mebendazole treatment is known to be of low efficacy, with a reported CR as low as 18% and ERR as low as 46% (35). Few data are available from Sub-Saharan Africa. So, the low CR and ERR in the treated groups may be due to a general low susceptibility of African hookworms to that drug regimen, as well as to local resistance. Also pyrantel, the control drug used, is known to have little activity against human hookworms (88). Furthermore, the Mali study relied on Kato-Katz slides from “overnight samples that were processed and examined on the same day” (33), which may have led to some overclearing of the slides and consequent underestimation of hookworm egg counts. The four-week interval between treatment and control survey was too long to distinguish treatment failure from rapid re-infection and/or maturing prepatent infections, particularly in a relatively high transmission area and for a drug as mebendazole, that does not affect immature infections.

Both a negative and a placebo group were included, showing ERRs of 37.5% and 32.5 %, respectively. This may be considered as suggestive of the poor efficacy of mebendazole, but also of statistical and methodological bias. The *in vitro* confirmation of the Mali results was based on the egg hatching technique, accepted in veterinary medicine but not yet standardised

for human hookworms. A 50% reduction of egg hatchability was found, as compared to a laboratory strain; it is unclear if this difference is statistically or biologically significant. Strain differences, processing of the field samples, delays during transport, etc., may have affected the results. Again, this study is at best suggestive, but does not provide conclusive evidence for reduced mebendazole efficacy. Afterwards this study has been repeated using a more rigorous study design, in which the efficacy of three anthelmintics (mebendazole, albendazole and pyrantel) against *N. americanus* was compared (121b). The participants were examined 10 days after treatment. After controlling for the drift in the faecal egg counts (opposite trends in male and female subjects) in the placebo treated subset, age, sex, fasting and intensity of infection, single dose mebendazole (500 mg) treatment showed efficacies (ERR) ranging from 60.9 to 89.9 % depending on the method used for the evaluation of the results. The efficacies obtained using albendazole (single dose of 400 mg) and pyrantel (12.5 mg/kg) were ranging from 92.1 to 99.7 and 4.8 to 89.7, respectively (121b). These results are more or less consistent with those reported elsewhere (35,88). It remains thus a matter of conjecture whether pyrantel and mebendazole lack efficacy against *N. americanus* or whether there is a beginning development of resistance.

In conclusion, AR in human hookworms might already occur, but the evidence to date is doubtful. Future studies should be carried out under well controlled conditions and using standardised methods for trial design, calculation of summary data relating to drug efficacies and for statistical analysis, in order to confirm the presence or absence of drug resistance in these or other human hookworms populations (121b). Ideally, clear hypotheses, standard protocols (*in vivo* as well as *in vitro*) and indisputable cut-off values should be established by a governing body and/or multidisciplinary groups of scientists, such as has been the case in veterinary medicine by the World Association for the Advancement of Veterinary Parasitology (WAAVP).

However, the doubts about the reported data should not lead to optimism or complacency. If anything, the critical review of these and earlier data shows that tolerance traits are indeed present in many hookworm populations. Even without taking into account the possibility of mutations, experience in veterinary practice suggests that these traits might quickly and irreversibly become dominant in helminths under drug pressure.

2.2. Drug resistance in schistosomes

2.2.1. Use of antischistosomal drugs

Praziquantel (PZQ) is the most common drug for the treatment of human schistosomiasis (32,89,154) being active against all species (*Schistosoma mansoni*, *S. haematobium*, *S. japonicum*, *S. intercalatum*, *S. mekongi*). In the field and particularly in community treatment, the usual dosage is 40 mg/kg body weight in a single dose; higher dosages or split regimens result in lower compliance (89). In hospitalised patients, particularly for *S. japonicum* and *S. mekongi*, and for heavy infections with the other species, the recommended dose is 2 x 30 mg/kg, up to three times daily (32,35,89). The drug is safe, with few or limited side effects; in heavy infections with *S. mansoni*, acute abdominal cramps and bloody diarrhoea are frequent but always transient. Cure rates with 40 mg/kg are usually between 70 and 90 %, egg reduction rates above 90% (32, 71, 89).

In endemic conditions, re-infection is the rule rather than the exception, particularly in children who are heavily exposed and appear to be (innately or immunologically) more susceptible to infection than adults (72). Nevertheless, when intensity and duration of infection decrease, treatment considerably reduces individual pathology and community morbidity (89,154).

Several brands and generic formats of PZQ are now on the market. Although there is no indication so far that substandard products are a problem (103), some are of unclear origin; it is advisable to select reputed production or wholesale companies complying with international quality control procedures. International competition has brought the initial high price back to about 0.40 \$ per average dose. WHO has therefore recently called for a major effort to bring the drug within reach of all primary health care systems (101).

In several major endemic countries the drug is not only widely available for treatments, but is also being actively distributed in order to prevent or control disease ("morbidity control"). Community-based treatment after active screening, through indiscriminate mass treatment, or in specific target groups is now the major control strategy in Egypt, China, Brazil, the Philippines, and several other countries (89,154). For example all school-aged children and millions of adults are screened and, if necessary, treated every six to twelve months in Egypt. In high-prevalence areas, treatment is now given indiscriminately to the entire population (46). Out of concern for the appearance of drug resistance under such high drug pressure, an elaborate national monitoring system has been set up, in which the stools from apparent treatment failures are referred to regional research centres and submitted to *in vivo* and *in vitro* tests.

Oxamniquine, used in a dosage of 15 to 40 mg/kg, is only active against *S. mansoni*, with cure (>80%) and egg reduction rates (>95%) usually somewhat higher than with praziquantel (71,154). Although by and large a safe drug, oxamniquine may have troublesome side effects in some individuals, such as drowsiness, severe dizziness, seizures. It is mostly used in Brazil and not on the market any more in most of Africa, because of the commercial dominance of praziquantel.

Metrifonate was a third, inexpensive drug available until recently, active only against *S. haematobium*, but it is no longer available for the treatment of schistosomiasis.

Thus, there is presently only one general schistosomicide available, praziquantel. The single available alternative, oxamniquine, is active only against to *S. mansoni*. The emergence of resistance is therefore a frightening prospect, not only for disease control or prevention, but also for curative use in clinical practice.

2.2.2. Reports on resistance to schistosomicides

As for nematodes, it should first be noted that cure and egg reduction rates in therapeutic trials with any drug for human schistosomes rarely reach 100%, even in situations where re-infection is excluded (32,71). Moreover, reported cure rates considerably overestimate real cure rates. Many light infections (with EPGs below the detection limit of the coprological

techniques) that persist after treatment are not detected by usual diagnostic methods but require repeated or very sensitive examinations (37,70). Thus, the recommended doses of schistosomicides should be considered as subcurative (41). In light of these data, it is safe to assume that in schistosome populations, some individual parasites are tolerant to the drug to some degree, at least at the usual dosages.

Unlike for nematodes, robust parasitological methods for the measurement of egg counts are available for schistosomes, such as the Kato-Katz method for faecal eggs and urine filtration for urinary schistosomiasis (83,106,154). Moreover, the detection and quantitation of circulating antigens in blood and urine have added another quantitative tool for the evaluation of drug efficacy (34). On the other hand, day-to-day variation of egg output and antigen levels is substantial - e.g. the coefficient of variation of EPGs in 7 repeated stool examinations varied between 28 and 245 % (50) -, and the relation between worm numbers in the blood and egg counts in excreta is even more indirect and statistically complex than for nematodes (37,70).

Resistance of schistosomes to oxamniquine is undisputably documented, both *in vivo* and *in vitro* (23,25). Epidemiologically, the phenomenon has remained remarkably limited to scattered areas in Brazil. Possibly, the resistance trait is disadvantageous to parasite survival and/or reproduction of schistosomes; also, the mutation may actually be induced by exposure of individual schistosomes to oxamniquine (16). Combined, these factors would explain a self-limiting process even under drug pressure. As the use of oxamniquine is by and large confined to Brazil, and it is being replaced by PZQ, oxamniquine resistance is not considered to be a major problem.

Recent reports on the possible development of resistance to PZQ have generated much more unrest, particularly as this drug is at the basis of current control strategies aimed at the reduction of morbidity through population-based treatment (151,152,154).

The first field report came from a new, intense and epidemic focus in Northern Senegal (72, 132). In a community with extremely high prevalences and intensities of infection, a cure rate of only 18% was observed using PZQ, much lower than usually reported from other (even comparably intense) foci (132). However, egg reduction rates were still over 80%.

Heavy initial infections, intensive transmission, prepatent parasites and immunological naivety were considered the most likely explanations for these low cure rates. The possibility of drug resistance or tolerance could not be ruled out, however. A possible hypothesis was also that in such an epidemic focus, a clonal parasite population may have sprung from a few tolerant worms.

The matter was further investigated in a systematic series of field studies, the results of which can be summarised as follows:

- The low cure rates with PZQ 40 mg/kg (18 to 36%) in the field were confirmed in four more study cohorts, various age- and intensity infection groups, in different seasons, with varying timings of follow-up surveys, and with circulating antigen detection (72,130,137).
- Cure rates remained abnormally low when the dose was increased to 2 x 30 mg/kg (73). Cure rates of oxamniquine at 20 mg/kg in a single dose, however, were normal (84%) (132).
- Cure rates with PZQ 40 mg/kg rose to normal when the treatment was repeated after two to four months, and were also normal in children originating from the endemic area but living in an urban area with no transmission (100,108).
- The efficacy of PZQ could be related to age and pre-treatment intensity, but not to other host factors, including behavioural or immunological parameters (137).
- Application of a statistical model relating egg counts more accurately to worm numbers showed that the poor cure rates could be explained by the initial high intensity of infection, even if over 95% of the worms were killed (36).

The overall conclusion of these observations is that there is no convincing field evidence of reduced susceptibility of *S. mansoni* to PZQ and that the observed low cure rates may be explained by the specific epidemiological situation. Unfortunately, there is no reliable *in vitro* test available to determine PZQ resistance. In fact, a major problem to develop such a test is precisely the lack of a reference schistosome strain that is resistant.

Several experimental *in vivo* studies have recently been conducted in order to unravel the problem in Senegal. In short, these studies have shown that

- It was possible to select from a mixture of *S. mansoni* strains kept for years in the laboratory a parasite population that was almost insensitive to PZQ treatment (51). However, it is probable that this result can be explained by the experimental protocol, in which mice were treated after 35 days of infection. Parasite lines with a slower maturation time would not yet be susceptible to praziquantel at that moment, and selected under drug pressure as a "resistant" strain (22).
- Submitted to the same protocol, a "wild" Senegalese strain appeared to be less susceptible to PZQ (53). Remarkably, this observation was not consistent with the high ERR observed in the field indicating reduced susceptibility at most. Again, it is quite probable that the result was an artefact, due to the early treatment of the infected mice. Subsequent studies with experimental treatment after 60 days of infection showed a markedly improved efficacy, albeit lower than in other geographical strains (52).
- In another laboratory, schistososomes isolated from Senegalese patients that had undergone several treatments but still (or again) excreted eggs did not show any reduction in susceptibility to PZQ (21,22).

The consistent field observation of low cure rates with PZQ can apparently be explained statistically by the high initial worm burdens and possibly heavy immature infections (against which praziquantel is not very effective) in combination with the inherent limits of the diagnostic system. Biologically, this hypothesis is supported by the high levels of circulating antigen (indicating heavy infections), and the results of repeated treatments and treatment in non-endemic areas, which gave normal cure rates. The "normal" results with oxamniquine can statistically be explained by a somewhat stronger inherent schistosomicidal effect.

The results of the mouse experiments are conflicting; the only methodologically indisputable observation on reduced susceptibility is the geographical strain difference (52). Although geographical differences in drug susceptibility have not been described for PZQ, they are well known for hycanthon and oxamniquine, leading even to region-specific dosage recommendations (4,32).

If anything, these studies lead to the conclusion that only a very substantial reduction in susceptibility can be detected reliably with current field methods. Laboratory confirmation is still compromised by the lack of standardisation and reference material. The international effort to establish at least some tentative protocols, and to co-ordinate the collection of data and material is therefore most welcome (114, 156).

Other, well documented clinical and experimental reports come from Egypt, an endemic area which, due to extensive drug usage would seem predestined for the appearance of praziquantel resistance. A nation-wide monitoring system was set up to detect and investigate cases in which praziquantel did not lead to cure, even after repeated treatment (9,46). From several dozen cases, largely clustered in one geographical area, parasites were isolated that showed a reduced lower susceptibility in mice and *in vitro* as compared to Egyptian reference strains (9,81, 81a). Again, the lack of standardised methods, particularly *in vitro*, do not yet allow definite conclusions. At the very least, however, the possibility that less susceptible strains are (and possibly always were) present, and emerging more prominently under drug pressure, cannot be excluded (9).

2.3. Conclusions

The recent reports on possible emerging drug resistance in human nematodes and schistosomes do not provide conclusive evidence, neither for the increase of innately tolerant strains nor for the appearance of newly mutated resistant strains. However, they strongly suggest that such tolerant or resistant strains can and do exist, and that these strains may emerge more prominently under drug pressure (hookworm in Australia, schistosomes in Egypt), or under specific circumstances (schistosomes in Senegal). Perhaps even more important, the published studies show that available tools, methods and reference materials are by far insufficient to detect problems of AR in a timely fashion, if at all.

Therefore, we will review in more detail the knowledge of the veterinary world, which has a longstanding experience with anthelmintic drug resistance, and analyse how they can be used to clarify and possibly remediate the situation in humans.

3. Drug resistance in livestock helminths and its relevance for human helminths

As described above, AR in livestock is now a well established fact. Several contributing factors have been identified and studied.

3.1. Contributing factors for the development of resistance

High treatment frequency

Barton et al. (6), and Martin et al. (97,98) have shown in well controlled trials that a high treatment frequency selects for resistance more strongly than less frequent dosing regimens. There is also strong evidence that resistance problems develop more rapidly in those regions where animals are dewormed regularly. Serious problems with AR in *Haemonchus contortus* were reported in some humid tropical areas where 10 to 15 treatments per year were used to control this parasite in small ruminants (42). Drug resistance, however, can also be selected at lower treatment frequencies, especially when the same drug is used over many years. Several authors (7,19,29,59) have reported the development of drug resistance even when only two or three treatments were given annually. This observation is important as similar treatment frequencies are advocated for the control of intestinal nematodes in humans (17,115,148,150).

Mono-drug regimens

Often a single drug, which is usually very effective in the first years, is continuously used until it no longer works. In a survey of sheep farmers in the USA, Reinemeyer et al. (112) found that one out of every two flocks were dosed with a single anthelmintic until it failed. Long term use of levamisole in cattle also led to the development of resistance, although the annual treatment frequency was low and cattle helminths seem to develop resistance less easily than worms in small ruminants (58,61). Frequent use of ivermectin

without alternation with other drugs has also been reported as the reason for the fast development of resistance in *H. contortus* in South Africa and New Zealand (127,139). In this light the frequent and continuous use of single drugs such as albendazole for the control of intestinal helminths, ivermectin for onchocerciasis or praziquantel for schistosomiasis in humans may raise concern. The quickness with which AR to benzimidazoles in livestock nematodes has spread was described above; if similar strategies are to be applied in humans, there is no reason why the same problems would not arise as well.

Because resistance of *H. contortus* in sheep and goats to ivermectin has been widely reported (31), Shoop (127) has warned of the risk of AR problems in the onchocerciasis control programs in Western Africa, which are increasingly based on periodic community-based treatment with ivermectin (113). Although the initial objectives of drug-based control strategies in schistosomiasis and helminthiasis were restricted to the reduction and prevention of disease in humans, they are now also advocated for the control and even interruption of transmission (17,113,155). Two ivermectin treatments per year during a period of at least ten years are recommended to interrupt transmission of *O. volvulus* among humans (155). In countries such as Egypt, active antischistosomal community treatment with praziquantel has been going on for more than a decade already, and will be continued, even intensified in the foreseeable future (46). AR may probably not develop as easily in helminths with an indirect life cycle (having the multiplicative part of their cycle in arthropods or molluscs) as in directly transmissible intestinal helminths. However, given sufficient time intensive treatment strategies such as in Egypt may provide opportunities for resistant strains to appear and/or become dominant.

Targeting and timing of mass treatment

Prophylactic mass treatments of domestic animals have certainly contributed to the widespread development of AR in helminths. Although no data are available from experimental studies, computer models (5) indicate that development of resistance is delayed when 20 % of the flock is left untreated. This approach would ensure that the progeny of the worms surviving treatment will not consist only of resistant worms. Given the well known overdispersed distribution of helminths, leaving part of the group untreated, especially that

carrying the lowest worm burdens, should not necessarily reduce the overall impact of the treatment.

In worm control in livestock, regular moving of the flocks to clean pastures after mass treatment, and/or planning treatment in the dry seasons is common practice to reduce rapid re-infection. However, these actions result in the next helminth generation that consists almost completely of worms that survived therapy and therefore might contribute to the development of AR (128,134). For example, Coles et al. (29) reported problems with AR in the helminths of sheep and goats on some small Greek islands which suffered from extended drought; in contrast, no AR developed under similar management and deworming practices on the mainland.

Contrary to livestock, where mostly 100 % of the animals of the herd or the flock are treated, population compliance is usually less than 80% in community-based mass treatment of humans: people are absent, not interested, ill or pregnant. Often, compliance decreases further after the first few treatments, if only because of the reduction of morbidity. Moreover, populations are often not stable and there may be an influx of neighbouring or travelling communities (47,48). Timing of treatment in dry, low transmission periods has been proposed (154). In some areas of China, synchronised treatment of cattle and humans is applied in the hope of reducing transmission (121a). However, such strategies are difficult to apply, if only because of organisational and logistical problems.

It may be hoped (but not guaranteed) that these typically human factors will delay (but not prevent) the occurrence and spread of AR in humans. However, if regular treatments are focused mainly on school children (intestinal worms) or in isolated communities (onchocerciasis), groups in which participation is well controlled and even reinforced, and in which transmission may occur in a relatively closed ecological system, the situation and risks may be not that different from livestock.

Underdosing

Underdosing is generally considered as an important factor contributing to the development of drug resistance, because subtherapeutic doses might allow the survival of heterozygous resistant worms (128). Several laboratory experiments have shown that

underdosing indeed contributes to the selection of resistant or tolerant strains (43,78). Some indirect field evidence further supports this assumption. Recently, it was shown that the bio-availability of benzimidazoles and levamisole is much lower in goats than in sheep, and that goats should be treated with dosages 1.5 to 2 times higher than sheep (77). For many years, however, sheep and goats were given the same anthelmintic doses. The fact that AR is very frequent and widespread in goats may be a direct consequence. Recent modelling exercises suggest that the field situation of AR is not always as simple (129). Depending on the initial frequency of the resistance alleles, there might be a range of dose levels where underdosing promotes resistance and a range of dose levels where it actually impedes resistance.

Although further research on the impact of underdosing on resistance development is necessary, current knowledge advises against the use of subcurative dosages. In order to reduce the costs of anthelmintic treatment campaigns in developing countries, nevertheless the use of lower dosages than the recommended therapeutic ones has been advocated (150). Such practices should clearly be avoided. As shown above, most of the currently applied anthelmintics are in fact subcurative in at least part of the population. This is considered acceptable in a perspective of morbidity control, but in the long run such strategies may contribute to the development of AR as well.

Underdosing in humans occurs widely in many developing countries. Drugs are commonly shared or used in half or lower the normal doses by cash-strapped families. Furthermore, generic products of substandard quality, repacked and/or reformulated products, and expired drugs are widespread in pharmacies and on general markets. Also, the presence of poor quality drugs has been documented in human as well as in veterinary medicine (104,126,141). Human drugs, especially antibiotics and anthelmintics, are produced by a large number of unlicensed companies all over the world. Quality control of these drugs is usually lacking.

3.2. Mechanisms of drug resistance

3.2.1. Benzimidazoles (BZ)

The best known mechanism of resistance is the one to benzimidazoles. No information

is available about the resistance mechanisms present in BZ-resistant hookworms of man, but veterinary helminthologists have studied in detail BZ resistance of *H. contortus*. The BZ exert their anthelmintic activity by binding to beta-tubulin, which interferes with the polymerisation of the microtubuli. Several authors (8,121) showed that there is an extensive polymorphism of the beta-tubulin gene in susceptible *H. contortus* populations. Roos et al. (121) proved that selection for resistance to BZ is accompanied by a loss of alleles at the locus of beta-tubulin isotype 1. Kwa et al. (91) nicely demonstrated that resistance to BZ is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1 (Phe being replaced by Tyr).

The same mutation was shown to occur in BZ-resistant fungi such as *Aspergillus nidulans* and *Venturia inaequalis* (82,85). The functional importance of this amino acid substitution was shown by heterologous expression of the beta-tubulin isotype 1 (isolated from BZ susceptible *H. contortus*) in BZ-resistant *Caenorhabditis elegans*. Expression of the *H. contortus* gene altered the phenotype of transgenic *C. elegans* from resistant to susceptible. Conversely, when Phe was replaced by Tyr at amino acid position 200 of this gene by *in vitro* mutagenesis, the reverting activity was lost (92).

A second resistance mechanism was identified in some *H. contortus* populations showing higher levels of resistance, and in which a deletion of the beta-tubulin isotype 2 locus was shown (121). However, Beech et al. could not confirm this in other BZ-resistant *H. contortus* populations (8). These authors also showed that changes in allele frequencies rather than novel rearrangements induced by exposure to the drug explained changes associated with BZ resistance. A similar stepwise selection of BZ resistance also occurs in some *Trichostrongylus colubriformis* and *Ostertagia circumcincta* populations (45,68).

Furthermore, Kerboeuf et al. (84) recently provided indirect evidence that P-glycoproteins (P-gp) also play a role in BZ resistance in *H. contortus*. P-gp are involved in multi-drug resistance (MDR) in mammalian tumor cells, *Leishmania* and *Plasmodium*, and in resistance to toxic compounds in *C. elegans*. Rhodamine 123, a P-gp transport probe, associated with the reversal agent verapamil (an inhibitor of multidrug resistance associated proteins), gave significantly higher levels of fluorescence in eggs from *H. contortus* resistant to BZ and IVM, than in susceptible eggs. These results confirm those obtained with biological drug assays using both anthelmintics and verapamil and reinforce the probability of a P-gp like dependent efflux in nematode eggs, which could be involved in resistance to xenobiotics. However, Kwa et al. (90) - using a P-gp gene probe from *H. contortus*, were not able to correlate

polymorphism to any of the (multi)-drug resistances examined in different *H. contortus* populations. It has to be noticed that the DNA used by Kwa et al. (90) was prepared from pooled L3-larvae and not from individual parasites, so no estimates of allele frequencies could be made (2). Since at least 14 P-gp genes seem to be present in *C. elegans*, it is also possible that P-gp other than those characterised by Kwa et al. (90) or MDR-associated proteins might be involved in drug resistance. Blackhall (11) recently found that the same gene, encoding a P-gp, which is responsible for resistance to ivermectin and moxidectin, is also involved in BZ resistance.

As specific BZ resistance seems to be due to similar point mutations in several fungi and nematodes of veterinary importance, it is not unlikely that it would be relevant for resistance in human nematodes as well. Especially since similar molecules are used in human and veterinary medicine, it would be worthwhile to look for the presence of these point mutations in human helminths as well.

3.2.2. Levamisole

Levamisole and the related anthelmintics pyrantel and morantel are cholinergic agonists with a selective action on nematode receptors. The mechanism of resistance to levamisole is not yet elucidated. Sangster (122) thoroughly reviewed the pharmacology of levamisole resistance. It is thought to be caused either by a reduction of the number of nicotinic acetylcholinesterase (nACh) receptors or by a decreased affinity of these receptors for the drug. Hoekstra et al. (79) were able to clone the gene *Hca 1* encoding the nACh receptor from *H. contortus*. Although polymorphism at the amino acid level could be demonstrated, these authors could not find evidence that alleles at this locus were involved in selection for resistance to levamisole. A similar gene *tar-1* was identified on the X chromosome in *T. colubriformis* (157). However, although statistical comparison of allele frequencies from individual male and female worms was consistent with sex linkage of *tar-1*, no correlation was found with levamisole resistance status.

3.2.3. Ivermectin (IVM)

IVM and other macrocyclic lactones affect gastro-intestinal nematodes by starvation

and/or paralysis by opening chloride channels, which are thought to be associated with alpha-subunits of glutamate-gated ion channels located on muscles of the pharynx and, possibly, the somatic musculature (122). Rohrer et al. (117) compared IVM-resistant and susceptible *H. contortus* populations and found that resistance is not due to an alteration in the binding of IVM to glutamate gated chloride channel receptors. Nevertheless, Blackhall et al. (13) did report that one allele of the putative alpha-subunit gene is associated with resistance to the drug.

Recently, Blackhall et al. (12) reported considerable genetic variation of a P-glycoprotein locus in *H. contortus*. In several drug selected strains of the parasite, selection for the same allele was observed. Using different approaches, Xu et al. (158) and Sangster et al. (124) came to the conclusion that P-gp might be involved in resistance to IVM in this helminth species.

Probably other mechanisms of resistance might be present as well, as suggested by Gill et al. (64) and Gill and Lacey (65). The latter described 5 possible types of resistance to IVM in *H. contortus* based on different behaviour in *in vitro* tests (larval development assay and L3 motility tests), different sensitivity to paraherquamide, an anthelmintic with a completely different structure and different binding sites than IVM and different inheritance (in at least 2 of the 5 resistance types). Gill and Lacey (65) also suggested that the mechanism of resistance to IVM might be different from one species of helminths to the other, because the critical events leading to expulsion have been shown to be different, e.g. when *O. ostertagi* is compared to *H. contortus* and *T. colubriformis*. Further research is needed to confirm these observations, of which the relevance to human *O. volvulus* is at present not clear.

3.2.4. Antischistosomal drugs (oxamniquine and praziquantel)

The mechanism of action of oxamniquine is closely associated with its irreversible inhibition of nucleic acid synthesis in schistosomes (23). Based on cross-breeding experiments using susceptible and drug-selected schistosome strains exhibiting stable resistance, Cioli et al. (24) suggested that oxamniquine is not bio-activated in resistant worms, which allow them to survive the drug action. The activating enzyme, which is present in sensitive and absent in resistant schistosomes, seems to be a sulfotransferase.

Up to now there is no clear understanding of the mode of action of praziquantel, which also hampers the elucidation of possible mechanisms of resistance to praziquantel. Redman et al

(111) have reviewed the existing knowledge and consider the praziquantel-induced Ca^{2+} influx across the tegument as vital in the drug's effect. However, the mechanisms leading to this alteration in Ca^{2+} homeostasis are not clear at all (22).

3.3. Genetics of drug resistance

3.3.1. Nematodes

Nematode parasite populations are genetically heterogeneous and thus able to respond to selective pressures, i.a. anthelmintic drugs (67). Widespread drug pressure will favour and select parasite lines carrying tolerance or resistance alleles. The rate at which resistance will spread in the parasite population depends on many factors. One key factor is the proportional contribution that helminths surviving therapy will make to the next generation. This contribution is influenced by the drug pressure (frequency and timing of treatment), the drug efficacy, the gene flow (the introduction of susceptible genotypes from elsewhere), the generation time and fecundity of the worms, the frequency of resistance alleles prior to drug use, the number of genes involved and the dominance or recessiveness of these genes. Since it is quite difficult to set up experiments to examine the influence of these different factors, several mathematical models have been developed to simulate the development of AR in gastro-intestinal helminths (5,63,128,129). Although these models have their limitations and must certainly be interpreted with caution (39), models such as the one of Barnes et al (5) concerning *Trichostrongylus colubriformis* in grazing sheep provide interesting insights. The model allowed up to 3 genes for drug resistance, each with 2 alleles, that were combined independently under random mating. Worms of all genotypes were assumed to be equally fit in the absence of anthelmintic. The initial frequency of resistance alleles in the worm population was assumed to be very low and set on 0.01 %. In order to examine the effect of using either mixtures of two drugs, or rotations of a single one, two independent genes for resistance to two drugs (with a different mechanism of action) were simulated, with resistance being co-dominant and each drug killing 99, 50 and 10 % of worms of homozygous susceptible (SS), heterozygous (RS) and homozygous resistant (RR) genotypes, respectively. The simulations were run for a period of 20 years with treatment being once a year for the ewes and 3 times a year for the lambs. These resulted in little development of resistance when

the two drugs were used together (mixture). Substantial resistance, however, developed for all rotation strategies, annual, 5- and 10 yearly, with slowest development of AR in the annual rotation strategy. Assuming equal initial drug efficacy and equal resistance allele frequency, resistance developed more rapidly if it was determined by a single gene than when two or more genes were involved. Furthermore, resistance evolved fastest when it was dominant, slower when co-dominant and slowest when recessive. When 20 % of the flock was never treated, resistance was delayed at the expense of worm control.

It has to be noticed, however, that this and most other models developed until now are deterministic, ignoring the overdispersed distribution of free living and parasitic helminth stages. Smith et al. (129) used a stochastic model to examine the effect of aggregated parasite distributions on parasite mating probabilities and the spread and maintenance of rare (resistant) genotypes. They concluded that spatial heterogeneity in transmission might be a significant force in promoting the spread of resistant genotypes, at least when infection levels are low.

When modelling exercises are compared with current knowledge of genetics of AR in helminths of livestock, the most striking and alarming observation is the high frequencies of resistance alleles observed in untreated populations of livestock helminths of veterinary importance. Beech et al. (8) analysed individual genotypes of susceptible *H. contortus* before any exposure to benzimidazoles and reported initial frequencies of resistance alleles of 46% and 12 % at the isotype-1 and isotype-2 β -tubulin loci, respectively. Anderson et al. (2) suggested that similar high frequencies associated with ivermectin resistance might occur in unselected lines of the same helminth species. The figures of Beech et al. (8) may be overestimations, but they indicate that resistance alleles in untreated helminth populations of livestock – and maybe also humans - might be much more common than usually assumed in the theoretical models.

Contradictory reports have been published regarding the number of genes involved in AR and their dominance or recessiveness. The available information, mainly on *H. contortus*, has been summarised by Anderson et al. (2). BZ resistance in this parasite seems to be polygenic; at least two, possibly three genes with recessive alleles are involved. Levamisole resistance in *H. contortus* and *Trichostrongylus colubriformis* is probably due to one single major gene or gene cluster, the alleles of which are autosomal recessive for the former and sex-linked recessive for the latter (2). Resistance to ivermectin in *H. contortus* appears to be mediated by

a single gene or gene complex with primarily dominant effects. Ivermectin resistance might thus develop quite fast, as appears to be confirmed by field observations in South Africa where ivermectin resistance in *H. contortus* developed after only 3 treatments (139). Avermectin/mylbemycin resistance is now widespread in *H. contortus* and *O. circumcincta* of small ruminants all over the world, but remarkably not for *T. colubriformis* (123).

Obviously, these veterinary experiences and findings are of considerable relevance to humans. The presence of tolerant strains to anthelmintics in any parasite population has been demonstrated; as far as biological observations and statistical extrapolations allow, the proportion of innately resistant helminths is in the order of percentages (10^{-2}), not of 10^{-3} or less as previously thought. Virtually all strategies proposed and implemented to date for human intestinal helminth control are based on a single-drug approach, without combination or rotation, and at a minimal frequency of once a year for a considerable length of time. Although the situation with livestock is different to that of humans, and the results or simulations cannot be automatically extrapolated, the biological, epidemiological and pharmaceutical similarities are of concern. Research should focus on genetic and related phenotypic similarities with relevance for AR in livestock and human helminths. Modelling and simulation studies, which have been applied to advance the cause for large-scale treatment programs in humans (17,113) should be used to project possible side-effects and AR in particular.

3.3.2. Trematodes

The genetics of resistance of schistosomes to oxamniquine are quite well known, but this is not the case for praziquantel. Contrary to the development of classical drug resistance in helminths, which spreads gradually through a population as a consequence of selection of resistant phenotypes present at low frequency, resistance to hycanthon/oxamniquine appeared universally in the first filial progeny of parasites exposed to the drug (16). This strongly suggests that resistance is induced rather than selected from pre-existing forms (16). The crossbreeding experiments of Cioli et al. (23,25) and Pica-Mattocia et al. (107) have clearly shown that oxamniquine resistance is controlled by a single, autosomal, recessive gene. Resistance to oxamniquine does not appear to spread easily within communities, but rather tends to remain limited to individual cases. According to Cioli et al. (25), this could be

due to a selective disadvantage of resistant schistosomes in the absence of drug pressure. The fact that resistance is induced rather than selected might also contribute to this phenomenon.

Little is known about the genetic or biochemical background of possible resistance to praziquantel. Recently, genetic differences have been demonstrated between a laboratory strain of *S. mansoni* selected for resistance to praziquantel and the parent-susceptible strain (105). Although these authors did not detect any major genomic rearrangements in these strains, they showed that mRNA encoding a fragment of the subunit 1 of cytochrome-c-oxidase was overexpressed about 5 to 10 fold in the resistant strain compared to the susceptible one. Further research is necessary to examine whether a similar phenomenon is also present in field strains suspected of resistance to praziquantel and whether other genes are also differentially expressed in resistant strains of *S. mansoni*.

3.4. Detection of drug resistance

3.4.1. Faecal egg-count reduction test (FECRT)

The most commonly used test to detect problems of anthelmintic resistance is the faecal egg-count reduction test (FECRT), which compares the egg count before and after treatment with an anthelmintic drug. A standardised protocol for the FECRT is available for the detection of anthelmintic resistance in nematodes of veterinary importance (27). In small ruminants faecal samples are taken from two groups of at least 15, preferably young animals, which have been bred on the farm and not treated in the previous 8 to 12 weeks. Animals are randomly distributed into a treatment and a control group. Faecal samples are collected 10 to 14 days after treatment. In order to reduce the workload no pre-treatment samples may be taken; it has been shown that comparing treatment and control groups post-treatment is as reliable as comparing pre- and post-treatment samples. Egg counts are carried out using a standardised McMaster method (27). The mean number of eggs per gram (EPG) in the faeces of the control group should be higher than 150 in order to allow valid comparison. The following formula is used to calculate the percentage reduction of the EPG: $ERR = 100 (1 -$

X_t/X_c), where X is the arithmetic mean EPG and c and t stand for the control and the treated groups respectively. According to the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) drug resistance in helminths of small ruminants is considered as present when ERR <95 % and the lower 95% confidence interval is below 90 %. If only one of both criteria is met, resistance is suspected (27).

This protocol could help guide the development of a standard approach for AR in humans. To start with, the objective is different: in livestock, the test is used as a routine local confirmation of known AR. In humans, the challenge is still to demonstrate that AR exists at all. Furthermore, study populations of humans are much more heterogeneous than animals, there is a loss of compliance in follow-up, sample collection is not evident, individual behaviour (concerning exposure as well as health seeking behaviour) can have an important impact on the test parameters. Finally, the infecting worm species are different and require other coprological methods.

Taking into account the methodological problems experienced in the past in defining drug resistance in human helminths (see 2.1.2.), possible elements for a standard protocol to detect AR in humans under field conditions could be:

Study groups

Studies to confirm suspected drug resistance, particularly for a compound for which this has not yet been convincingly reported, should include at least a treatment group (with the compound under study) and a non-treated group (possibly placebo). Preferably, also a "positive" control group, treated with another, non-related and presumably efficacious drug should be included. Drugs used should be of undisputable origin and quality, and adequate dosages should be used, i.e. those recommended for clinical use, not subcurative doses applied for community-based morbidity control with individual dosages adapted to actual body weight. The tablets must be swallowed under direct observation; particularly in young children appropriate syrup or suspension formats should be used. People who vomited or have severe diarrhoea shortly after treatment should be excluded from the cohort. Apart from toxicity reasons, pregnant women and people with systemic illnesses should also be excluded as pharmacological and immuno-parasitological dynamics may be disturbed.

Pharmacodynamical studies are not essential from the start, but should be conducted before conclusions about drug resistance are made.

Sample sizes should be determined using a statistical power analysis based on a quantified hypothesis; i.e., for each tested anthelmintic a "Normal" and an "Abnormal" CR and ERR should be defined beforehand. As is made clear by the above review, there are currently no generally accepted "Normal" rates. An international concerted action to determine reference data would be useful.

Study group composition must be statistically non-different for age, sex ratio and pre-treatment mean egg count, and this regarding averages as well as distribution. Children and adults should in any case be considered as different populations. Other possible confounding factors which may lead to differential exposure patterns such as e.g. socio-economic class, occupation, school attendance, religion, must be avoided as well. The groups should ideally be selected from one more or less homogeneous population (e.g. one village) and studied simultaneously, in order to avoid spatial and temporal variations of transmission. None of the study subjects should have received treatment with the drug or a related compound in the previous three (nematodes) to twelve (schistosomes) months, as such subjects may be in the process of "rebuilding" their parasite load.

Given these requirements, and the unavoidable drop out of study subjects, initial sample sizes should probably be not less than 50 children or adults in each study group, if only to validly test the distribution pattern of the egg counts.

The pre-treatment egg counts should be sufficiently high to allow meaningful statistical interpretation, taking into account the detection level of the coprological method.

Obviously, all ethical conditions must be met: fully informed consent of subjects and/or their parents; treatment of negative controls immediately after follow-up, or earlier if clinically necessary; monitoring and management of side effects; clearance of local and national health authorities.

Parasitological methods

A standardised egg counting technique should be used to determine individual egg counts. In the case of schistosomes, *Ascaris* and *Trichuris*, the Kato technique can be used in

a standard way, as described by Katz et al.(83), Peters et al. (106) or Polderman et al. (109). Slides should preferably be stored for later reference and quality control.

For hookworms, utmost care must be taken to validate and standardise the Kato technique. Martin and Beaver (99) recommended reading the slides after 30 minutes, and not later than after 60 minutes. This was based on only a few clinical samples, however. In the field, stool consistency and transparency can vary widely between individuals and communities. In any case, Kato-slides based on stool samples of more than 25 mg, such as the standard Kato-Katz, can hardly be read after only one hour (106) and are thus not suitable for standardised, quantitative hookworm research. Reading all slides within a narrow window of time after preparation requires a rigidly organised and supervised field set-up. Ideally, an adapted Kato technique in which hookworm eggs are preserved, or alternative methods comparable to the veterinary FECRT should be developed. There is a great need for the development, optimisation and validation of a standard protocol, without which further field studies on AR in hookworms will remain severely handicapped.

Faecal helminth egg counts show strong day-to-day, inter-individual and intra-individual variations, both for nematodes and schistosomes (50,69,75). To obtain somewhat accurate schistosome egg counts at the individual level, a minimum of three stool samples must be examined (49,50).

If the focus is on cure rates (e.g. in order to establish fully curative doses), then the most sensitive coprological (qualitative) methods should be used in conjunction with the quantitative ones, such as glycerine sedimentation for schistosomes, and cultures for hookworms (7a). The latter is anyway essential in at least a sub-sample to determine the exact species involved.

The statistical interpretation of mean egg counts is complicated. Scientific accuracy demands the use of models which relate the egg count to worm burdens, the underlying outcome parameter of treatment. Direct use of EPG assumes a proportional relationship, which is far from the biological and statistical truth. Practical statistical tools to that end are not readily available, and have so far only been developed for schistosomes (38). For simplicity, mean EPGs can be used for a first and crude analysis and can be sufficient to reject the hypothesis of resistance. As shown by the Senegalese experiences with PZQ, however, more sophisticated analysis is essential before drawing definite, and far-reaching conclusions.

In veterinary science, arithmetic mean egg counts are preferred over geometric means, because they are more sensitive and allow an earlier detection of resistance (102). This may be justified in situations where AR is known to exist and needs only to be confirmed in a particular situation. Statistically, however, arithmetic means are by no means valid due to the strongly aggregated helminth egg counts, which usually follow a negative binomial distribution (3). Geometric means are more appropriate though not yet ideal, as the distribution patterns change after intervention.

The interval between treatment and sampling should be adapted to each parasite species and to the drugs used. For the evaluation of the efficacy of hookworms to BZ for example, a period of about two weeks is appropriate. A longer period would allow immature or even new infections to become patent, while a shorter one may overestimate efficacy, as some drugs temporarily suppress egg production without killing the worms.

For schistosomes the problem of distinguishing active from immature or even past infections is somewhat more complicated. As the worms live in the blood vessels, eggs follow a long and difficult path from the intravascular location of the worms to the outside world, and may be excreted up to 6 to 8 weeks or even longer after their production. The Kato method does not distinguish dead from live eggs. On the other hand, premature infections which are not affected by praziquantel can become patent days after successful cure of adult worms. Newly contracted infections may result in egg excretion within 4 to 6 weeks. The ideal solution for this dilemma would be to consider only patients outside the endemic area, and to evaluate cure after 8 to 12 weeks or even longer. In practice, this can only be done in tourists who usually carry uncharacteristic light infections. A pragmatic and generally accepted compromise is to evaluate cure in an endemic area after 5 to 6 weeks of treatment (71,72,114). However, the results will always have to be interpreted in the light of possible re-infection (including maturation of prepatent infections) in high transmission areas. If possible, treatment trials should take place in a non- or low transmission season. The quantification of circulating antigens, particularly in serum, can be a useful complementary tool (34). Cure can already be assessed within a few days to a week after treatment, so it is much less sensitive to rapid re-infection. Yet, antigen detection cannot fully replace egg counts as 5 to 30 % of the infections are still missed (34); the assay is not commercially available and requires much more laboratory infrastructure than egg counts.

It may be clear from the above that valid data to confirm anthelmintic drug resistance in the field requires considerable expertise in parasitology and epidemiology, well trained field teams, careful organisation and strict quality control; and that it is vital for further studies to improve and establish appropriate methods and standard protocols (156).

3.4.2. Laboratory tests

3.4.2.1. Laboratory tests for the detection of resistance in livestock helminths

A variety of different laboratory tests has been described for the detection of AR in helminths of livestock (31). Those which are most commonly used and which might be applied to detect AR in human helminths are briefly described here.

Egg hatch test

The egg hatch test is an *in vitro* test, which is only used for the detection of BZ resistance in livestock helminths, based on the ovicidal activity of this group of molecules. The original test was described by Le Jambre (94); a standardised protocol was adopted by the WAAVP (27). Freshly collected faeces (within 3 hours of being shed) are needed to obtain reliable data. If this is not possible, samples must be stored anaerobically; this storage does not influence the outcome of the test, at least for the major gastrointestinal helminths of small ruminants (80). Helminth eggs are purified and incubated with a series of concentrations of thiabendazole (TBZ). This compound has been selected, because it dissolves readily in DMSO and side-resistance is usually present with other members of the BZ-group. After 24 h, the number of hatched larvae is counted. When resistance develops, the ovicidal activity decreases, which results in a higher percentage of hatching of the eggs. Based on vast experience with the test, WAAVP considers resistance to be present when the $ED_{50} \geq 0.1 \mu\text{g/ml}$ (27). This *in vitro* test has the advantage of requiring only one faecal sample. However, several authors have reported poor correlations between the results of the FECRT and the egg hatch test for helminths of livestock (14,42). Unfortunately, the FECRT and the egg hatch assay detect resistance only when at least 25 % of the worm population carries resistance genes, as shown by artificial infection of animals

with mixtures of helminth populations with a known level of AR (96). Since reversion to susceptibility is considered to be possible only as long as resistance genes are present in less than 5% of the helminth population (120), FECRT and egg hatch assays thus allow to detect AR only when it is too late to interfere. Field and experimental data for helminths of livestock indicate indeed that reversion to susceptibility to anthelmintic drugs in livestock helminths rarely occurs, once resistance has been confirmed (31).

Larval development assay

The larval development assay (LDA) is more laborious and time consuming than the egg hatch test, but allows the detection of resistance to the major broad-spectrum anthelmintic classes, including the avermectins/mylbemycins. It was originally described by Coles et al. (30), further improved by several others (66,93), and is now commercially available (DrenchRite ®, Horizon Technology, Australia). In the LDA nematode eggs or L1 are exposed to different concentrations of anthelmintics incorporated into agar wells in a microtitre plate. The effect on the subsequent development into L3 larvae is measured. The results correlate well with those of *in vivo* tests. It is claimed that this test is more sensitive than FECRT and egg hatch test, and detects AR when about 10 % of the worm population carries resistance genes (40), but this remains to be proven.

Larval motility or paralysis tests

Several *in vitro* assays to detect resistance to BZ, macrocyclic lactones or levamisole/morantel have been described which are based on the motility of larvae (31). For the latter group of anthelmintics, a clear-cut distinction between susceptible and resistant strains is not always possible (60,142). A similar motility test has been used to evaluate the sensitivity of *O. volvulus* microfilariae to ivermectin (135). In order to render the interpretation more objective, a micromotility meter has been developed (10). Folz et al. (55,56) used this apparatus to detect drug resistance in *H. contortus* and *T. colubriformis*, but other authors found it less reliable (57,142).

Polymerase chain reaction (PCR)

The first specific primers to detect drug resistant parasitic nematodes were developed by Kwa et al. (91). These primers discriminated between heterozygous and homozygous BZ-resistant *H. contortus* for the alleles in question (beta-tubulin isotype 1), even when these genotypes are phenotypically indistinguishable, and could also identify BZ-resistant *T. colubriformis*. According to Roos et al. (121), PCR detected 1 % of resistant individuals within a susceptible worm population, a tremendous improvement over other *in vivo* and *in vitro* tests.

Recently, Elard et al. (44) developed a more simplified method for the diagnosis of BZ resistant *O. (Teladorsagia) circumcincta*. Using four primers (2 allele-specific and 2 allele-non-specific ones) in the same PCR, adult worms were characterised as to the mutation of the residue 200 of the isotype 1 beta-tubulin. The technique has now been refined for use on a single worm, egg or larva (119). Since the frequencies of alleles associated with anthelmintic drug resistance might be quite high even in susceptible populations, it is indeed important to examine DNA from individual parasites. If DNA is prepared from pooled parasites, the association between particular alleles is likely to be obscured (2).

As the same mutation is responsible for BZ resistance in many parasitic nematodes, this method may provide a means for investigation of the frequencies of alleles bearing it in a wide range of animal and human intestinal nematodes.

Another interesting development is the availability of a P-gp gene probe for *Onchocerca volvulus* (90). Since it has been shown that P-glycoprotein plays a role in resistance to BZ and IVM in *H. contortus* (12, 84, 158), it can be expected that the same resistance mechanism might develop in many other helminths, including *O. volvulus*.

3.4.2.2. Laboratory tests for the detection of resistance in human helminths

Apart from the use of the egg hatching test for hookworms in the Mali study (33), *in vitro* tests for AR in human nematodes have so far not been developed, adapted or validated. A major problem is obviously the lack of reference resistant strains. If these were available, the egg hatch test and the larval development assay, as well as the promising new PCRs, could probably easily be validated for human hookworms.

Laboratory tests for schistosomicide resistance, in particular PZQ, consist mainly of

measuring worm count reduction after treatment in experimentally infected mice. First of all, it must be stressed that white mice are highly unnatural hosts for schistosomes; these large blood-dwelling worms are giant foreign bodies in the tiny murine blood vessels. Proportionally, a single schistosome in a mouse (blood volume 5 ml) corresponds to 10,000 worms in an adult (5 l blood volume). Few mice survive high worm counts long enough to allow therapeutic trials, so the statistical power is inherently limited. Mouse-based experiments are laborious, subject to considerable methodological pitfalls, among others with respect to different strain maturation times (9,22,52). Laboratory strains are usually maintained using eggs derived from livers of mice that have been infected for 5 to 6 weeks, resulting in the selection of parasites which mature much more rapidly, and become susceptible to PZQ much earlier, than natural strains. As mentioned above, such bias probably explains the first reports on induced and "natural" PZQ resistance in the laboratory (51,53). Also, it is not easy to isolate homogeneous parasites, resistant or not. Usually, mice are infected with a mixture of cercariae from at least five snails, in order to obtain bisexual, productive infections. These snails have in turn usually been exposed to 3 to 5 miracidia, resulting often in mixed infections. These miracidia, even if isolated from stools of one person not responding well to treatment, stem from an unknown number and variety of adult worm couples of which only one or a few may be (partly) tolerant to the drug. Confirming and assessing drug resistance in such a model is thus a most tedious and tricky task. The standard protocol proposed by Fallon et al (54), based on procedures and recommendations of Cioli in a series of EC-supported consensus meetings in Leiden (the Netherlands), is a valuable basis for better standardisation, but this mouse model remains difficult to handle and interpret.

There is thus a great need for *in vitro* tests. Adult schistosomes can be cultured in artificial media, providing an excellent opportunity for straightforward *in vitro* exposure tests for individual worms. Such tests are much more accurate, reproducible and feasible than mouse experiments, and allow the screening of a great number of individual worms and well defined isolates. It has allowed the in-depth research of resistance to hycanthon and oxamniquine (16,23). However, for praziquantel the test cannot be established as long as there is no convincing resistant reference strain (21).

Therefore, the main priority in research on AR in human schistosomes and nematodes is to conduct field studies in communities where clinical and/or epidemiological suspicion

warrants the investments needed, to isolate as many individual parasites as possible from non-cured patients, and to confirm the results in animal models. Once such strains are established and consolidated, *in vitro* tests can be validated. These will then in turn allow much wider and faster testing of field isolates, and in-depth research of the biology and genetics of AR in human helminths.

4. Conclusions and recommendations

There is as yet no unequivocal evidence that resistance to commonly used anthelmintics in humans is an emerging problem, either through new mutations or by the selection of innately tolerant strains. However, experiences with other infectious agents and particularly those with the quick and dramatic spread of AR in livestock should guard the medical world against the widespread use of anthelmintics for the control of helminths.

The projected conditions in drug-based human helminth control may be different than in livestock: the transmission dynamics are more complex (particularly for schistosomes and filariae); treatments may be less frequent, and coverage lower; different strategies can be proposed to reduce the appearance or selection of resistant helminth strains. However, these are all hypothetical and optimistic assumptions, which may delay but probably not avoid the appearance of AR. The biological, epidemiological and pharmaceutical similarities between human and livestock helminths are so great that optimism may amount to complacent neglect. In livestock, the problem is mainly economical, which is bad enough. In humans, widespread AR would be a serious public health problem. At present, our only certainty is the striking lack of adequate tools to detect AR in human helminths, and the inability to remedy the problem once it is detected. The perspective is indeed extremely worrying. For major helminths affecting man there are a few drugs available which are both safe and efficacious; as the commercial benefits are low there is little or no investment in research on new molecules.

If drug-based strategies are implemented, the following guidelines may delay the development of resistance:

- Target and justify the intervention

Indiscriminate mass treatment (without any previous screening of the population) should be applied only in areas and groups where the impact of helminths and the benefits expected outweigh the costs and burden on the health system, and where it can be integrated in a sustainable package of health care. Such a cost-benefit calculation must be made by local and national health authorities, taking into account a whole range of qualitative and quantitative parameters, for which no clear-cut model is available.

- Incorporate other control measures

Although health education programmes, construction of latrines, improved water supply, etc. are much more difficult to implement than treatment programmes, they have a much wider impact on public health, improve the sustainability of the helminth control and allow the reduction of the number of treatments in the long run. Mass treatment is easy and popular, but can reduce the commitment to more fundamental advances in the improvement of the living conditions of the local population.

- Reduction of the number of treatments

The most efficient way to delay the development of drug resistance remains the reduction of the selection pressure by the drugs, in particular the number of treatments, preferably to one per year at most. It is obvious that a reduced treatment frequency should be combined with other control measures (see above) in order to maximise its effect. Two or three treatments a year, as advised by Albonico et al. (1), were already sufficient to induce the development of AR in some livestock helminths.

- Avoid the exposure of the whole parasite population to the drug

As suggested by simulation models, limiting the exposure of the whole helminth population should delay the development of AR. Targeted treatment e.g. aimed at schoolchildren is preferable over indiscriminate mass treatment, though even in such programmes over 50 % of the parasite population may be exposed to anthelmintics (2). Timing of treatment in low-transmission seasons may seem efficient in terms of re-infection, but may contribute to the development of AR.

- Use the correct dosage

The use of lower dosages of anthelmintics for morbidity control programmes has been advocated to reduce costs, but should be avoided in order to prevent or delay AR. In fact, the costs of drugs make up only a minor part of treatment programmes (87). Some of the currently recommended drug dosages, including praziquantel 40 mg/kg, ivermectin 150 µg/kg, mebendazole 500 mg, albendazole 200 mg and even 400 mg, are actually subcurative. Although the administration of higher doses might increase costs, the useful life of the drugs may be extended, a worthwhile investment.

Incorrect dosages due to substandard or counterfeit anthelmintics must and can be avoided by imposing adequate quality standards on wholesale suppliers for national health care systems and special control programmes. Obviously, there is also an urgent need for drug quality control systems in the private and public curative sector.

- Simultaneous or rotational use of different drugs

The simultaneous use of two or more drugs with a different mechanism of action is able to postpone the development of resistance to each of the drugs used (15,76,133). The cost increase is a serious obstacle, however. A less effective alternative is the rotation of drugs belonging to different classes. In any case strategies which depend exclusively on one single drug administered during many consecutive years, as in current onchocerciasis and schistosomiasis control programmes, seem bound to result in resistance problems.

- Monitor the development of drug resistance

Monitoring the development of AR should be an obligatory part of large scale worm control programs. As made clear in this paper, standardised reliable tests to detect AR are not yet available.

The most appropriate strategy would therefore seem *not* to embark on control strategies based on the widespread and frequent use of anthelmintics, and to restrict their use to curative medicine and possibly targeted interventions in very high risk groups or areas, which can be identified through rapid appraisal methods or through the regular health information system. To that end (and many others) reinforcement of the general primary

health care systems should be the first priority in the control of human helminths. Meanwhile, the most important scientific challenge is to develop the appropriate tools, methods and protocols to reliably and timely detect the appearance of drug resistance in human helminths.

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Table 1. Main helminth species of livestock for which drug resistance has been reported.

Host	Parasite	BZ	LEV/MOR	AVM/MIL
Sheep/goat	<i>Haemonchus contortus</i>	+		+
	<i>Ostertagia</i> spp.	+	+	+
	<i>Trichostrongylus</i> spp.	+	+	+
Horse	Cyathostomes	+		

BZ: benzimidazoles; LEV/MOR: levamisole/morantel; AVM/MIL: avermectins/milbemycins

Table 2. Important features of reports on treatment failures of human hookworm infections

	Mali	Australia
Helminth species	<i>N. americanus</i>	<i>A. duodenale</i>
Initial prevalence / transmission	High	Moderate
Previous drug exposure	in health centres	community treatment
Anthelmintic drug	Mebendazole (Vermox®)	Pyrantel (Combantrin ®)
- dose (mg)	500 mg/person	10 mg/kg
- treatment regimen	single	single
Study design		
- no. of subjects	103	29
- random selection of subjects	yes	yes
- control group, other drug	Pyrantel	Albendazole
- control group, no treatment	yes	no
- placebo	yes	no
- coprological method	Kato-Katz	ZnSO4 flottation + Kato
- EPG after treatment (weeks)	4	1
Cure rate(CR %)		
- treated group	22.9	13.3
- control group, no treatment	25.0	ND
- control group, other treatment	44.8	100
- placebo group	22.6	ND
Egg reduction rate (ERR %)		
- treated group	- 6.5 (increase)	- 46.1 (increase)
- control group, no treatment	39.5	ND
- control group, other treatment	75.0	100.0
- placebo group (vitamin C)	32.7	ND
In vitro assay (drug resistance)	egg hatch test	ND

ND: not done.

EPG: eggs per gram faeces.

ERR: % reduction of EPG after treatment as compared to EPG before treatment

CR: % of treated (infected) persons becoming negative after treatment

References: 33,116.

