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Anthelmintic resistance: the song remains the same

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ABSTRACT

The prevalence of resistance to broad-spectrum anthelmintics among veterinary helminths has dramatically increased and has evolved from a scientific curiosity into a serious crisis facing small ruminant production in many countries. It also poses a veritable threat to other livestock and human helmiths. Both the molecular mechanisms of action and mechanisms of resistance of anthelmintics are poorly understood. Benzimidazoles bind to nematode β-tubulin, preventing microtubule aggregation and leading to paralysis and death. Levamisole acts through nicotinic acetylcholine receptor of the parasite muscle, causing membrane depolarization and contraction resulting in paralysis. Macrocyclic lactones modulate L-glutamate-gated chloride channels present on the pharynx and somatic muscle membrane of the parasites, thereby paralyzing them. Modes of action of schistosomicides and fasciolicides remain incompletely defined. A number of helminths have developed multiple resistance making their control difficult or almost impossible. As chemotherapy remains the mainstay of helminth control, ways of preserving the efficacy of the anthelmintics must be sought. Complete grasp of the molecular events and the underlying Darwinian selection in the parasites is the ultimate challenge if the persistent dilemma is ever to be alleviated, and that goal is yet an unforeseeable vista.

Key words: Anthelmintics; benzimidazoles; helminths; imidazothiazoles; macrocyclic lactones; parasites; resistance; schistosomicides.

INTRODUCTION

Helminthiasis caused by nematodes, trematodes and cestodes is the most important cause of lost production in small and large ruminants in many parts of the world. In addition, it has been estimated that helminths infect a quarter of the world's population and are a major cause of morbidity, anemia, malnutrition and immunosuppression in the tropics and some temperate regions.[1,2] Irreparable physical and physiological damages, sometimes exacerbated by cognitive retardation, loss of productivity among the workforce, and maintenance of poverty are often the indubitable consequences.[3] There are compelling reports that helminthiasis may further impair the immune response to human immunodeficiency virus (HIV) and tuberculosis (TB),

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and possibly contribute to their spread.[4]

Control has largely relied on the use of pharmaceutical anthelmintics, which can represent the single largest part of the expenditure on animal health in many countries. Almost inevitably the worms have developed resistance to the anthelmintics rendering helminth infections rampant as ever. This has become critical for sheep production in parts of South Africa and Brazil, with isolates of *Haemonchus contortus* failing to respond adequately to any available drugs. Goat production has been abandoned in some regions and in western Europe it is normal to zero graze dairy goats because of the lack of effective anthelmintics and the problem of drug residues in milk. It must be expected that increasing numbers of sheep farmers will have to go out of production unless novel drugs are introduced.

In addition resistance could become a serious problem in human helminths as mass drug administrations are being launched to eradicate worms or to treat almost all the local population. There are the first suggestions of ivermectin (IVM) not working properly in Ghana against *Onchocerca volvulus*, the cause of river blindness, and cases of reduction of activity of both benzimidazoles and pyrantel against human hookworms. Resistance has developed to both oxamniquine (OXA) and praziquantel (PZQ) in the tropical blood fluke, *Schistosoma mansoni* although how far this will spread is not clear.[5] Three major strategies are required to address the issue of resistance, the production of new sensitive tests to detect resistance, the determination of the extent of the problem, and the design and application of strategies to slow the development and spread of resistance.

Resistance to Anthelmintic Drugs Defined

Drug resistance was first defined in nematodes by Prichard *et al.* in 1980: 'resistance is present when there is a greater frequency of individuals within a population able to tolerate doses of compound than in a normal population of the same species and is heritable'.[6] Coles and Kinoti[7] took it further in respect to schistosomes stressing the difference between tolerance and resistance. Tolerance is when the drug does not work the first time used and resistance is any significant increase in concentration of drug required to remove worms when compared with the most sensitive isolate of that particular parasite. Tolerance can occur in larval stages, e.g. in immature *S. mansoni* against PZQ or in larval cyaththstomins in horses against IVM or may be found between species, e.g. OXA kills *S. mansoni* but not *S. japonicum*.

Therefore, anthelmintic resistance can be understood 'as a decline in the effieciency of an anthelmintic against a population of parasites that is generally susceptible to that drug'.[8] Reduction in the sensitivity to the drug is reflected by the decrease of the frequency of individual worms as they are exposed to chemotherapy, compared to the frequency observed in the same population before introduction of the treatment. However, the statements are expressed under general assumptions that the degree of susceptibility prior to drug regime and the underlying genetic selection are known, but not exact in practice. To resolve this conception it was reconciled that, for most commonly employed anthelmintics, resistance is present if the percentage reduction in egg count (of the parasite in the excreta of the host) is less than 95%.[9]

Drug Resistance in Veterinary Parasites

There are only three broad-spectrum anthelmintics available for the control of helminths: group 1, the benzimidazoles (BZs) (albendazole, cambendazole, ciclobendazole, fenbendazole, flubendazole, mebendazole, oxibendazole, ricobendazole, thiabendazole and triclabendazole); group 2, the imidazothiazoles (morantel, pyrantel and levamisole,

Table 1. Major reported resistances to commonly used anthelmintics.

Host	Helminth Parasite	Broad-spectrum anthelmintic						Group-specific anthelmintic					
		BZs	IZs		MLs			SNs		RXN	OPP	ONQ	PPZ
			M/P	LEV	IVM	MXD	DMT	MBC	CST				
Sheep	*Trichostrongylus* spp.	+		+	+	+		+	+		+		
	Haemonchus contortus	+	+	+	+	+			+	+			
	Teladorsagia spp.	+		+	+	+		+	+				
	Cooperia curticei												
	Nematodirus sp.			+									
	Fasciola hepatica	+							+				
Goat	*Trichostrongylus* spp.	+		+									
	Haemonchus contortus	+	+	+	+				+		+		
	Ostertagia spp.	+											
Cattle	*Trichostrongylus axei*	+											
	Haemonchus contortus	+		+	+			+					
	Haemonchus placei	+	+		+	+	+						
	Oesophagostomum spp.							+					
	Trichuris spp.	+			+			+					
	Ostertagia ostertagi	+						+					
	Cooperia spp.	+			+	+	+	+					
	Fasciola hepatica												
Horse	*Strongylus* spp.	+	+										+
	Cyathostomes	+	+		+						+		
Pigs	*Oesophagostomum* spp.	+	+	+									+
	Trichuris suis			+									
Dog	*Ancylostoma caninum*		+										

BZs = benzimidazoles; IZs = imidazothiazoles [M = morantel, P = pyrantel]; MLs = macrocyclic lactones [IVM = ivermectin, MXD = moxidectin, DRM = doramectin]; SNs = salicylanilide [MBC = milbemycin; CST = closantel]; RXN = rafoxanide; OPP = organophosphate; OXA = oxamniquine; PPZ = piperazine.

LEV); and group 3, the macrocyclic lactones (MLs) (abamectin, doramectin, eprinomectin, ivermectin, moxidectin and selamectin). The earliest reports of anthelmintic resistance in sheep involved BZs, followed by resistance to LEV and finally resistance to MLs. Resistance has been reported from all the four corners of the world, to all available drugs, in all classes of helminths, and extensive reviews are available.[8,10-15] Reports on the prevalence of anthelmintic resistances are summarized in Table 1.

Drug Resistance in Human Helminths

Human parasites are inherently more difficult to study than veterinary parasites because there are no other satisfactory hosts for the worms such that direct experimental investigations are impossible. It is not a matter of simplicity to predict which helminth species is most likely to develop resistance to drugs. Human helminths that have complex life cycles, such as nematodes, may be assumed less likely to develop resistance because there are several stages in their life cycles at which environmental selection, which tends to mitigate against selection for resistance, can occur.

Even then several authors have already warned of the development of resistance of the nematode filaria, *O. volvulus* to IVM,[15,16] a drug of choice in the treatment of human onchocerciasis (river blindness). Recently, Grant[17] has drawn the attention to the potential incidence of resistance to IVM in adult macrofilariae of *Onchocerca*, which would even be more disastrous than the resistance in microfilariae. Albendazole is being co-administered with an antifilarial drug, diethylcarbamazine, in lymphatic filariasis elimination programmes. Genetically, the filarial worms are ascertained to have the potential of developing resistance to albendazole, and hence it is extremely important to monitor drug sensitivity in regions like India where high incidence of filariasis is observed.[18]

Notwithstanding arguments to the evidences, failure of treatment of two human hookworms, *Necator americanus* with mebendazole,[19] *Ancylostoma duodenale* with pyrantel[20] are reported, which should be considered evocative for the development of resistance.

Among trematodes, which also have indirect life cycles, resistance to OXA is unequivocally documented in *S. mansoni*, both *in vivo* and *in vitro*.[21,22] Recent reports on the possible development of resistance to PZQ have created much more anxiety, particularly since the drug is regarded as the drug of choice for treatment of schistosomiasis caused by different species of human schistosomes (*S. mansoni*, *S. haematobium*, *S. japonicum*, *S. intercalatum*, and *S. mekongi*), and is approved for current control strategies aimed at the reduction of morbidity through population-based treatment.[23,24] Significantly low response and possible resistance to PZQ have appeared largely from Senegal[25] and Egypt,[26] indicating drastic reduction in the cure rates and recurrence of *S. mansoni* infection in PZQ-treated patients. Notable tolerance to PZQ has been reported even in Brazil, where it is rarely used.[27]

Even though conclusive authentication may not be drawn from these reports, and the fact that decreased cure rates may have resulted form other factors in these high schistosomiasis prevalent regions, the dangers of resistance to PZQ should not be ruled out as tolerance traits are undoubtedly developing.[14,15] Results of *in vivo* and *in vitro* tests on several isolates of *S. mansoni* obtained from PZQ-treated, but uncured, patients in Egypt and Senegal, and on laboratory-maintained isolate that has been subjected to drug pressure during passage in mice, demonstrated that resistance to PZQ can undoubtedly develop.[27-29] In any event, situations now undeniably exist where schistosomiasis is not effectively treated with the recommended drugs.

Specific Mechanisms of Resistance

The biochemical mechanisms underlying

anthelmintic resistance are poorly understood, but appear to be complex and vary among different helminth species correlated with the type of drug employed. Much of the molecular events involving the mechanisms by which the parasites develop drug resistance remain to be investigated. Understanding their mechanism of action is thus, critical for predicting resistance in the target organism. Different drugs employed for the different parasites exhibits variations in their modes of action. Hence, the discussion is limited to the major groups of anthelmintics.

Macrocyclic lactones

The major mechanisms helminths use to acquire drug resistance appear to be through receptor loss or decrease of the target site affinity for the drug. MLs are established to modulate the L-glutamate-gated chloride (GluCl) channels that are found on membranes of the pharynx, somatic muscle and particular neurons of the helminths.[17,30,31] One way the worms die is as a result of starvation leading to paralysis caused by the inhibition of pharyngeal pumping.[32] Of the MLs, mode of action of IVM is best studied. The entry of IVM into the nematode is facilitated by sensory (amphidial) neurons located in the cephalic end of nematodes.[33] Once inside the cuticle, it specifically targets three families of the α-subunits of GluCl channels.[32,34] GluCl channels found in insects, nematodes and crustacea, are not present in vertebrates, and are similar in sequence, and presumably analogous to the subunit A of γ-aminobutyric acid ($GABA_A$) receptors;[35] thus, clarifying the fact that MLs are non-toxic to mammalian hosts. It is also reported that IVM acts on the nicotinic acetylcholine receptor (nAChR) subunit α7 as a positive allosteric effector of the neuronal nAChR.[36] Detections of several isolates of IVM receptors make it conjecturable that resistance to MLs is the result of genetically determined alteration in the receptors preventing appropriate activation of the receptor on binding of the drugs, and that the mechanism of resistance might be different from one helminth species to another.

Imidazothiazoles/tetrahydropyrimidines

Resistance to LEV and the related imidazothiazoles/tetrahydropyrimidines, such as pyrantel and morantel, is a more complex issue. The target site of these nicotinic agonists is a pharmacologically distinct nAChR channel in nematodes.[37] LEV is known to be a more potent agonist than acetylcholine at nematode muscle nAChRs.[38] The LEV receptors of nematodes, like those in vertebrates, are understood to be composed of five subunits that surround a central non-selective cation pore.[39] At therapeutic concentrations, LEV produces depolarization and contraction of nematode somatic muscle, which leads to paralysis and elimination of the parasite without affecting the host nicotinic receptors.[40] Calmodulin-dependent (CaM) kinase II and tyrosine kinases have been demonstrated to be involved in supporting the opening of acetylcholine/LEV receptors on *Ascaris suum* somatic muscle.[41] Resistance to LEV is presumably produced by changes in the averaged properties of the receptor population, with some receptors from sensitive and resistant isolates having indistinguishable characteristics. For instance, loss of *G35* subtype of the receptor population is probably responsible for resistance in the nematode *Oesophagostomum dentatum*.[40]

Benzimidazoles

BZs are extensively used against veterinary parasites and the mechanism of resistance to this class is primarily established, with these studies confirming the mode of action of these drugs.[42] Unlike other anthelmintics so far studied, passive diffusion is a major mechanism of BZs penetration into the parasites, where lipid solubility is a determinant factor influencing the diffusion of these molecules

through the parasite tegument.[43] BZs exert their effect by binding selectively and with high affinity to the β-subunit of helminth microtubule protein, tubulin, leading to subsequent disruption of the tubulin–microtubule dynamic equilibrium.[44] By binding to free β-tubulin, BZs inhibit the polymerization of α- and β-tubulin molecules and the microtubule-dependent uptake of glucose, resulting in paralysis and death. Molecular modifications in the β-tubulin of the parasite are apparently the reason for resistance to BZs.[45] Some tubulin isotypes were found to be lost during selection for resistance resulting in the reduction of high affinity BZ-binding sites.

Antischistosomal drugs

Both the primary mechanism of action and resistance mechanisms of the antischistosomal drugs, OXA and PZQ has not yet been reasonably elucidated.[21,22] OXA is particularly effective against male *S. mansoni* and its mechanism of action is accounted to be closely associated with its irreversible inhibition of nucleic acid synthesis in schistosomes.[46] It has an anticholinergic effect, which increases the parasite's motility and inhibits nucleic acid synthesis.[47] Based on cross-breeding experiments using susceptible and drug-selected schistosome strains exhibiting stable resistance, it has been suggested that OXA is not bioactivated in resistant worms, allowing them to survive the drug action. The activating enzyme, which is present in sensitive and absent in resistant schistosomes, seems to be a sulfotransferase.

PZQ affects mainly the female parasite of all species of human schistosomes, and causes tegument changes and a reduction in the glutathione.[48,49] Recent experiments signifying that components of Ca^{2+} ion channels are the molecular target of PZQ are of considerable interest, as this drug appears to interfere with calcium homeostasis causing rapid influx of Ca^{2+} and a Ca^{2+}-dependent muscle contraction, resulting in flaccid paralysis of the adult parasites.[49] The β-subunits of Ca^{2+} channels of schistosomes are reported to have different structural motifs from those of other known β-subunits, implying that β-subunits are possibly the target sites, and structural alterations render resistance to PZQ.[50] Further molecular insight to the role of the structurally altered β-subunits is required that will probably reveal a more lucid knowledge on the mechanism of action and resistance to PZQ.

At Molecular Crossroads

Presently our knowledge about the genetics of drug resistance in helminths has a lot of gaps. Although some argument is still ongoing about the number of genes involved in resistance to the different anthelmintics, as a number of molecular events are obviously involved, there is a general consensus that reversion to susceptibility is rare once resistance has developed, even when other drugs with completely different working mechanisms are used for prolonged periods.[51] Fascinatingly, drugs exhibiting multiple sites of action, referred to as MISER (multiple independent sites of action evading resistance) anthelmintics,[30] are presumably expected to produce resistance more slowly than those that have only one gene coding for its target site. It can be inferred from the following assessment that to develop a high level of resistance, a simultaneous mutation of several genes is necessary.

Resistance to BZs is best understood in terms of molecular genetics. Several reports showed that there is an extensive polymorphism of the β-tubulin gene in susceptible *H. contortus* populations, and it has been proved that selection for resistance to BZs is accompanied by a loss of alleles at the locus of β-tubulin isotype 1.[44] It has been clearly demonstrated that resistance to BZs is correlated with a conserved mutation at amino acid 200 in β-tubulin isotype 1, with phenylalanine

(Phe) being replaced by tyrosine (Tyr).[52,53] The functional importance of this amino acid substitution was shown in which heterologous expression of the β-tubulin isotype 1 gene altered the phenotype of transgenic *Caenorhabditis elegans*, a free living nematode, 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.[54] Another mutation of Tyr in position 167 of isotype 1 β-tubulin gene has also been observed in different nematodes.[55]

It is well known that the rate at which resistance develops in a given helminth population depends on many factors, among them the frequency of resistance alleles in the initial untreated population.[56] Usually this frequency is estimated at a very low level. However, in untreated *H. contortus* populations the initial frequencies of alleles responsible for resistance to BZs at the isotype 1 and 2 β-tubulin loci were 46 and 12%, respectively, which is surprisingly high.[44] The analysis of 3rd-stage larvae (L3) showed a decrease of the homozygous TTC/TTC genotype and an increase in heterozygous TTC/TAC and homozygous TAC/TAC individuals. The results of the molecular analysis lead to the proposal that polymorphism within codon 200 of β-tubulin gene is not the only reason for the development of BZs resistance, although it definitely is the major mechanism.[56]

IVM resistance appears to be mediated by a number of genes. In *C. elegans,* simultaneous mutation of three genes, *avr-14, avr-15*, and *glc -1*, encoding GluCl channel α-type subunits reportedly confers high-level resistance, suggesting that both target mutation and transport alteration can lead to resistance in worms. In contrast, mutating any two channel genes confers modest or no resistance.[32] It was also noticed that the genes *unc-7* and *unc-9*, which encode innexins (gap junction proteins), and the *dyf* gene, *osm-1*, were connected and involved in resistance.[30] It is evident form *C. elegans* and parasitic species that there are several genes encoding the α-subunits of these ion channels,[57,58] implying that resistance to these compounds will be polygenic and will require the simultaneous presence of more than one gene for the whole helminth to show resistance.

LEV resistance seems to be caused by one gene or gene cluster, the alleles of which are autosomal recessive. Mutants resistant to LEV define 11 genes and fall into three classes: *uncs*, *pseudo-wild types*, and *twitchers*.[58] Mutants in six genes with the *unc* phenotype exhibit extreme LEV resistance, uncoordinated motor behavior, and resistance to other cholinergic agonists. *lev-1* is the only locus for which the predominant mutant phenotype is that of partial resistance but for which two rare *unc* extreme resistance alleles, *x21* and *x61*, have also been found. These two alleles are the only extreme LEV resistance mutations that show any dominance.[39] Other mutant types that might identify additional genes important to receptor function, such as revertants or suppressors of the levamisole-resistant mutant phenotype, have yet to be sought extensively.

The genetic background of resistance to PZQ remains largely unknown. Recent work demonstrated the genetic differences between a laboratory strain of *S. mansoni* selected for resistance to PZQ and the parent susceptible strain.[59] The genes for the β-subunits of Ca^{2+} channels of schistosomes are reported to have different sequences from those of other known β-subunits, and transfection of a schistosome β-subunit into non-schistosome cell lines rendered the latter considerably more sensitive to PZQ. It is also demonstrated that cells expressing the structurally unusual schistosome β-subunit, *Sm*$Ca_v\beta1$ in their voltage-operated Ca^{2+} channels, exhibit increased current amplitude in the presence of PZQ. The low susceptibility to PZQ noted in some *S. mansoni* strains could be due to some mutation(s) in the gene coding for this protein, and the different sensitivity of schistosomes to PZQ action could be due to the expression of different β-subunits in the parasite.[60]

FACTORS IMPLICATED TO DEVELOPMENT OF RESISTANCE

The most important factor in the development of resistance in veterinary helminths to anthelmintics is the contribution that the worms, which survive treatment, make to the next generation. This in turn depends on the number of worms in refugia, that is, the numbers of worms that are not exposed to the drugs.[61,62] There are three main factors that influence the population of refugia – the numbers of larvae on pasture, the number of treated animals, and the extermination of all developmental stages within the host. Moving treated animals to rested pastures to minimize exposure to infective larvae has been recommended a useful method in endemic areas.

However, these actions result in the next helminth generation that consists almost completely of worms that survived therapy, and this practice is certainly responsible for the development of resistance. For example, problems with resistance are reported in the nematodes of sheep and goats on some Greek islands, which suffered from extended drought; in contrast, no resistance developed under similar management and deworming practices on the mainland.[63] Especially the drench-and-move system, in which all animals in a flock are treated before they are moved to clean pastures containing few or no worms in refugia, is a strong selector of resistance. Only recently, it is realized that a balance has to be found between treatment efficacy and delaying the development of resistance. Only treating some animals on a farm has been proved to be very successful in delaying the development of resistance, although this might have some consequences on productivity.[64] It implicates that for anthelmintic efficacy to be maintained, the number of worms in refugia must be sufficiently large and this should be considered above all else when worm management in both livestock and human is planned.

Treatment frequency is certainly another important factors in the selection of resistance. There are reliable evidences that a high treatment frequency selects for resistance more strongly than do less frequent dosing regimens, and that resistance develops more rapidly in regions where animals are dewormed regularly. Serious problems with resistance in *H. contortus* were reported in some humid tropical areas where 10 to 15 treatments per year were used to control this parasite in small ruminants, and most often the number of treatments is limited to 1-3 per year. Even at these lower treatment frequencies, many cases of resistance have been reported, especially when the same drug is used over many years.[7,61]

Long-term use of LEV 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 do worms in small ruminants.[10] Frequent use of IVM without alternation with other drugs has also been reported as the reason for the fast development of resistance in *H. contortus*. *S. mansoni* is reported to have a great capacity to develop resistance to therapeutic doses of a determined drug, especially when the parasitic population is under continuous pressure from schistosomicides.

Finally, underdosing is another important factor in the development of resistance. Underdosing, which can occur through improper administration of drugs, underestimation of weight, dilution of the drug for economic reasons, use of substandard drugs, enhanced drug metabolism by some types of animals, such as goats, or prolonged drug persistence, can contribute to selection for resistance. As is shown by the models,[65] 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, there are dose levels where underdosing promotes 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 other genotypes. On the contrary, when the initial frequency of the allele for resistance is high, the dose that promotes resistance most strongly is that kills all susceptible homozygotes and all heterozygotes, but none of the resistant homozygotes.

CONCLUSION

In spite of remarkable achievements in the discovery and development of anthelmintics, helminthiasis remains the major constrain to successful livestock production and the primary health problem in developing countries.[1-3,10] Since the first reports of resistance to the broad-spectrum anthelmintics were made some three decades ago, the phenomenon drastically worsen the blow to animal industry and human health. Escalating development of multiple resistances emphasizes an urgent reexamination of the present helminth control practices, even ban on the use of certain drugs are strongly advocated. Ironically, people in general are not paying their attention to the recommendations and schemes that are designed to minimize the upsurge.[66]

Although novel classes of anthelmintics (e.g. parahequamide, cyclooctadepsipeptides, amino-acetonitrile derivatives, etc.)[67,68] and some promising vaccine candidates have been discovered, in particular cathepsin L proteases from *F. hepatica*, aminopeptidases from *H. contortus*, and aspartic proteases from schistosomes and hookworms,[69] the problem is still relentless. This is partly a result of the complex immunological interactions occurring during helminth infections, which are not yet fully understood, especially regarding the immune mechanisms conveying protection, and possibly the greatest restrains in their commercial development are the enormous costs involved.[70] Considering the ever-increasing demand of livestock products, researchers have focused on non-chemotherapeutic alternatives and a variety of approaches have been the subject of intense investigations. A relatively recent innovation is the biological control approach to nematode parasites; however, commercialization is still a limiting factor.

It is apparent that unless novel therapeutics is available on large-scale employment, helminth parasites are going to cause inexorable economic and health problems. Therefore, the immediate scientific challenge would be the development of appropriate tools and protocols to reliably and quickly detect the appearance of drug resistance and its biological basis, and to systematically control the current prophylactic use of anthelmintics.

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