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Molecular diagnosis of anthelmintic resistance

Georg von Samson-Himmelstjerna *

Institute of Parasitology, University of Veterinary Medicine Hannover, Bünteweg 17, 30559 Hannover, Germany

Abstract

Conventional and real time polymerase chain reaction-based tests have been developed for the diagnosis of anthelmintic resistance (AR) in populations of several small and large ruminant as well as horse gastro-intestinal nematode species. To date, molecular markers that correlate well with AR are available only for the detection of benzimidazole resistance. Recently, however, a single nucleotide polymorphism was found in vitro to be of functional relevance for reduced drug efficacy to macrocylic lactones. The focus of the present review, therefore, is the molecular mechanism of action of these two drug classes and potential applications of this knowledge to the diagnosis of AR. It is argued that a prerequisite for future molecular diagnosis will be tests providing reliable and exact quantification of resistance related alleles in DNA extracted from representative pools of parasites.

Keywords: Anthelmintic resistance; Benzimidazoles; Macrocyclic lactones; PCR; SNP; Allele-quantification

1. Introduction

Several recent investigations have demonstrated a progressive spread of anthelmintic resistance (AR), mainly in nematode populations of livestock animals (Hughes et al., 2004, 2005; Kaplan, 2004; Mejia et al., 2003; Sargison et al., 2005; Tandon and Kaplan, 2004; Wirtherle et al., 2004). AR has already attained clinical and economical significance in some areas of the world, particularly in trichostrongyle species of sheep and goats. The problem appears to be reaching even more serious levels with the development of multi-drug resistant isolates in small ruminants. More recently, resistance against macrocyclic lactones (MLs) has been found in cattle trichostrongyle species in Europe (Coles et al., 2001) as well as South and North America (Mejia et al., 2003; Gasbarre et al., 2004). Two studies even report multi-drug resistance against benzimidazoles (BZs) and an ML (Anziani et al., 2004; Mejia et al., 2003). In horses, resistance is currently found most often in small strongyles against BZs and, to a far lesser extent, pyrantel (PYR) (Kaplan, 2002). Despite these alarming findings, it has to be stated that these drugs still perform satisfactorily in a high proportion of stables (Comer et al., in press; Kaplan, 2004; Wirtherle et al., 2004). This apparent contradiction between increasing evidence of AR in the field, on one hand, and uncertainty about the actual status of the nematode population in any particular group of animals, on the other, is a concern that needs to be addressed by those responsible for parasite

* Tel.: +49 5119538557; fax: +49 5119538555.
E-mail address: gvsamson@tiho-hannover.de.

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control on the premises. The same is true for worm control in cattle, and also for small ruminants in those parts of the world where AR is not already highly prevalent.

It is important to maintain the efficacy of currently available anthelmintics wherever resistance has not emerged and to prevent the further selection of resistance where it has already started to become apparent. This is only possible if adequate means are available for the diagnosis of AR. In vivo tests, like the controlled test, which involves necropsy of treated and untreated animals, or the faecal egg count reduction test (FECRT) are expensive and laborious. Alternative in vitro tests have been established, like the egg hatch test (EHT) and the larval development test (LDT). These are faster, less labour intensive and have found broad acceptance, mainly for surveys of AR prevalence. The best evaluated and most often used in vitro tests are those for the detection of BZ resistance, while so far those developed for use with PYR or the MLs often lack sensitivity or fail to agree with other tests like the FECRT (Craven et al., 1999; Lind et al., 2005; Tandon and Kaplan, 2004; Varady and Corba, 1999).

Molecular tools offer the opportunity to overcome at least some of these shortcomings. As in numerous other fields of veterinary parasitology (see the review by Gasser, 2006), the polymerase chain reaction (PCR) technique has opened new perspectives for AR research. Several PCR protocols have been developed which provide high accuracy and sensitivity when used to investigate single worms. However, meaningful results depend on testing representative number of individuals (e.g. 100) from the isolate under investigation. This is expensive and consequently such tests have only been used in epidemiological surveys or fundamental studies. This represents a major drawback of the current technology. One of the most important challenges is to develop molecular tests suitable for routine use that will assess the resistance level of a parasite population on the basis of a pooled DNA sample.

This review discusses the pros and cons of currently available test protocols and gives an outlook into future perspectives for the molecular diagnosis of AR. The focus will be on BZ resistance as, to date, a greater body of knowledge exists for this than for other anthelmintic classes. The molecular mechanism by which the BZs are believed to act is briefly reviewed to provide a basis for understanding how resistance might function. Although introduced into the market nearly two decades after the BZs, the MLs have become the most important and most frequently used anthelmintic group due to their broad spectrum of activity, their high efficacy and low toxicity. Recent work on bovine gastrointestinal nematodes has provided new insights into the effect of distinct molecular changes on the ML target molecule, the glutamate-gated chloride channels (GluCl) (Njue et al., 2004). The potential relevance of these data to other parasite species and future options for molecular diagnosis of ML-resistance will also be discussed.

2. Mechanisms of action and resistance of benzimidazole anthelmintics in parasitic nematodes

Friedman (1979) showed that BZs selectively bind to tubulin in parasitic nematodes leading to inhibition of microtubule formation. Alpha- and beta-tubulin molecules are soluble proteins which aggregate to form heterodimers, the building blocks of the insoluble polymeric microtubules. These undergo a constant process of proliferation and dissociation at their opposite ends (Lacey, 1988). Microtubules play a fundamental role in several important cell functions, like cell division, shape and motility or intracellular substrate transport. When alpha-/beta-tubulin with bound BZ is incorporated into the growing end of the microtubule, further heterodimers are prevented from being associated. This process was termed ‘capping’ (Lacey, 1988). Since dissociation continues at the opposite end, the microtubules become degraded and finally completely disappear. The role of tubulin as the BZ target molecule was further substantiated by Russell and Lacey (1992) who found that BZ-binding was significantly reduced in BZ-resistant as compared with BZ-susceptible *Haemonchus contortus* and *Trichostrongylus colubriformis* isolates. Genetic analysis showed that the level of beta-tubulin polymorphism was reduced in resistant *H. contortus* isolates, presumably due to the selection of resistant genotypes during repeated drug exposures (Kwa et al., 1992; Roos, 1990). Furthermore, by comparing

the beta-tubulin coding sequences obtained from BZ-susceptible and -resistant isolates of sheep trichostrongyles, it was discovered that certain beta-tubulin sequence polymorphisms that lead to changes in amino acid sequence were linked with resistance. A single nucleotide polymorphism (SNP) at codon 200 of the beta-tubulin isotype 1 which results in the expression of tyrosine (encoded by TAC) in resistant worms instead of phenylalanine (encoded by TTC) in susceptible worms, is considered to be most closely linked with BZ-resistance (Wolstenholme et al., 2004). This association was found in the sheep trichostrongyles *H. contortus*, *T. colubriformis* and *Telodorsagia circumcincta* (Elard et al., 1996; Elard and Humbert, 1999; Geary et al., 1992; Kwa et al., 1993, 1995; Silvestre and Cabaret, 2002). The same SNP has been observed in other species like *Cooperia oncophora* from cattle or some small strongyle species from the horse, but its significance for BZ-resistance is either not yet thoroughly investigated or appears to be of lesser importance than in the sheep parasites (Drogemuller et al., 2004a; Njue and Prichard, 2003; Samson-Himmelstjerna et al., 2003). Further SNPs, also resulting in phenylalanine to tyrosine exchanges at codon 167 of the beta-tubulin isotypes 1 and 2 in sheep trichostrongyles, or only isotype 1 in small strongyle species, respectively, were also found to be potentially associated with BZ-resistance (Drogemuller et al., 2004b; Prichard, 2001). These findings support the proposal that the BZ-binding site involves alpha- and beta-tubulin domains between the beta-tubulin phenylalanine 167 and 200 residues. This configuration would allow the planar BZ ring to be locked between the two phenyl rings and to form a covalent bond between the cysteine201 and the BZ carbamate (Prichard, 2001). However, this model of BZ-binding was questioned, by arguing that neither the intercalation of the BZ molecules between the phenyl rings, nor the formation of a covalent bond, can be brought in agreement with the structural data of the tubulin dimer obtained by electron crystallography (Nogales et al., 1998; Robinson et al., 2002). Furthermore, it was stated that the specific residues suggested as contributing to BZ-binding appear to be located in an inaccessible region of the beta-tubulin. An alternative model of BZ-binding to helminth beta-tubulin was provided by Robinson et al. (2004) through the generation of an *H. contortus* beta-tubulin model structure utilizing the bovine alpha–beta-tubulin dimer atomic structure (Lowe et al., 2001) and the bacterial beta-tubulin homologue FtsZ (Lowe and Amos, 1998). This model suggests that by dissociation of the heterodimer, conformational changes may result in a widening of the cleft between the N-terminal and intermediate domains within the beta-tubulin monomer, where the BZ-binding region, including the above-mentioned sites, is believed to be located. By this means, a surface area became solvent accessible and allowed the docking of the molecular structure for albendazole–sulphoxide, including the formation of hydrogen-bonds and hydrophobic interactions between the drug and those residues found to be linked with BZ-resistance (Robinson et al., 2004). Furthermore, these authors argue that tyrosine instead of phenylalanine at residue 200 would facilitate the formation of an H-bond at the base of the binding area, potentially preventing drug binding by closing the cleft.

Apart from the target-associated mechanism of BZ-resistance, non-specific resistance pathways, mainly involving effects evoked by transmembrane efflux pumps, i.e. P-glycoprotein (P-gp), were found to contribute to reduced drug susceptibility (for review see Kerboeuf et al., 2003a). Similar observations have been made in other areas of drug resistance as well, and for different drug classes in the same organism. Therefore, these proteins are classified as multi-drug resistance (MDR) proteins. The level of P-gp expression in *H. contortus* eggs was found to differ between susceptible and anthelmintic resistant isolates and can be used to evaluate the resistance status (Kerboeuf et al., 2003b; Xu et al., 1998). However, to date, these procedures have not been applied in the field, possibly due to sophisticated equipment and consumable requirements.

3. Molecular diagnosis of BZ resistance

3.1. Conventional PCR

In the past, molecular tools for the analysis of AR have been confined to BZ-susceptible and resistant genotypes. These tests utilised the resistance-related SNP in codon 200 of the beta-tubulin isotpye 1 described in the previous section and were initially

developed for the analysis of BZ-resistance in small ruminant trichostrongyles (for review see Humbert et al., 2001). Allele-specific PCR-based tests were described to determine the genotype of *H. contortus* (Kwa et al., 1994) and *Telodorsagia circumcincta* (Elard et al., 1999) adult worms. A set of four primers allows potential amplification of three beta-tubulin fragments: (1) a pair of universal flanking primers generates a nonBZ-resistance specific fragment, (2) a BZ-susceptible specific reverse primer together with the universal primer detects the BZ-susceptible allele and (3) the BZ-resistant forward primer together with the universal reverse primer identifies the resistant allele. Since these fragments all differ in size, the test can be performed in one reaction, the non-specific PCR fragment providing an internal positive PCR control. In an extension to this work, Silvestre and Humbert (2000) combined the allele-specific beta-tubulin codon 200 PCR with a PCR-restriction fragment length polymorphism (PCR-RFLP) procedure allowing the genotyping and identification of single *H. contortus*, *T. colubriformis* and *Telodorsagia circumcincta* third stage larvae. More recently, a similar approach was followed to establish an allele-specific beta-tubulin codon 200 PCR for several cyathostomin species (Samson-Himmelstjerna et al., 2002b). In this, a forward primer for the BZ-susceptible and another one for the BZ-resistance allele are combined in separate reactions with a universal reverse primer. In contrast to the trichostrongyle allele-specific PCR described above (Silvestre and Humbert, 2000), no internal control is involved and so it is important to run positive controls, for example, by using DNA of previously genotyped adults. This procedure was found to be suitable for genotyping the DNA isolated from single third stage larvae and adults of at least seven different cyathostomin species. However, as indicated above the observations made by using this test on experimentally selected and field selected BZ-resistant cyathostomin isolates clearly differed from those reported repeatedly for small ruminant trichostrongyle isolates. In phenotypically highly BZ-resistant equine isolates the frequency of the BZ-resistance related beta-tubulin codon 200 allele was only comparatively moderately elevated and often did not even dominate (Pape et al., 2003; Samson-Himmelstjerna et al., 2002a, 2003).

3.2. Real time PCR

Alvarez-Sanchez et al. (2005) describe the development of a real time PCR method for determining allele frequencies of beta-tubulin isotype 1 codon 200 in nematode DNA samples. Allelic differentiation was achieved by using two allele-specific reverse primers in combination with a universal forward primer in separate reactions. A non-specific detection system based on the double-stranded DNA intercalating dye SYBR® Green I, which fluoresces once bound to the DNA, was used. Relative quantification was performed by the measurement of fluorescence at each cycle and the determination of a cycle threshold (ct) value, indicating the cycle number at which the fluorescence exceeds the background threshold. For example, a sample in which the susceptible allele detecting primer combination reaches the threshold one cycle earlier than the resistance detecting combination would be considered to contain the respective alleles in a ratio of 2:1. Thus, the difference in ct-values can be used to calculate allele frequencies. Alvarez-Sanchez et al. (2005) initially investigated the suitability of their procedure by analysing the allele frequencies of PCR products obtained by allele-specific beta-tubulin codon 200 nested PCR fragments (Silvestre and Humbert, 2000). These fragments were obtained following amplification of 10 single larva DNA samples from one BZ-susceptible and one BZ-resistant *Telodorsagia circumcincta* isolate, respectively. Each of the 10 larvae from both isolates was found to be homozygous for the respective allele showing allele frequencies of >99%. Subsequently, the authors determined the allele frequencies in BZ-susceptible and -resistant isolates of *H. contortus*, *Telodorsagia circumcincta* and a susceptible *T. vitrinus* isolate using DNA obtained from representative pools of parasites. Using DNA extracted from pools of 10,000 larvae of each isolate, they compared the real time PCR data with the results of the conventional PCR and reproducibly observed very similar allele frequencies. Furthermore, it was shown that the BZ-resistant *Telodorsagia circumcincta* isolate, which gave the lowest susceptible-allele frequency of only 13.7% in real time PCR, was also characterized phenotypically as the most resistant according to egg hatch test and faecal egg count reduction results. This new method, for the first time,

offers the opportunity to analyse allele frequencies based on samples of pooled larvae, thus, allowing the inexpensive, sensitive and rapid molecular diagnosis of BZ-resistance in trichostrongyles. However, this method has not yet been used on mixed species or field samples and thus it remains to be seen to what extent further optimisation will be required. SYBR® Green-based real time PCR procedures are potentially adversely affected by the generation of primer artefacts, which also contribute to the detected level of fluorescence, and, therefore, care has to be taken to prevent non-specific amplification (Bustin and Nolan, 2004). To monitor possible primer–dimer generation a melting curve at the end of the reaction was performed, confirming that only specific products with a melting temperature of over 80 °C were generated (Alvarez-Sanchez et al., 2005).

Real time PCR formats, employing fluorescent labelled oligonucleotide probes, usually promote a high degree of specificity since fluorescence is only achieved following hybridization of the probe within a region flanked by the PCR primers. Therefore, non-specific PCR products do not have any impact on the detected signal. Various formats of probes are available including hybridization probes such as molecular beacons (Tyagi and Kramer, 1996) or hydrolysis probes like the so-called TaqMan® probes (Heid et al., 1996). More recently Scorpions primers have been introduced (Whitcombe et al., 1999), which are comparatively long oligonucleotides with a fluorescence and quencher containing stem-loop 5′-tail followed by the primer sequence. Following primer extension the loop sequence hybridizes with its complement within the same strand. Scorpions-based real time PCR reactions result in faster signalling compared to hydrolysis probes, have improved allelic discrimination capabilities, and were found to provide good multiplexing opportunities, as shown for *Giardia lamblia* genotype specification (Ng et al., 2005). Comparing the SYBR® Green I, Scorpions® and TaqMan® chemistries on different real time PCR instruments, Terry et al. (2002) reported that the TaqMan® chemistry gave the most precise and accurate results. By addition of a minor groove binder (MGB) molecule at the 3′-end of the TaqMan® probes, the binding intensity between probe and template is significantly enhanced (Afonina et al., 1997; Kutyavin et al., 2000). This allows the design of shorter probes with higher specificity and was shown to be particularly suitable for analysis of A/T rich regions (Walburger et al., 2001). The latter attribute accords with the beta-tubulin codon 200 region in cyathostomin species (Pape et al., 1999). Consequently, a comparison of molecular beacon and TaqMan® MGB-probes revealed that only the latter reproducibly provided the required specificity to allow differentiation of the TTC/TAC beta-tubulin codon 200 SNP (von Samson-Himmelstjerna et al., 2003). This assay was shown to be suitable for the reproducible and reliable real time PCR-based genotyping of single third stage larvae and adults of seven cyathostomin species. However, the TaqMan® MGB-probe based method did not allow accurate analysis of allele frequencies in DNA samples isolated from pooled third stage cyathostomin larvae. This is in contrast to observations made by using the SYBR Green I-based real time PCR procedure for beta-tubulin codon 200 genotyping in small ruminant trichostrongyles (Alvarez-Sanchez et al., 2005). Although probe-based real time PCR assays are generally considered to be more precise than SYBR Green I-based procedures (Bustin and Nolan, 2004), there are exceptions to this rule (Schmittgen et al., 2000). Hypothetically, the high A/T content (65%) in the probe sequence corresponding to the beta-tubulin codon 200 region, could lead to higher instability in probe binding compared with PCR primer binding in the less A/T rich (60–50%) flanking region during SYBR Green I real time PCR. The annealing temperature during the TaqMan® MGB-probe based assay was more than 10 °C higher than that of the SYBR Green I technique. This may furthermore result in reduced probe-template binding intensity and thus increased variability in the quantitative detection of template. Therefore, it would be worthwhile to investigate if the non-specific chemistry-based real time PCR would allow beta-tubulin codon 200 allele-quantification in cyathostomins.

4. Mechanism of ML type drugs and potentially resistance-related molecular changes

Worms treated with MLs show reduced pharyngeal pumping, paralysis of body muscles and uterus leading to failure of movement and egg laying (for review see Yates et al., 2003). Glutamate- and GABA-gated chloride channels are considered to be

the target of the MLs (Cully et al., 1994, 1996; Holden-Dye and Walker, 1990). The heterologous expression of nematode glutamate-gated chloride channels (GluCls) in *Xenopus* oocytes allow in vitro pharmacological studies of the effects of MLs (Arena et al., 1995; Njue et al., 2004). Although P-gp has been shown to be involved in ML- as well as BZ-resistance (for review see Kerboeuf et al., 2003a), sequence polymorphism correlated with ML-resistance has so far only been identified in the GluCl-gene (Njue et al., 2004). In this study on the cattle nematode *C. oncophora*, the expression of an alpha-GluCl subunit with phenylalanine instead of leucine in the N-terminal domain at site 256 resulted in reduced sensitivity of homomeric and heteromeric alpha-/beta-channels. This reduced sensitivity is related to the natural ligand glutamate as well as to IVM and moxidectin. However, the same polymorphism has not yet been identified in ML-resistant isolates of other species, like *H. contortus* (Hejmadi et al., 2000). The significance of the alpha-GluCl subunit was further underlined by analysing the genetic variability of GluCl alpha-genes in ivermectin-susceptible and -resistant *C. oncophora* isolates (Njue and Prichard, 2004). Based on single stranded conformational polymorphism (SSCP) analysis (see the review by Gasser, 2006) of a fragment of the *C. oncophora* GluCl alpha3 gene, these authors observed significant allele-frequency differences between IVM-susceptible and -resistant isolates, indicating an association between this gene and IVM-resistance. The amplified gene sequence included coding and non-coding areas located N-terminal of the 256 site. Sequencing the PCR products used in this SSCP analysis failed to reveal differences in deduced amino acid sequences. Therefore, no correlation could be found between the GluCl-256 SNP and one of the alleles known to be of increased frequency in the resistant isolate. A causative mutation outside the fragment analysed by SSCP but genetically linked (i.e. in close enough proximity to maintain coinheritance) to it, however, could also lead to the changes in allele frequencies seen in this analysis. Further research is necessary to investigate the significance of the GluCl-256 SNP to the ML-resistance phenotype in parasitic nematodes, before it can be considered as marker of utility for molecular diagnostic tools.

5. Future opportunities

The assessment of allele frequencies based on the analysis of SNPs appears to be a key issue in the future development of tools for the diagnosis of anthelmintic resistance. Pyrosequencing, a recently introduced de novo sequencing strategy offers accurate, reproducible and high-throughput allele-specific SNP quantification opportunities (Alderborn et al., 2000; Ronaghi et al., 1996, 1998). This technology is based on the quantitative detection of light signals which are generated as the result of a multi-enzyme reaction. Following the addition of nucleotides to the sequencing primer bound to the complementary target DNA strand, pyrophosphate is released in a quantity equimolar to the amount of incorporated nucleotides. The pyrophosphate is then converted to ATP which finally is used by firefly luciferase to produce a light signal which is detected by a charge coupled camera device. Through the addition of the four possible nucleotides one at a time in a predetermined sequence, the nucleotide sequence of the template is determined. Furthermore, the template quantity can be assessed by the measurement of the light signal intensity. Compared with alternative techniques, including real time PCR, minisequencing and MALDI-TOF mass spectrometry, pyrosequencing gave the most precise average allele frequencies for three SNPs (Shifman et al., 2002). Furthermore, it allows multiplexing (Lotsch et al., 2003; Pourmand et al., 2002), so that more than one SNP can be quantified on one target sequence, which could be of advantage particularly for the analysis of BZ-resistance where apparently at least two beta-tubulin SNPs are involved (see above). Several additional techniques have recently been developed for the molecular assessment of allele-frequencies, including polony amplification (Butz et al., 2004; Mitra and Church, 1999) or microarray-based systems (Liljedahl et al., 2004; Lindroos et al., 2003). During PCR colony, or polony, amplification the highly diluted template DNA molecules are immobilized in a acrylamide gel containing all necessary PCR components on a microscope slide. Gene expression levels can then be performed by using differently labelled sequencing primers in two consecutive sequencing reactions. By microarray-based minisequencing quantitative multiplex genotyping of up to 100 SNPs can be achieved in a similar

number of samples per microscope slide. However, both these procedures are not mutation tolerant, since they will produce a false negative result if a new mutation occurs within the SNP.

In conclusion, it can be stated that a range of promising new molecular technologies is currently available which can be exploited for the development of improved tools for the molecular diagnosis of anthelmintic resistance. However, this will only prove useful if further fundamental research on the mechanisms and characteristics of anthelmintic resistance provide meaningful molecular resistance markers.

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