Invited review

Understanding anthelmintic resistance: The need for genomics and genetics

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Abstract

Anthelmintic resistance is a major problem for the control of many parasitic nematode species and has become a major constraint to livestock production in many parts of the world. In spite of its increasing importance, there is still a poor understanding of the molecular and genetic basis of resistance. It is unclear which mutations contribute most to the resistance phenotype and how resistance alleles arise, are selected and spread in parasite populations. The main strategy used to identify mutations responsible for anthelmintic resistance has been to undertake experimental studies on candidate genes. These genes have been chosen predominantly on the basis of our knowledge of drug mode-of-action and the identification of mutations that can confer resistance in model organisms. The application of these approaches to the analysis of benzimidazole and ivermectin resistance is reviewed and the reasons for their relative success or failure are discussed. The inherent limitation of candidate gene studies is that they rely on very specific and narrow assumptions about the likely identity of resistance-associated genes. In contrast, forward genetic and functional genomic approaches do not make such assumptions, as illustrated by the successful application of these techniques in the study of insecticide resistance. Although there is an urgent need to apply these powerful approaches to anthelmintic resistance research, the basic methodologies and resources are still lacking. However, these are now being developed for the trichostrongylid nematode Haemonchus contortus and the current progress and research priorities in this area are discussed.

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1. Introduction

Anthelmintic resistance is widespread in parasitic nematodes of sheep and horses and is an emerging problem in cattle throughout the world (Kaplan, 2004). The most serious problems have been with trichostrongylid nematode parasites of sheep and consequently most research has been conducted on this group of parasites (Sangster and Gill, 1999; Prichard, 2001; Kaplan, 2004). Resistance to the three major classes of anthelmintics – benzimidazoles, tetrahydropyrimidines/imidazothiazoles and macrocyclic lactones – is common (Table 1). Although some progress has been made in elucidating the molecular mechanisms of resistance, there are still many more questions than answers. Our understanding of benzimidazole resistance is more advanced than for the other major anthelmintic classes and it is generally accepted that mutations in the isotype-1 β-tubulin gene are a major determinant of resistance in many parasitic nematode species. However, there is increasing evidence that the situation is more complex and other loci may also be involved (Prichard, 2001). Our understanding of resistance to tetrahydropyrimidines/imidazothiazoles and macrocyclic lactones is less advanced and although a number of genes have been implicated as contributing to resistance, their relative importance is not known. In addition, it is still unclear how resistance alleles arise, are selected and spread in parasite populations and also whether the mechanisms of resistance differ between...
different parasite species or different isolates of the same species. It will be important to gain a detailed understanding of these issues if parasite control schemes are to be designed and implemented in such a way as to minimise the development of resistance and maintain the useful life of the currently available drugs.

A major research priority that has emerged in recent years is to develop sensitive molecular diagnostic assays of resistance to enable accurate assessment of the resistance status of parasite populations (von Samson-Himmelstjerna, 2006). This has been fuelled by rapid progress in high-throughput, sensitive genotyping technologies which, although once the preserve of specialist laboratories, are becoming cheaper, simpler and more widely available. With this trend likely to continue, molecular diagnostic tests are an ever more attractive and realistic proposition. However, the major limiting factor to such tests becoming a reality is not one of technology or cost but our poor understanding of the molecular and genetic basis of anthelmintic resistance. This review article discusses how genomic and genetic approaches are needed if significant further progress is to be made in this field.

### 2. Current methodology: The candidate gene approach

Practically all of our present knowledge of the possible mechanisms of anthelmintic resistance has been based on candidate gene studies. This involves making an “educated guess” as to which genes might be involved in resistance and then conducting experimental work to test the hypothesis. The majority of this experimental work has involved searching for associations of the resistance phenotype with polymorphisms in candidate genes. This is done either by comparing susceptible and resistance field populations or by artificially selecting for resistance during experimental infections. The most notable success of this approach has been identification of the isotype-1 β-tubulin locus as a major determinant of benzimidazole resistance in *Haemonchus contortus*. This has led to many subsequent studies implicating this locus as a key cause of benzimidazole resistance in other parasitic nematode species. However, the results have been less clear when similar approaches have been applied to macrocyclic lactone and levamisole resistance. To appreciate the need for genomic and genetic studies, it is useful to first review the candidate gene approach and speculate on the reasons for its differing success when applied to these different anthelmintic classes. Benzimidazole and ivermectin resistance will be used to illustrate the key issues. Levamisole resistance will not be discussed in detail as the same broad principles described for ivermectin resistance apply.

### 3. The candidate gene approach applied to benzimidazole resistance

The demonstration that mutations in a β-tubulin gene could give rise to benzimidazole resistance in fungi (Sheir-Neiss et al., 1978) and in the model nematode *Caenorhabditis elegans* (Driscoll et al., 1989) provided the original impetus to investigate this possibility in parasitic nematodes. Evidence for selection on the β-tubulin locus was first shown for *H. contortus* by probing Southern blots with the an isotype-1 β-tubulin cDNA and demonstrating a reduction in the number of hybridising fragments in resistant compared with susceptible populations (Roos et al., 1990; Lubega et al., 1994). Similar results were subsequently found for *Trichostrongylus colubriformis* (Grant and Mascord, 1996). Sequence analysis found that a phenylalanine to tyrosine substitution at the P200 position of the β-tubulin polypeptide was the only polymorphism that was consistently different between susceptible and resistant populations (Kwa et al., 1993a,b). An allelespecific PCR was then used to demonstrate that the Tyr200 polymorphism could be easily detected in several resistant populations but not in susceptible populations (Kwa et al., 1994). This provided strong circumstantial evidence that the P200 Phe-Tyr substitution was an important determinant of benzimidazole resistance in *H. contortus*. However, association studies of this type cannot directly demonstrate that a particular polymorphism is capable of conferring a resistance phenotype since it is possible that a polymorphism in a genetically linked gene could be responsible for resistance. The establishment of a causal relationship between a mutation and the resistance phenotype is a major challenge in the case of parasitic nematodes since there is a lack of reverse genetic and molecular tools to study gene function in these organisms. However, in the case of benzimidazole resistance, this hurdle was overcome by some elegant experiments in which the capacity of different alleles of the parasite β-tubulin
gene to modulate benzimidazole sensitivity was tested using *C. elegans* as a heterologous expression system (Kwa et al., 1995). Transgenic expression of *H. contortus* isotype-1 β-tubulin alleles that encoded a Phe residue at P200 increased the drug susceptibility of a *C. elegans ben-1* mutant whereas alleles which encoded a Tyr residue at P200 did not. The functional significance of the P200 substitution was directly demonstrated by site-directed mutagenesis experiments. Replacing the Phe P200 codon with Tyr removed the ability of a β-tubulin allele from a susceptible strain to confer susceptibility to the *C. elegans ben-1* mutant (Kwa et al., 1995). Hence, in the case of the isotype-1 β-tubulin locus, persuasive association studies have been backed up with conclusive functional studies. Subsequent work has consistently shown an association of the P200 Phe to Tyr substitution with the benzimidazole resistance phenotype for a variety of parasite species including *Teladorsagia circumcincta*, *Trichostrongylus colubriformis* and *Cooperia oncophora* (Grant and Mascord, 1996; Silvestre and Humbert, 2002; Winterrowd et al., 2003). Quantitative PCR and pyrosequencing assays have now been developed to detect this polymorphism in parasite populations, providing the first example of a molecular-based diagnostic test for anthelmintic resistance (von Samson-Himmelstjerna and Blackhall, 2005; Coles et al., 2006). Clearly, the elucidation of the isotype-1 β-tubulin locus as a common determinant of benzimidazole resistance is a success story for the candidate gene approach and it is interesting to consider why this is the case. The key factor is probably that the hypothesis on which the candidate gene was chosen was particularly robust; β-tubulin was known to be the main target of benzimidazoles in a wide range of organisms from fungi and mutations in this locus, including the P200 substitution, were already known to give high levels of resistance in these organisms (Sheir-Neiss et al., 1978; Driscoll et al., 1989). Furthermore, exhaustive mutagenesis experiments that isolated 28 independent mutations giving rise to high levels of resistance to benzimidazoles, all mapped to the *ben-1* β-tubulin locus (Driscoll et al., 1989). This suggested that a β-tubulin gene was by far the most likely resistance gene for parasitic nematodes, albeit with the caveats of extrapolating model organism data to parasite populations in the field. As will be discussed below, the background information for choosing candidate resistance genes for macrocyclic lactones, or for that matter levamisole, is less clear cut.

In spite of the success story outlined above, it is clear that the P200 mutation in the isotype-1 β-tubulin gene is not the only mechanism involved in resistance to benzimidazoles. The earliest evidence for this involved a series of in vitro selection experiments with *H. contortus* (Kwa et al., 1993a,b; Roos et al., 1995). A susceptible population (SE) was exposed to benzimidazole in vitro such that 20% of the population survived and L3s derived from these were used to experimentally infect a sheep to produce the next generation, named RE1, which was then used for the next round of selection. This was repeated four times to produce a series of selected populations (RE1-RE4). Egg hatch assays showed that the level of benzimidazole resistance continued to increase at each round of selection with the ED50 (µg/ml) for each population being SE = 0.06; RE1 = 0.16; RE2 = 0.33; RE3 = 0.9; RE4 = 1.8. Since there was no significant change in the level of polymorphism of the isotype-1 β-tubulin gene, as determined by restriction fragment length polymorphism (RFLP) analysis or in the frequency of the P200 mutation between the RE2 and RE4 populations, other mechanisms must be involved to produce higher levels of resistance (Kwa et al., 1993a,b; Roos et al., 1995). Interestingly, a deletion of a second β-tubulin gene, isotype 2, was observed to occur between the RE3 and RE4 populations implicating this as a mechanism for higher levels of benzimidazole resistance and a similar observation was also made by independent workers (Lubega et al., 1994). Significantly, an isotype-2 β-tubulin deletion was also observed in a population of worms isolated directly from the field that had very high levels of benzimidazole resistance (Kwa et al., 1993a,b; Beech et al., 1994). However, at present, this is the only published example of a field population for which an isotype-2 β-tubulin deletion has been described and so its general importance is yet to be determined. Subsequent to these studies, another amino acid substitution in the isotype-1 β-tubulin polypeptide has been associated with the resistance phenotype; a Phe to Tyr substitution at P167 in *H. contortus*, *T. circumcincta* and equine cyathostomins (*Cyathostominae*) (Prichard, 2001; Silvestre and Cabaret, 2002; Drogemuller et al., 2004).

Examination of the *H. contortus* genome sequence (4.5-fold coverage) reveals there to be at least two β-tubulin genes in addition to isotype 1 and 2 which have yet to be investigated for a potential role in benzimidazole resistance (G. Saunders, C. Britton, J.S. Gilleard, unpublished data). In addition, recent work has shown that inhibitors of P-glycoprotein and cytochrome-P450 can increase the sensitivity of trichostrongyloid nematodes to benzimidazoles raising the possibility that drug metabolism and drug efflux mechanisms could also play a role in benzimidazole resistance (Kerboeuf et al., 2003) (L. Stenhouse, P. Skuce, J. Gilleard, F. Jackson, unpublished data). Hence, before molecular diagnostics can be meaningfully applied to the diagnosis and monitoring of benzimidazole resistance, further research is needed to determine the relative importance of different β-tubulin gene mutations and to investigate other novel resistance mechanism. In summary, the candidate gene approach has successfully identified several mutations in two different β-tubulin genes that appear to contribute to benzimidazole resistance in the field. However, alternative approaches will be needed to assess the quantitative contribution of these and to identify, or rule out, additional mechanisms of resistance.

4. The candidate gene approach applied to ivermectin resistance

The candidate gene approach has also been applied to investigate the mechanism of resistance to the macrocyclic
lactones, e.g., ivermectin and moxidectin, and the results of
these studies have been reviewed in detail elsewhere (Prichard,
2001; Wolstenholme et al., 2004). The same approach of
choosing genes considered likely to be involved in resist-
ance and examining the association of polymorphisms
with the resistant phenotype has been pursued. However,
the results have been much more ambiguous than those
for benzimidazole resistance. An increasing number of
genes has been implicated in ivermectin resistance in H.
contortus, predominantly by showing a reduction in poly-
morphism or selection of particular alleles in resistant pop-
ulations. These include the GluCl glutamate-gated
chloride channel subunit (Blackhall et al., 1998a,b), a γ-
aminobutyric acid (GABA) channel (Blackhall et al.,
2003), P-glycoproteins (Blackhall et al., 1998a,b; Sangster
et al., 1999) and a putative amino-acid gated anion channel
subunit HG1 (Prichard, 2001). More recently, it has been
suggested that a number of Onchocerca volvulus genes
may be under selection by ivermectin treatment including
P-glycoproteins (Eng and Prichard, 2005; Ardelli et al.,
2006a,b), β-tubulin (Eng and Prichard, 2005) and a variety
of adenosine 5′-triphosphate (ATP)-binding cassette trans-
porter genes (Ardelli and Prichard, 2004; Ardelli et al.,
2006a,b). Consequently, the overall impression is that
the mechanism of ivermectin resistance is complex with alleles
at several loci contributing to the resistance phenotype.
However, it is important to be aware that functional stud-
ies have not yet been used to confirm the importance of the
loci listed above, except in one case; the GluCl glutamate-
gated chloride channel subunit of the cattle nematode C.
onchophora. Differences in allele frequencies of this gene
were observed between a resistant and susceptible parasite
isolate but no such differences were observed for a GluClβ
subunit (Njue and Prichard, 2004). GluClz cDNAs, differ-
ing by three non-synonymous substitutions, were cloned
from ivermectin resistant and ivermectin sensitive C.
onchophora strains and expressed in Xenopus laevis oocytes (Njue
et al., 2004). The GluClz from the resistant strain was 2.5-
fold less sensitive to ivermectin on whole cell voltage clamp
recordings. Site-directed mutagenesis experiments
suggested that just one of the three substitutions (L256F)
was responsible for the loss of sensitivity. These detailed
experiments certainly implicate this polymorphism in the
GluClz subunit as a mechanism of ivermectin resistance
but the relatively modest changes in the sensitivity to iver-
mectin make its quantitative contribution unclear. Also,
this particular polymorphism has yet to be demonstrated
in other species of nematode showing ivermectin resistance.
Hence, in spite of a lot of independent studies, the
candidate gene approach has yet to identify a clear “front
runner” as the major gene contributing to ivermectin
resistance.

It is interesting to speculate on why this may be the case.
Perhaps the most important reason is that for ivermectin
resistance, unlike benzimidazole resistance, there is no sim-
ple single hypothesis on which to base an “educated guess”
about the identity of the most important resistance gene(s).
The key evidence on which predictions are based comes
from an understanding of the mode of action of ivermectin
and a knowledge of those genes in C. elegans which, when
mutated, can give rise to ivermectin resistance. Our current
understanding of the mode of action of ivermectin has been
reviewed in detail elsewhere (Yates et al., 2003) and so only
the points directly relevant to this discussion will be consid-
ered here. It has been demonstrated that the macrocyclic
lactones can interact with a number of different ion chan-
nels including glutamate-gated chloride channels (Cully
et al., 1994), GABA receptors (Adelsberger et al., 2000), n7 nicotinic acetylcholine receptors (Krause et al., 1998)
and P2X4 receptors (Khakh et al., 1999). For a number of
years, electrophysiological studies suggested that the
GABA receptor might be the major target (Martin and
Pennington, 1989; Holden-Dye and Walker, 1990). Howev-
er, both electrophysiological and genetic studies in C. ele-
gans now strongly suggest that it is the glutamate-gated
chloride channels which are the major target in nematodes
(Dent et al., 2000; Yates et al., 2003). In contrast to benz-
imidazole resistance, in which saturation mutagenesis of
C. elegans has only identified a single locus conferring resis-
tance (ben-1), the genetics of ivermectin resistance in C. ele-
gans are complex. Mutagenesis experiments have
uncovered over 30 different loci that can give rise to resis-
tance but at relatively low levels (Starich et al., 1995).
In a seminal piece of work, it was demonstrated that simulta-
eous mutation of three different C. elegans glutamate-gated
chloride channels, avr-14 (GluClα3), avr-15 (GluClα2)
and gc-I (GluClα1), confers extremely high levels of resis-
tance to ivermectin whereas mutating any two of these
genes does not (Dent et al., 2000). This suggests that each
of these receptor subunits are involved in parallel pathways
and it is proposed that avr-15 acts in pharyngeal muscle
whereas avr-14 and gc-I act in neurons regulating phary-
ngeal function (Dent et al., 2000). Since interaction of
ivermectin with any one of these subunits can inhibit phary-
ngeal pumping, each of the GluCl channels independently
confers ivermectin sensitivity and consequently
mutations in any one cannot confer resistance. Ivermectin
binding studies on C. elegans mutants showed that the
GluCl subunits encoded by these three genes accounted
for all of the high-affinity binding to ivermectin in C. ele-
gans extracts (Dent et al., 2000). Hence, both genetic and
biochemical studies suggest that these are the key targets
of ivermectin. However, it is clear that a variety of other
genes, that do not encode the targets of the drug, can
confer resistance in the C. elegans model. Two genes encoding
innexins, unc-7 and unc-9, confer resistance to ivermectin
(Boswell et al., 1990; Sedensky et al., 1994; Dent et al.,
2000). Innexins are components of invertebrate gap junc-
tions that are involved in the connectivity of the nervous
system and it is proposed that they allow ivermectin
induced hyperpolarisation to spread between different cells.
Hence, in unc-7 and unc-9 mutants the effects of ivermectin
may be localised to those cells expressing the targeted
GluCl receptors. Interestingly, when the unc-7 mutation
is combined with the \textit{avr-14}, \textit{avr-15}, \textit{gcl-1} triple mutant an even higher level of resistance is achieved suggesting the presence of at least one additional ivermectin receptor, albeit of lower affinity, which acts independently to the other major targets (Dent et al., 2000). Another category of mutants known to give rise to relatively low level ivermectin resistance are the Dyf ('Dye-filling') mutants (Starich et al., 1995; Dent et al., 2000). These are mutations which prevent the uptake of hydrophobic fluorescent dyes via the amphid sensory neurones and are thought to be resistant to ivermectin due to reduced drug uptake (Starich et al., 1995; Dent et al., 2000).

In summary, the information from \textit{C. elegans} mutagenesis experiments suggests a large number of genes can be mutated to give rise to low levels of ivermectin resistance but it requires multiple mutations in independent target genes to give high levels of resistance. Consequently, using \textit{C. elegans} studies and knowledge of drug mode-of-action to choose candidate resistance genes in parasites is far more complex for ivermectin than for benzimidazoles. Furthermore, the absence of fully sequenced genomes for the key parasitic nematode species makes it difficult and laborious to systematically test the large number of candidate genes implicated from \textit{C. elegans} studies.

5. Relevance of \textit{C. elegans} studies to ivermectin resistance in parasites

Chemical mutagenesis of \textit{C. elegans} and selection of resistant mutants in the laboratory is a very different process to the selection for anthelmintic resistant parasites by prophylactic and therapeutic drug use in the field. For example, mutations that can be tolerated by \textit{C. elegans} under optimal laboratory conditions may have severe fitness consequences for parasites in the field. Also, the genetic variation that is the raw material for selection in the parasite consists of polymorphisms arising naturally in the population rather than mutations “forced” by chemical mutagenesis. Consequently, whilst the candidate genes suggested by the \textit{C. elegans} work provide a useful starting point, it is quite possible that completely different mechanisms are important in parasites “in the field”. At present there is no clear view on the genetic complexity of ivermectin resistance in parasitic nematodes. Studies of different ivermectin-resistant parasite isolates suggest that there are phenotypic differences which may imply that there are several different mechanisms involved (Gill et al., 1998; Gill and Lacey, 1998; Sangster and Gill, 1999). For example, several different ivermectin-resistant \textit{H. contortus} isolates showed differences in the ability of avermectin to inhibit in vitro development and motility. These isolates also showed differences in their relative sensitivity to different members of the avermectin/milbemycin group. It has been suggested that a key factor in determining the mechanism of resistance might be the nature of the selection pressure used to produce resistance (Gill et al., 1998; Sangster and Gill, 1999). Gradual selection using serial experimental infections in the face of sub-therapeutic drug concentrations may be more likely to select for a multigenic basis of resistance. In contrast, more rapid selection using therapeutic concentrations of drug might be more likely to select for a single gene effect. At present this is largely speculation but the limited genetic analysis that has been undertaken on ivermectin resistant parasites supports this. Notably, genetic crossing experiments with the Chiswick avermectin resistant (CAVR) strain, which was isolated from the field as a resistant isolate and then subsequently further selected, are consistent with a single locus conferring resistance in a genetically dominant fashion (Dobson et al., 1996; Le Jambre et al., 2000). Reciprocal crosses between the CAVR and susceptible isolates showed that 100% of the F1 generation were fully resistant and larval development assays were consistent with 25% of the F2 generation being resistant (Le Jambre et al., 2000). However, more work is required to confirm the genetic basis of resistance in the CAVR strain and analysis on other resistant isolates is required to determine if resistance determined by a single major locus is a common phenomenon in parasitic nematodes.

6. Limitations of candidate gene association studies

There are several potential reasons why candidate gene studies have not yet identified a single locus that is clearly the major single determinant of ivermectin resistance in parasitic nematodes. It could be that the mechanism of ivermectin resistance is complex and multigenic, as appears to be the case in \textit{C. elegans}, with high levels of resistance being dependant upon the co-occurrence of mutations in several, or even a large number of, genes. Alternatively, it could be that only one or two loci are important but they have not yet been considered as candidate genes. This demonstrates the limitation of the candidate gene approach; by its very nature it will not identify “novel” genes responsible for resistance i.e., those genes not predicted by current hypotheses. In addition, if the wrong candidate genes are being studied, the data generated do not take us any further forward in understanding the genetics of resistance, e.g., how many loci are involved, their dominance relationships or differences in the genetic basis of resistance between isolates/species. There is also a significant problem in interpreting genetic association studies if our approach is limited to looking for associations of polymorphisms in candidate genes with resistance phenotypes in the absence of an understanding of the basic genetics and population genetics of the organisms in question. For example, it is important to know the extent to which genetic variation, based on neutral markers across the genome, is partitioned between the isolates being examined before the significance of differences in candidate genes can be determined. Also, it is important to know the extent of linkage disequilibrium surrounding the loci of interest in order to determine whether polymorphisms associated with resistance are closely linked to the resistance-conferring mutation or are a large physical distance away in the genome.
Consequently, whilst ongoing candidate gene studies are certainly worthwhile, new approaches are needed; namely the application of genomic and genetic approaches. These do not rely on a prior “educated guess” as to the identity of putative resistance genes and provide additional information to verify the significance of the results obtained. Research on insecticide resistance provides a very persuasive example of the power of these approaches, not only to identify resistance genes, but also to assess their relative importance to the resistance phenotype.

7. Lessons from insecticide resistance studies

7.1. Combining forward genetics and functional genomics

The elucidation of the molecular basis of drug resistance has progressed furthest in those systems that have good genomic resources and have forward and reverse genetic techniques to study gene function. The insect *Drosophila melanogaster*, like *C. elegans*, is a model organism predominantly used to study animal development that has also been used to study the molecular basis of insecticide resistance (ffrench-Constant et al., 2004). Although *Drosophila* itself is not a major pest, it has been exposed to insecticides in the field in a similar manner to many economically and medically important arthropod species. Consequently, *Drosophila* is a particularly valid model for the evolution and spread of insecticide resistance in natural insect populations. The study of insecticide resistance in *D. melanogaster* provides illuminating insights into the power of an integrated genetic and genomic approach to study drug resistance in a metazoan organism.

An early example of the value of forward genetics in studying insecticide resistance came from the identification of the GABA receptor as a major determinant of resistance to cyclodiene insecticides (ffrench-Constant et al., 1993). Field populations of *Drosophila* were isolated that were 4000-fold resistant to dieldrin and classical genetic studies revealed that resistance was conferred by a single genetic locus (ffrench-Constant et al., 1990; ffrench-Constant and Roush, 1991). Detailed genetic mapping determined that the gene responsible was *Rdl*, a GABA receptor widely expressed in the insect nervous system, and an alanine to serine or glycine substitution at position 302 was shown to be functionally capable of conferring resistance (ffrench-Constant and Roush, 1991; ffrench-Constant et al., 1993). Subsequent studies showed that the same mutation was present in resistant isolates of many different insect species including the whitefly (*Bemisia tabaci*), a cockroach (*Blatella germanica*), several species of beetle, a mosquito (*Aedes Aegypti*) and an aphid (*Myzus persicae*) (ffrench-Constant et al., 1998). Interestingly, this amino acid substitution is the only one in the Rdl gene that has been implicated in resistance (ffrench-Constant et al., 1998). This situation has some similarities to that of benzimidazole resistance in parasitic nematodes, where the β-tubulin P200 is considered to be a key mutation in a number of different species. However, there are some important differences to emphasise. First, it is known that the Rdl mutation is the single major cause of resistance in natural field populations of *Drosophila* due to the original genetic mapping studies. In contrast, although the P200 β-tubulin mutation is clearly an important determinant of benzimidazole resistance, its quantitative contribution to the resistance phenotype has not been assessed due to a lack of forward genetic methodologies in the parasites concerned. Second, the *Drosophila* work did not rely on any prior assumptions as to the identity of the resistance gene but instead, by the very nature of the genetic mapping approach, was capable of identifying the major genetic determinant of resistance no matter what its molecular identity.

An even more persuasive example of the benefit of using forward genetics and functional genomics is the identification of a major determinant of dichloro-diphenyl-trichloro-ethane (DDT) and multi-drug resistance in *Drosophila* (Daborn et al., 2002). In a situation reminiscent of the current ivermectin resistance research in parasitic nematodes, a large number of different insect genes had been implicated as being involved in DDT resistance (ffrench-Constant et al., 2004). However, classical genetic mapping of a mutagenesis-induced imidacloprid/DDT resistant strain and two field isolated DDT resistant strains of *D. melanogaster* demonstrated that resistance was conferred in a dominant fashion by a locus to the right of chromosome 2 at 64.5 cM (Daborn et al., 2001). Intriguingly, a cluster of genes encoding cytochrome P450 enzymes – molecules involved in detoxification of xenobiotics – were present in this region. Northern blot analysis revealed that just one of these genes, Cyp6g1, was overexpressed in several resistance strains examined. A genomic approach was then utilised to verify the significance of these results; a microarray was used to compare the levels of transcription of all the cytochrome P450 genes identified in the *Drosophila* genome between field isolated DDT-resistant and susceptible *Drosophila* strains (Daborn et al., 2002). Out of 90 cytochrome P450 genes on the array, only Cyp6g1 was upregulated in two independent DDT-resistant strains. Quantitative RT-PCR was used to demonstrate that the Cyp6g1 mRNA was 10- to 100-fold higher in 12 resistant strains compared with six susceptible strains. Further forward genetic mapping studies were then used to demonstrate that nitempyram and lufenuron resistance also mapped to the same locus. In order to determine if overexpression of cyp6g1 was both necessary and sufficient to confer DDT resistance, the protein was overexpressed in transgenic flies using the GAL4/UAS system. Transgenic overexpression, leading to an approximately 100-fold increase in Cyp6g1 transcript levels, conferred resistance of flies to DDT. It is clear that both a system of forward genetics and the availability of an annotated, largely complete, genome sequence was essential for the identification and verification of Cyp6g1 as a major determinant of multi-insecticide resistance.
7.2. Importance of population genetic studies

There are additional important lessons to be learned from these DDT resistance studies in insects. Sequencing of Cyp6g1 alleles from two DDT resistant field strains revealed no polymorphisms within the coding regions of the gene but identified an insertion of an Accord transposable element in the 5' flanking sequence of the gene. This is a fascinating, and largely unanticipated, mutational basis of resistance. The importance of this transposon insertion to DDT resistance in the field was verified by a diagnostic PCR assay which found the Accord insertion in the Cyp6g1 locus in all of the 28 resistant strains examined. Sequencing of the regions flanking the insertion revealed that all these resistance alleles belonged to a single clade, in contrast to alleles from susceptible populations which belonged to several different clades (Daborn et al., 2002). This suggests a model of a single mutational event producing the resistance allele which then spread by migration as opposed to arising from multiple independent origins. The population genetics of this association of the Accord transposable element with DDT resistance was then examined in further detail (Catania et al., 2004). More than 90 lines derived from 27 different populations of separate geographical origin were tested for DDT resistance and the presence of the Accord insertion. In all cases, the resistant lines contained the Accord insertion and the susceptible lines lacked it. The extent of linkage disequilibrium around this locus was then tested by examining microsatellite polymorphism along a 180 kb genomic region encompassing the Cyp6g1 gene. A significant reduction in variability was observed extending at least 20 kb downstream of the gene (Catania et al., 2004). This part of the work is noteworthy for several reasons. First, it illustrates the need for caution when trying to interpret associations of polymorphic markers with a resistance phenotype. If resistance has emerged relatively quickly, genetic recombination will have had insufficient time to dissociate linked markers from the resistance conferring mutation. Hence, markers adjacent to the resistance mutation, or in some cases even quite distant markers, may “hitchhike” and show reduced polymorphism and/or associations of particular alleles. This has implications for anthelmintic resistance studies in which single nucleotide polymorphisms (SNPs) in candidate genes show associations with the resistance phenotype. If resistance has emerged relatively quickly, genetic recombination will have had insufficient time to dissociate linked markers from the resistance conferring mutation. Hence, markers adjacent to the resistance mutation, or in some cases even quite distant markers, may “hitchhike” and show reduced polymorphism and/or associations of particular alleles. This has implications for anthelmintic resistance studies in which single nucleotide polymorphisms (SNPs) in candidate genes show associations with the resistance phenotype. In order to interpret such data, polymorphic markers covering large stretches of contiguous sequence around the locus need to be examined to determine the extent of linkage disequilibrium. This in turn requires a physical or genetic map, or even better, a fully sequenced genome. The second point worth noting from this study is that, if a fully sequenced genome is available, the “hitchhiking” phenomenon can be potentially used as a tool for identifying resistance conferring genes. By examining polymorphic markers across the genome in resistant and susceptible field populations, areas of reduced polymorphism may indicate regions of the genome under selection. This has been a valuable approach to study the genetic basis of chloroquine resistance in Plasmodium falciparum (Walliker, 2005). However, it is worth noting that, due to the huge population sizes of the trichostrongyloid nematodes, regions of linkage disequilibrium around a resistance locus may be relatively short which would mean very large numbers of densely spaced markers would needed for this approach. On the other hand, for parasites such as human filarial worms which have smaller population sizes and relatively long generation times, hitch-hiking mapping with wider spaced markers may be feasible. In any case, significant genomic resources will be needed for any such approaches to be applied to parasitic nematodes.

7.3. Resistance-conferring polymorphisms are not always SNPs

A final important lesson from the DDT resistance work is the identification of transposable elements as mutators that can give rise to insecticide resistance. Most of the current research on anthelmintic resistance works on the understandable assumption that SNPs, or deletions, in the coding regions of target genes are likely to be the major mutational mechanisms of resistance. However, nematode genomes are full of many different types of transposon (Vos et al., 1993; Le et al., 2001; Witherspoon and Robertson, 2003) and their potential involvement as potential mutator mechanisms in anthelmintic resistance has yet to be investigated. In the case of the Cyp6g1, the Accord transposon inserted near a regulatory element caused upregulation of expression of a metabolic enzyme. However, there are many potential mechanisms by which a transposon could alter the expression of resistance-associated genes (ffrench-Constant et al., 2006). These include a change in the expression pattern of a gene due to disruption of enhancer/suppressor elements, removal of regulatory elements by transposition elsewhere in the genome, insertion of a transposon in the 3' untranslated region affecting mRNA stability or alteration in the splicing pattern of a gene. A recent illuminating example of this latter mechanism was the demonstration that an insertion of a long interspersed element-like Doc transposable element into a predicted gene of unknown function (CHKov1) was associated with organophosphate resistance in D. melanogaster (Aminetzach et al., 2005). This transposon insertion results in an alteration of gene splicing such that two truncated transcripts are produced instead of one longer transcript. Since the function of the gene is unknown, the mechanistic basis of resistance is as yet unclear. In addition to alterations in gene expression, the open reading frame of genes can be disrupted either by insertion of a transposon or deletion following imprecise excision of a transposon near the target gene (Zwaal et al., 1993). All these data serve to illustrate that a thorough knowledge of an organism’s genome is essential to unravelling mechanisms of drug resistance and that completely unanticipated processes and genes may be involved. Once again a candidate
gene approach, based on association studies using SNP markers, is unlikely to unearth such phenomena.

The discussion of work on insecticide resistance in \textit{D. melanogaster} illustrates the power of genetics and genomics in identifying resistance genes and also investigating and confirming their importance. Much of this work was conducted on lines derived from \textit{D. melanogaster} populations that originally developed resistance to insecticides in the field. Consequently, it is likely that at least some of the mechanisms uncovered will be relevant to more economically and medically important species and current evidence certainly supports this (\textit{ffrench-Constant et al., 2004; Hemingway et al., 2004; David et al., 2005; Wondji et al., 2005}). The situation of anthelmintic resistance in parasitic nematodes is somewhat different since the only nematode with significant genomic resources and genetic tools at present is \textit{C. elegans}. Although this free-living nematode is an extremely useful tool for parasitic nematode research, it is not exposed to selection pressures by anthelmintics in its natural environment that are equivalent to those experienced by parasitic nematodes. Consequently, in order to apply the approaches outlined above in a way that is likely to identify resistance-conferring mutations relevant in the field, genomic and genetic resources need to be developed for some of the important parasitic nematode species themselves. This should be a major current research priority and is a prerequisite for significant future progress in anthelmintic resistance research. One parasite species that lends itself to such resource development is the sheep parasite \textit{H. contortus}. The important features of this parasite and current progress on the development of genomic and genetic resources will now be discussed.

8. \textit{Haemonchus contortus} as a model parasitic nematode for the study of anthelmintic resistance

There are a variety of reasons why \textit{H. contortus} lends itself to being a good model parasite system in which to study the phenomenon of anthelmintic resistance. It is one of the parasitic nematode species for which anthelmintic resistance is most widespread and most intensively studied to date (Prichard, 2001). It is also a close relative of many of the other important parasitic nematode species of grazing animals where anthelmintic resistance is a growing problem (Kaplan, 2004). In addition to this, it is one of the more experimentally tractable parasitic nematode species of economic or medical importance. Being a parasite of sheep and goats, it is possible to undertake experimental work in its natural host rather than rely on in vitro work or the use of “artificial” laboratory animal hosts. In contrast, studies of human or cattle helminth parasites in their natural hosts are limited by ethical and cost considerations, respectively. \textit{H. contortus} is extremely fecund, with a single female producing up to 4000 eggs per day, making it easy to generate large amounts of parasite material and to undertake genetic crosses (Coyne and Smith, 1992). The relatively large size of the adult allows DNA prepared from a single worm to be used as template for thousands of PCR-based genotyping assays which is a crucial requirement for molecular genetic studies. Furthermore, there are simple methods for L3 cryopreservation in which viability and infectivity are retained allowing parasite isolates, inbred lines and genetic cross progeny to be archived (Gill and Redwin, 1995). Another key strength of \textit{H. contortus} is its relatively close phylogenetic relationship with \textit{C. elegans} (Blaxter et al., 1998). This is important for two reasons. First, \textit{H. contortus} is well placed to benefit from comparative genomic studies with \textit{C. elegans}, which should help both in genome annotation and in extrapolation of experimental data from \textit{C. elegans}. Second, \textit{C. elegans} has proven utility as a heterologous system for the functional expression \textit{H. contortus} genes (reviewed in Gilleard, 2004). This means that genes implicated in conferring anthelmintic resistance can be functionally tested using the \textit{C. elegans} system (Kwa et al., 1995). This is a crucial element since tools for studying gene function are very limited for parasitic nematodes. Although there has been some recent progress in developing RNAi for \textit{H. contortus} there are still formidable problems for reverse genetic analysis in this and other parasite species (Geldhof et al., 2006). Hence, it is likely that functional expression of genes in \textit{C. elegans} will remain an important research tool for parasitic nematodes in the foreseeable future. The \textit{H. contortus} research community is one of the largest in parasitic nematode research and studies a wide a variety of aspects of basic and applied biology. Consequently, \textit{H. contortus} is one of the most attractive parasite species for the development of genomic and genetic resources, particularly for the study of anthelmintic resistance.

9. \textit{Haemonchus contortus} genetics: Current status and research priorities

The development of forward genetic analysis in \textit{H. contortus} would provide a powerful approach to study the mechanisms of anthelmintic resistance. It would allow the genetic basis of resistance to be investigated, including the number of loci involved, their degree of dominance and their relative contribution to the resistance phenotype. More importantly, once detailed genome maps and the full genome sequence are available, forward genetic mapping of resistance loci by linkage analysis will be possible allowing the identification of resistance conferring mutations in a manner similar to that described for insecticide resistance. Such genetic mapping is routinely used in model organisms such as \textit{C. elegans} and \textit{D. melanogaster} for which robust genetic methodologies and associated genomic resources are well established. The challenge is to develop such approaches for parasitic nematodes. Although it would be naïve to suggest that parasite genetics is likely to reach the same levels of sophistication as model organism genetics, there is considerable potential once a number of technical issues have been addressed. Genetic crossing of trichostrongylid nematodes is certainly possible and has
been practiced, albeit to a limited extent, for at least 30 years (Le Jambre, 1977, 1981; Le Jambre et al., 1979, 1999, 2000; Martin et al., 1988; Martin and Pennington, 1989; Sangster et al., 1998). Most of these studies have been performed using *H. contortus* (Le Jambre, 1977, 1981; Le Jambre et al., 1979, 1999, 2000, 2005; Sangster et al., 1998) but successful crosses with *T. colubriformis* have also been achieved (Martin et al., 1988; Martin and McKenzie, 1990; Le Jambre et al., 2005). These studies have predominantly been performed to assess the degree of dominance of the resistance phenotype and to determine whether resistance appears to be conferred by a single locus. In general, mating methods have involved surgical implantation of adult male worms from one strain and adult female worms from another into the abomasum of a recipient sheep host. F1 progeny are then assessed for their resistance phenotype by either in vitro and or in vivo assays and then used to orally infect recipient sheep to produce the F2 generation. Back crosses between F1 progeny and parental strains have also been performed (Le Jambre et al., 1979, 1999). Differing results have been obtained with different parasite species and drug classes which has led to a variety of hypotheses; thiabendazole resistance of *H. contortus* is suggested to be an autosomal multigenic semi-dominant trait (Le Jambre et al., 1979), ivermectin resistance a dominant trait (Roos et al., 2004). Although this is clearly an important limitation of these early genetic studies is that they were “ahead of their time” because there were few genetic markers with which to characterise parental strains, monitor and analyse crosses and there were certainly no genomic resources with which to develop genetic mapping approaches. Nevertheless, these studies provide an extremely valuable “proof of concept” and have developed the basic approaches and information that should allow more sophisticated forward genetic approaches to be developed.

As well as genetic crossing methodologies, there are several other key resources that need to be developed before forward genetic analysis of *H. contortus* becomes a reality. First, large numbers of robust polymorphic genetic markers need to be identified and mapped onto the genome. Some microsatellite and SNP markers are already available (Hoekstra et al., 1997; Otsen et al., 2000a,b, 2001). However, there are only a small number of these and there is no information regarding their relative positions in the genome. One of the aims of the *H. contortus* mapping and sequencing project, discussed in the final section of this review, is to place large numbers of polymorphic markers onto the physical map of the genome. As well as the development of these genetic marker resources more information about the genetic variability of the parasite is needed. A number of studies suggest that there is a tremendous amount of genetic variation both within and between *H. contortus* populations (Otsen et al., 2000b, 2001; Prichard, 2001; Troell et al., 2003). However, many of the laboratory strains have yet to be genetically characterised and there is no published system of monitoring their integrity during experiments or for comparing strains between different experiments and laboratories. A standardised approach to this is urgently needed. Recently, the high level of genetic variation between strains has enabled us to develop a system of rapid genetic fingerprinting of strains using microsatellite markers (J.S. Gilleard, E. Packard, V. Grillo, F. Jackson and L. Redman, unpublished data).

The high level of genetic variation between different *H. contortus* isolates bodes well for forward genetic analysis in this parasite since it suggests that the parental strains of a genetic cross will be sufficiently divergent to enable maternal and paternal alleles to be distinguished in F2 progeny. However, one problem that needs to be surmounted is the high level of genetic variation within each strain. This is a confounding issue for linkage analysis, particular if mass matings are to be used. One potential solution is to develop inbred strains and an approach which has been previously adopted is to infect a sheep with the L3 progeny from a single female *H. contortus* adult worm and repeat the process through several generations (Otsen et al., 2000b, 2001; Roos et al., 2004). Fifteen generations of inbreeding significantly reduced the level of polymorphism as measured by amplified fragment length polymorphism (AFLP), RFLP of several loci and a microsatellite locus (Roos et al., 2004). Although this is clearly an important step towards developing strains suitable for genetic crosses there are still remaining problems to solve. The approach is very labour intensive and protracted; even after eight generations of inbreeding the level of polymorphism was only minimally reduced. Also, the level of polymorphism within each strain, even after 15 generations of inbreeding, is still likely to cause problems with analysis of genetic crosses particularly when based on mass matings. The relative inefficiency of this inbreeding approach is probably due to the fact that a single adult *H. contortus* female generally contains progeny from several different males which will tend to maintain the levels of polymorphism in spite of inbreeding (V. Grillo, E. Packard, J.S. Gilleard, F. Jackson, unpublished data). One solution to this would be to develop systems of single pair mating which would make inbreeding strategies much more efficient and this should be a research priority. In addition, if genetic crosses were based on single pair matings, many
of the problems of within strain polymorphism would be avoided.

A final requirement to make forward genetic analysis of anthelmintic resistance a reality is the ability to accurately determine the resistance phenotype of individual worms. Most studies at present rely on assays such as the egg hatch assay and larval development test that define resistance status at a population level; an ED_{50}, an ED_{90} or a discriminating dose for the population of worms being examined is the result of these assays. Reliable methods that can identify individual worms as having a susceptible or resistant phenotype are needed so that marker genotypes and resistance phenotypes can be reliably correlated in F2 populations of genetic crosses.

The above discussion has focussed on current research priorities that are necessary to enable meaningful genetic characterisation of anthelmintic resistant parasite strains and to undertake genetic mapping studies to identify resistance loci. Although it is beyond the scope of this review, it is important to note that genetic resources are also needed for population genetic studies on the parasite. As discussed earlier, a detailed understanding of the geographical variation of the parasite and the extent of linkage disequilibrium in different populations is needed in order to interpret candidate gene association studies and to study the origin, evolution and spread of anthelmintic resistance.

10. *Haemonchus contortus* genomic resources

The major effort in parasitic nematode genome resource development over the last decade has focused on expressed sequence tag (EST) sequencing (Parkinson et al., 2004). Although these are extremely cost-effective and valuable resources that provide a massive amount of information for gene discovery, they inevitably represent an incomplete and biased sample of the genome. Consequently, full genome sequence is needed if functional genomic approaches such as microarray analysis, proteomics, comparative genomics and forward genetics are to be fully exploited. Unfortunately, full genome sequencing projects for parasitic nematodes lag behind those of most other pathogen classes. The majority of the most important bacterial and viral pathogens now have fully sequenced genomes and this is increasingly the case for protozoal pathogens (Gardner et al., 2002; Berriman et al., 2005; Pain et al., 2005). Consequently, it is imperative that full genome resources are developed for many of the important human and veterinary helminth parasites so that parasitic helminth research is not left behind in the post-genomic era. In the case of anthelmintic resistance research, it should be apparent from the above discussion that genomic and genetic approaches are entirely dependant on the availability of full genome sequence and associated bioinformatic and genetic resources.

The large genome size of parasitic helminths has often been considered a disincentive to full genome sequencing projects. However, one could argue that this has been overstated. Parasitic nematode genome sizes vary from $0.5 \times 10^8$ to $3 \times 10^8$ bp with the trichostrongylid nematodes such as *H. contortus* being at the lower end of this range (Leroy et al., 2003). Consequently, the technical and economic considerations for parasitic helminth sequencing should be no more daunting than for some of the larger protozoal genomes; the *Eimeria tenella* and *P. falciparum* genomes are $0.65 \times 10^8$ and $0.23 \times 10^8$ bp, respectively (Gardner et al., 2002) (http://www.sanger.ac.uk/Projects/E_tenella/).

The human filarial nematode parasite *Brugia malayi* is the parasitic nematode with the most advanced genome sequencing project to date (Ghedin et al., 2004) with approximately 8-fold coverage of shotgun sequence completed (http://www.tigr.org/tdb/e2k1/brma/new.shtml). The *H. contortus* genome project is the next most advanced and is being undertaken by the Pathogen Sequencing Unit of the Wellcome Trust Sanger Institute, Cambridge, UK (http://www.sanger.ac.uk/Projects/H_contortus/). The aim of the project is to produce a fully finished (no gaps), assembled and annotated *H. contortus* genome sequence by a combination of shotgun sequencing and clone-by-clone BAC/fosmid sequencing. The ISE strain, designated mHco1, has been used to prepare template DNA for genome mapping and sequencing (supplied by Dr. Marleen Roos and Dr. Fred Borgsteede). This strain was chosen because it was the most highly inbred genetically characterised strain available (Roos et al., 2004). Use of an inbred strain is potentially extremely important to minimise problems of genome assembly, particularly for an organism with high levels of polymorphism. 4′-6-Diamidino-2-phenylindole (DAPI) staining of metaphase spreads of cells from early embryos suggests that the ISE strain karyotype consists of 12 evenly sized chromosomes (J.S. Gilleard and J. Smith, unpublished data). The presence of only 11 chromosomes in some embryos is consistent with an XX/XXO basis of sex determination and so the karyotype appears similar to that of *C. elegans*. Genomic DNA from a single worm, or a small number of worms, has been used to make short insert plasmid libraries and approximately 300 Mb of shotgun sequence is now available for Blast searching or ftp downloading at http://www.sanger.ac.uk/Projects/H_contortus/. Bac and fosmid libraries have been constructed and physical mapping by BAC fingerprinting is in process. Approximately 10,000 BAC-end sequences are also available for downloading or Blast searching. Assembly data on the 300 Mb of shotgun sequence suggests this represents approximately 4.5-fold genome coverage which would make the *H. contortus* genome size approximately 80 Mb.

An independent approach to physical mapping is also being undertaken in the form of HAPPY map construction (J. Pachebat, J. Gilleard and P. Dear, unpublished data). This method – so-called because it uses haploid equivalents of DNA and the polymerase chain reaction – allows construction of a map based on the relative position in the genome of sequenced tagged sites (STS) without the need for DNA cloning (Dear and Cook, 1993). This avoids a variety
of potential artefacts and is ideal for an organism with few pre-existing genomic resources. The method involves random breaking of DNA and the determination of linkage of STS markers. Sheared DNA is diluted into aliquots that each contain a haploid genome equivalent and each aliquot is then screened for the presence or absence of STS markers using PCR. Linked markers tend to occur more frequently in the same aliquots than non-linked markers. Hence, if a “mapping panel”, consisting of 96 aliquots of sheared DNA, is screened with several thousand STS markers, the map order and distance between markers can be calculated from the frequency with which the different markers “co-segregate”. This approach has been successfully used to map the genomes of several organisms and human chromosomes (Dear et al., 1998; Piper et al., 1998; Konfortov et al., 2000). The \textit{H. contortus} HAPPY map being constructed aims to comprise of approximately 3000 STS markers. These include over 2000 BAC end sequences, several hundred ESTs, several hundred microsatellites and a variety of genes of interest including candidate genes potentially involved in anthelmintic resistance. The HAPPY map will give an overview of synteny between the \textit{H. contortus} and \textit{C. elegans} genomes and provide valuable mapping data to aid the assembly of the genome sequence. It will also provide an important resource for the forward genetic mapping approaches described earlier in this review as well as for population genetic studies.

The aim of these current efforts is to provide a complete, fully annotated genome sequence for \textit{H. contortus}. This will allow functional genomic approaches, already making a massive impact in other areas of biology, to be applied to \textit{H. contortus} and related parasites. In addition to the applications to anthelmintic resistance research that have been described in this review, genomic resources are clearly central to other areas of research on these parasites, including vaccine and drug discovery, diagnostics, molecular epidemiology and the study of basic parasite biology. Consequently, development of these resources should be a major research priority for the parasitology research community and research funding agencies if parasitic nematode research is to benefit from the tremendous promise of the post-genomic era.

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