Chapter 84
Drug Resistance Assays for Parasites

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1 Introduction

Drug resistance has become an increasingly serious and widespread problem worldwide, impacting across a broad phylogenetic range of important medical and veterinary parasite species. These include parasites from the groups of the Apicomplexa, the flagellates and the helminths. Accurate measurement of resistance is vital for several reasons. In addition to making decisions on effective drug use for parasite control, resistance assays (sometimes called sensitivity assays) are also used to monitor prevalence, severity and dissemination of resistance and to evaluate the impact of control regimes on resistant parasites. In some cases they are used to assist the choice of drugs in clinical situations. A review of the assays currently available and knowledge of the information they provide gives a vital signpost of our current situation across a variety of parasite species and also as a guide in the design of future assays for resistances yet to develop.

Resistance assays consist of various in vivo and in vitro tests. The in vivo tests include various assays with the definitive host species and within experimental host species. There is in an even greater variety of in vitro assays available. These include assays which measure survival of parasites in the presence of drug, phenotypic assays (that measure a mechanism of resistance) and genetic assays (using DNA probes that rely on knowledge of the genetic basis of resistance). The selection of an appropriate assay type and design depends on several factors. One important factor is the prevalence and severity of resistance.

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1.1 Establishment, Development and Dispersal of Resistance

Sutherst and Comins (1) describe three components to the genesis of resistance. The first is establishment. This is largely a random event influenced by the population size and diversity and the mutation rate for the gene(s) in question. The second step is development. In this process the use of the selective agent (e.g. the chemical) allows resistance to develop but the prevalence of resistant alleles is too low for resistance to be clinically apparent. In the third step, dispersal, there is further selection and spread of the resistance genes through the wider population of the organisms. At this phase clinical resistance (also termed field resistance) first appears. The processes of development and dispersal are influenced by biology, management and chance events such as linkage disequilibrium and gene dispersal via an intermediate host. These processes are driven by drug selection, reflected in survival and subsequent reproduction of parasites following drug treatment.

1.2 Sensitivity, Specificity and Level of Resistance

Fundamental parameters for all diagnostic assays are their sensitivity and specificity (not to be confused with drug sensitivity). The sensitivity of an assay is defined as the probability that the presence of resistance would be correctly identified, in resistance studies sensitivity is then used to refer to how early or at what level of gene frequency resistance is detected. The sensitivity that is required of an assay depends on the purpose. When resistance is emerging, assays with high sensitivity (e.g. 0.99) are required to detect small increases in prevalence.

Specificity is the probability that a test can correctly identify that resistance is not present. In practice, species identification in resistance assays is often the factor that limits

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specificity (and sensitivity) and drives the need for simultaneous resistance assays and species identification.

The level of resistance is the degree of resistance in a parasite population. It might be quoted as a percentage of drug efficacy or an EC_{50} on a test (see below).

1.3 Challenges

To be useful, a resistance assay must be able to accurately detect and reflect the frequency of resistance in a population. Various challenges arise in attempting to achieve this aim.

The first important challenge is to be able to detect resistance in the development phase when prevalence of resistant alleles is low but still at a point where further development may be avoided by management. Genetic tests offer the possibility of detecting resistance at this early stage but these require detailed knowledge which is rarely available, especially before resistance is widespread.

Another important challenge is to validate in vitro assays, against the in vivo environment to ensure they provide meaningful clinical data.

Even when good tests are available, the source of the resistant isolates for testing and validation remains an important issue. This is relevant when comparisons of isolates from different geographical regions or between laboratory and field isolates are being made. Resistant lines may be developed in the laboratory by subjecting the parasites to increasingly higher concentrations of drug with each generation, often by in vitro exposure. These isolates are sometimes referred to as 'laboratory' isolates. Possible pitfalls in this approach are that resistant laboratory isolates may differ from field isolates and one field isolate may differ from another, which may lead to the development of inappropriate assays and validation against atypical isolates.

Another challenge is that the nature of resistant populations may differ. For example, some parasitic infections, especially the protozoa that divide asexually, may become near clonal, because all progeny develop from the few survivors of drug treatment. On the other hand, populations of parasitic worms are highly genetically diverse.

Finally, tests have to be available in a timely fashion. There is little point in developing and applying a test for resistance when the prevalence of resistance is greater than 90%.

1.4 Mechanisms of Resistance

A summary of the drug actions and resistance mechanisms of the more important parasites for which resistance is a clinical or field problem appears in Table 1.

2 Assay Methods to Detect Resistance

The general properties of assays follow. Specific assays are listed in Table 2 and some are highlighted in further sections.

2.1 In Vivo Bioassays

2.1.1 In Vivo in Definitive Host

This is the traditional approach to resistance detection and most direct as the data reflects the therapeutic response to a drug. Responses (or efficacy) are usually calculated from numbers of the parasite in a host sampled both before and after the application of treatment. For animal herds, an untreated control group can be compared with an equivalent treated group.

Limitations of this approach include the need for knowledge of efficacy of the drug at the dose rate used in the host species against susceptible and resistant isolates, the cost of maintaining hosts or sampling of patients, the preliminary creation of parasite-free hosts and, in the case of malaria, differentiating between resistance and recrudescence caused by reinfection. An additional concern is the ethics of euthanasia of mammals (say, for worm counts) and the ethics of sampling humans, especially if some individuals remain untreated.

2.1.2 In Vivo in Experimental Hosts

If available, experimental host species can provide greater convenience and cost effectiveness when testing for drug resistance, especially in determining dose–responses. Essential steps in developing such assays are to validate the similarity of the host/parasite relationship between the host and experimental host and the congruence of drug pharmacokinetics.

2.2 In Vitro Assays

2.2.1 Survival (Effect) Assays

In vitro assays have the advantage of being useful to detect resistance without interference by host factors. Generally, they involve exposing individuals of a certain stage of the parasite's life cycle to a set concentration within a range of concentrations of the drug and observing an endpoint, reflecting parasite survival after an appropriate incubation time.

Table 1 Major drug treatments, mode of action and known resistance mechanisms

Parasite and host	Drug/example (abbreviation)	Mode of action	Putative mechanism of resistance
Plasmodium in humans	Aminoquinolones/chloroquine, quinine, mefloquine	Alkalises parasite food vacuoles and prevents lysosomal activity that is essential for haemoglobin degradation	For chloroquine resistance – *Pfcrt* gene – K76T[a] transition has the most consistent correlation with resistance – *Pfmdr1* gene – N86Y transition leading to active efflux pump Mefloquine resistant parasites have multiple copies of *pfmdr1* alleles and a drug efflux phenotype
	Antifolates: Dihydrofolate reductase inhibitors/pyrimethamine proguanil – synergistically with dihydropteroate synthase inhibitors/sulphadoxine, dapsone	Synergistic combinations of antifolates inhibit the utilisation of folate by inhibiting dihydrofolate (dhfr) reductase and dihydropteroate synthase (dhps) Folates are essential for synthesis of pyrimidines and thus DNA	Linked with mutations in *dhfr* and *dhps* *Dhfr* A S108N transition is the initial step rendering the enzyme insusceptible to the drug *Dhps* Transitions S436F, A436F, A437G, K540E, L540E, A581G, A613S, A613T and a mutation at 586 have been reported in resistant parasites
	Sesquiterpenes/artemisinin	Concentrated in RBCs, action may involve damage to the parasite membrane by free radicals or covalent alkylation of proteins Artemisinin inhibits the Plasmodium SERCA ortholog (2)	Multidrug resistance (drug efflux) like phenomenon
	Diamidines/pentamidine	Accumulation within food vacuole where it binds avidly to ferriprotoporphyrin (FPIX), blocks haemozoin crystallization	
	Tetracyclines/doxycycline	Binds ribosomes to inhibit protein synthesis	
Eimeria in chickens	Ionophores/salinomycin	Act on trophozoite and sporozoite. Opens ion channels in cell membranes leading to collapse of membrane potential and nutrient transport	Unknown
	Sulphonamides/sulphadimidine and trimethoprim	Act on second stage and later schizonts Act on nucleotide synthesis as p-aminobenzoic acid (PABA) agonists (synergised with dhfr inhibitiors) and inhibits DNA synthesis (see Sect. 3.1 for details)	Unknown
	Quinolones/decoquinate	Acts on sporozoite. Dihydro-orotate DH inhibitors that prevent synthesis of purines required for DNA replication	
	Pyridones/clopidol	Acts on sporozoites through disruption of electron transport	
	Other anticoccidials	Robenidine – interrupts guanine use in DNA synthesis, inhibits oxidative phosphorylation Nicarbazin – energy metabolism in mitochondria Toltrazuril – inhibition of mitochondrial respiration and nuclear pyrimidine in DNA synthesis	
Trypanosoma in ruminants	Phenanthridinium compounds/ Isometamidium	Cleavage of kinetoplast DNA – topoisomerase complexes	– Changes in the mitochondrial electrical potential – Nucleoside transporters involved
	Diamidines/diminazene	Binds to kinetoplast DNA and inhibits topoisomerase II and RNA editing (3)	Point mutations in P2 adenosine transporter
	Homidium salts	Interference with: – Glycosomal functions – The function of an unusual AMP binding protein – Trypanothione metabolism – Replication of kinetoplast minicircles (3)	Unknown but may be a change in nucleoside transporters

(continued)

Table 1 (continued)

Parasite and host	Drug/example (abbreviation)		Mode of action	Putative mechanism of resistance
Trypanosoma in humans	*Trypanosoma brucei*	Organic arsenical/ melarsoprol	Not yet established	Loss of a P2 amino-purine transporter (4, 5) and probably other mechanisms
		Ornithine analogue/ eflornithine	Suicide inhibitor of ornithyl decarboxylase (ODC) (6) which is the rate-limiting enzyme for the synthesis of polyamines from ornithine (7)	Unknown but various mechanisms suggested (7)
		Diamidines/ pentamidine	Binds to kinetoplast DNA and inhibits topoisomerase thus interfering with kinetoplast replication (8) May cause inhibition of multiple cellular targets (9)	Changes in drug transporters, including the P2 aminopurine transporters and the High Affinity and Low Affinity Pentamidine transporters HAPT1 and LAPT1(4, 5, 10–12)
		Naphthalene derivative/ suramin	Target uncertain although has been shown to inhibit many enzymes (13)	Unknown but several mechanisms suggested (7)
	Trypansoma cruzi	Nitroimidazole/ benznidazole	Covalent modification of macromolecules by nitroreduction intermediates (reductive stress) (14)	
		Nitrofuran/nifurtimox	Reduction of the nitro group creates a highly reactive free radical followed by a reactive oxygen species then leads to death (7)	Changes in the enzyme or transporter (7)
Leishmania in humans	Polyene antibiotic/amphotericin B (used as a second-line drug)		Acts preferentially on ergosterol – the predominant sterol in membranes	Resistant parasites showed increased membrane fluidity with changes in lipid composition including an ergosterol precursor
	Pentavalent antimonials/sodium stibogluconate (SbV) Meglumine antimoniate		Activated in vivo. May be associated with inhibition of glycolytic enzymes but also depends on effective host immunity	Depends upon: – Transformation of pentavalent compound to trivalent compound – Formation of a thiol conjugate by unknown conjugase/transferase – Drug extrusion by elevated levels of MRP
	Diamidines/pentamidine		Accumulate within parasites leading to disintegration of the network of kinetoplast DNA and collapse in the mitochondrial membrane potential	Efflux pumps appear to operate in removing cytosolic or membrane-associated drug
	Paromomycin/aminosidine (aminocyclitol-aminoglycoside antibiotic)		Binding to the endoplasmic reticulum may be involved	
	Azoles/itraconazole Ketoconazole		Inhibitors of cytochrome P-450-dependent lanosterol C14 α-demethylase (a step in ergosterol biosynthesis)	Overexpresion of squalene synthase confers itraconazole resistance
	Metronidazole		Itraconazole inhibits an enzymatic activity several steps downstream of squalene synthase in ergosterol biosynthesis	
	Nucleoside analogues pyrazolopyrimidines/ allopurinol			Differences in the affinity of enzymes of the purine salvage pathway
	Alkyllysophospholipids/miltefosine		Lipid biosynthetic enzymes may represent a target	For miltefosine resistance – Point mutations in a P-type *ATPase* (15)
	Diminazene and Homidium Salts – see *Trypanosoma*			
	5-Nitroimidazoles, bezimidazoledrugs and substituted acridines – see *Giardia*			

Giardia in humans	5-Nitroimidazoles/metronidazole, tinidazole, secnidazole	Prodrug reduced by ferredoxin to active nitro compound in the parasite. Toxicity via depletion of SH groups and DNA strand breaks (16)	Multifactorial – Reduced drug activation through decreased activities of pyruvate:ferredoxin oxidoreductase and/or ferredoxin essential for activation (16) – Drug transport changes (16) – Gene rearrangements (17)
	Benzimidazole drugs/albendazole (ABZ)	Binds tubulin, inhibiting microtubule assembly which is important for the adhesive disk (18)	– ABZ-resistant cells have enlarged median bodies suggesting increased microtubule synthesis – No evidence for mutation in *Giardia* tubulin genes (18)
	Substituted acridine/quinacrine HCl	– Taken up and accumulated – Acts on membrane (16) perhaps by inhibition of the activity of cytoplasmic NADH oxidase (19)	– Drug transport changes (membrane blockade or active efflux) – Actively excluded from resistant trophozoites (16)
	Nitrofuran/furazolidone	Reduction of drug by NADH oxidase to toxic nitro radical (19)	
Trichomonas in humans	5-Nitroimidazoles/metronidazole tinidazole	See *Giardia* and (20)	Multifactorial – (Laboratory isolates) down-regulation of hydrogenosome pyruvate:ferredoxin oxidoreductase and ferredoxin transcription – (Clinical isolates) altered drug transport pathways (20)
Entamoeba in humans	Emetine	Unknown	Multidrug resistant transporters involved (21)
	Metronidazole	Prodrug activated by reduction of nitro groups by ferredoxin (22)	Increased superoxide dismutase activity may detoxify active radicals (23)
	Iodoquinol, diloxanide furoate, paromomycin, tetracycline, chloroquine	Tetracycline is presumed to act via inhibition of protein synthesis	
Trichostrongyloids in sheep	Benzimidazole (BZ)/albendazole	Disrupts cellular integrity by specifically binding to parasite tubulin	Selection of alleles of β-tubulin in two steps: (a) Isotype 1 – involving a F200Y transition (F167Y is also implicated) (b) Loss of isotype 2
	Imidazothiazoles and tetrahydropyrimidines/levamisole (LEV)	Cholinergic agonists	Unknown, but may include alterations in receptor subunits, selective expression of subunit genes or modulation of receptor
	Macrocyclic lactones (ML)/ivermectin (IVM)	Opens glutamate-gated Cl^- channels on muscles to inhibit pharynx and the somatic musculature	Several candidate genes including *GluCl* receptors
	Salicylanilides/closantel (CLS)	Uncoupling oxidative phosphorylation, lowering cytoplasmic pH and inhibiting glycolysis	
Fasciola in sheep	Salicylanilides/closantel	See Sect. 3.7	
	Benzimidazoles/triclabendazole	Thought to bind to fluke β-tubulin with effects on tegumental synctium and cellular integrity	
Schistosoma in humans	Praziquantel (PZQ)	Putatively acts on voltage-gated Ca^{2+} channels (β subunits) causing increased intracellular [Ca^{2+}] and paralysis	May involve residues at the interface between the α and β domains of the Ca^{2+} channel (24)

[a]Within this table amino acid substitutions are written in the form K76T meaning Lysine is replaced by Threonine at residue number 76

Table 2 Examples of available resistance assays

Parasite and host	Drug/example	In vivo	Survival (dose–response)	Phenotype (site of action)	Genotype
Plasmodium in humans	4-Aminoquinolines/ chloroquine	In vivo drug trial with recommended dose rate (defined by WHO in terms of parasite clearance). See text	Inhibition of *P. falciparum* growth and multiplication in presence of antimalarial drug in vitro: 1. WHO microtest: schizont maturation 2. Metabolic endpoint such as isotope incorporation, colorimetric enzyme assays which reflect parasite numbers: – Parasite lactate dehydrogenase – DELI assays – Histidine-rich protein 2 assay 3. Dipsticks based on immunoquantative Plasmodium proteins: – LDH – DELI – Histidine-rich protein 2 assay		AS-PCR and PCR-RFLP – *Pfcrt* base transition causing K76T[a] is the most consistent finding Also S72C, M74I, N75E, H97Q, A220S, E271Q, N326S, N326D, I356T, I356L, R371I, R371T – Pfmdr1 gene including substitutions at positions 1034, 1042, 1246
	Antifolate combination: e.g. Pyrimethamine/ sulphadoxine				AS-PCR and PCR-RFLP *dhfr* transition S108N plus some of: A16V, S108T C50R, C50I, N51I, C59R, V140L, I164L, *dhps* S436F, A436F, A437G, K540E, L540E, A581G, A613S, A613T and a change at 586
	Mefloquine				1. TC-PCR for increased (e.g. twofold) gene-copy number of the *Pfmdr1* gene 2. *Pfmdr1* substitutions in various alleles, e.g. N86Y, 184, 1034, 1042
Eimeria in poultry	Anticoccidials: – Isometamidium (ISMM)	Feed efficiency trials measuring growth rates of infected birds versus infected and treated birds			
Trypanosoma in ruminants	– Diminazene – Homidium salts	– Test ability of drugs to protect cattle under experimental or natural challenge using parasitaemia as endpoint – 'Block treatment test' of cattle under natural challenge – Test in mice (single or multi-dose test)	– *T. brucei:* incubate metacyclic or bloodstream *T. brucei* in vitro with drugs: survival endpoints used – All trypanosomes: drug incubation followed by Glossina infectivity – Metabolism of the dye Alamar Blue by live cells to generate both a colorimetric and a fluorescent signal (31)		PCR-RFLP *TcoAT1* gene (resistance to diminazene)

	Only for isometamidium (ISMM)	ELISA for isometamidium in combination with parasite detection tests (32)		Mitochondrial electrical potential (MEP) assay (33)	PCR-RFLP *TbAT1 gene* and other gene(s)
Trypanosoma in humans	Antitrypanocidal drugs	In vivo assessment of drug sensitivity in mice	– Metabolism of the dye Alamar Blue by live cells to generate both a colorimetric and a fluorescent signal (31)		
Leishmania in humans	Anti*leishmania* drugs especially pentavalent antimonials (SbV)	BALB/c mice infected with *L. donovani* treated with 1. Miltefosine ED_{50} = 7.5 µM 2. SbV ED_{50} = 7.6 µg SbV/ml (no data published on resistant strains) (34)	Amastigote/macrophage culture assay for SbV resistance *L. donovani* RF = 3 (35) Also promastigote assay and the axenic amastigote culture assay. See text		
Giardia in humans	5-Nitroimidazoles/ metronidazole tinidazole	Shift of ID_{50} for metronidazole based on parasite numbers in infected mice (36). RF = 2–4	MIC (metronidazole) for survival in culture over 2 days (37) RF = 5–8	Assay for decreased PFOR activity in resistant isolates (38) RF = negative 2–3	Associated chromosome duplications
	Quinacrine	–	ID_{50} 0.2–2.93 µM (39) for susceptible	Drug excluded from resistant trophozoites	
	Benzimidazoles/ albendazole (ABZ)	ID_{50} for ABZ in mice from 9–53 mg/kg (susceptible) (36). RF = 2–10	MIC of 3 µM (susceptible) (37) RF = 4	Drug causes cytoskeleton distortion; resistant trophozoites have enlarged median body but otherwise appear normal	
	Nitrofuran/furazolidone	ED_{50} furazolidone in mice 13.5 mg/kg in mice (susceptible) (40)	ID_{50} 0.3–1.7 µM furazolidone (39)		
	Nitazoxanide		MIC on culture of 12 µM nitazoxanide over 2 days, 50 µM (susceptible) (16) – IC_{50} of 8 µM for nitazoxanide compared with 15 µM for metronidazole (41)		
	Paromomycin	ED_{50} of 7.6 mg/kg (susceptible) (40)			
Trichomonas in humans	Nitroimidazoles/ metronidazole		Aerobic assay over 2 days[b] – metronidazole MIC 25–50 µM (susceptible) (37) RF = 4–8		Down-regulation of hydrogenosome function
Entamoeba in humans	Emetine		IC_{50} for survival in culture >2 µM (42)	Threefold increased expression of *Pgp* in resistant lines (43)	Up-regulation of Pgp mRNA levels
	Metronidazole		MIC in culture of 12.5 µM (susceptible) (37) RF = 2	Increased superoxide dismutase activity in resistant lines (23)	Up-regulation of superoxide dismutase mRNA levels

(continued)

Table 2 (continued)

Parasite and host	Drug/example	In vivo	Survival (dose–response)	Phenotype (site of action)	Genotype
Trichostrongyloids in sheep	Anthelmintics benzimidazole (BZ)	– FECRT[b] <95% efficacy and <90% LCL scored as resistant	– Larval development assay. [b]RFs for BZ = <70; LEV = >50; IVM = <10) (28)	Radioactive BZ-benzimidazole tubulin binding (BZ)	Benzimidazole – F200Y β-tubulin transition by AS-PCR and pyrosequencing
	levamisole (LEV)	– Treat and slaughter test	– Egg hatch Assay (LEV)		F167Y is also screened
	Macrocyclic lactones (ML)		– Egg embryonation (BZ)		
	Salicylanilides (SAL)		– L3 motility (BZ, MLs)		
			– Larval paralysis (LEV)		
			– Larval migration assay (SAL) RF = <10		
Fasciola in sheep	Salicylanilides	Rat infection, sheep treat and slaughter trial			
		RF = 2–4			
	Triclabendazole	Treat and slaughter trial			
		RF = >7			
Schistosoma in humans	Schistosomicidal Drugs especially praziquantel (PZQ)	Clinical cure rate	Miracidial morphology (46) and survival analysis in the presence of PZQ	Muscle contraction studies (47) and [$^{45}Ca^{2+}$]-uptake (48)	Subtracted PCR – overexpressed mRNA coding subunit 1 of the cytochrome *c* oxidase (49), significance uncertain
		Worm count in treated rodents (44)	RF = 2		
		RF = 3 (45)			

MIC minimum inhibitory concentration; RF resistance factor; PCR polymerase chain reaction; LCL lower confidence limit

[a]Within this table amino acid substations are written in the form K76T meaning Lysine is replaced by Threonine at residue number 76

[b]Assays applied in field for treatment decisions

Examples of end points are maturation, evidence of multiplication, viability or motility, measured by techniques such as parasite enumeration (e.g. visual counting), fluorescent activated cell sorting (FACS) or a feature linked to viability or parasite mass. Where ranges of drug concentrations are employed, a dose–response line can be generated and parameters such as effective dose to kill 50% (ED_{50}) calculated and compared with values from known susceptible parasites. Valid application of in vitro assays relies on co-expression of resistance traits in the life-cycle stages used in vitro and the parasitic stages.

2.2.2 Phenotypic Assay

Phenotype assays are those that measure a resistance phenotype related to the site of resistance. Unlike survival assays, phenotype assays require knowledge of the mechanism of resistance. Some of the types of phenotypes that could lead to resistance are

1. *Alteration or loss of a drug receptor* such as benzimidazole resistance in trichostrongyloids.
2. *Reduced drug levels* through decreased uptake or increased removal (or sequestration) of drug, e.g. mefloquine resistance in *Plasmodium falciparum*.
3. *Reduced concentration of active drug* could occur through decreased drug activation or increased drug metabolism, e.g. metronidazole resistance in *Giardia*.
4. *Alteration of a protein with an action upstream or downstream of the target site* – may result in physiological changes that prevent transduction of the effect, e.g. *Leishmania* resistance to itraconazole.

In parasitology, few of these types of tests are practical. However similar tests are commonly applied in insecticide resistance where detoxifying enzyme activity is a common mechanism of resistance.

2.2.3 Genetic Assay

Genetic tests offer the potential for very sensitive detection of resistance but in order to design and use gene probes, the genetic basis of resistance must be known. Moreover, the mechanisms must be the predominant one for a particular parasite/drug combination.

A number of PCR-based methods have been developed to identify resistance genotypes. The types of assays that are used in this chapter are described below:

1. For single base pair transitions (point mutations) allele-specific PCR (AS-PCR) have been developed. At its simplest, this depends on designing one of the two primers used in the PCR with the 3′ base complementary to one (e.g. resistant) and not the other (e.g. susceptible) allele. An amplification product is obtained only when polymerisation is initiated on both strands (in this case for resistant parasites).
2. PCR-restriction fragment length polymorphism (PCR-RFLP), involves PCR amplification of the region flanking the polymorphism followed by the cleavage of the PCR product with a restriction enzyme that cleaves only the specific (e.g. resistant) allele.
3. PCR-sequence specific oligonucleotide (PCR-SSO) probing involves PCR amplification of the region flanking the polymorphism. The resulting DNA fragments are spotted onto membranes, and resistant sequences are identified by hybridisation with short probes corresponding to resistance sequences (25).
4. Tandem Competitive PCR (TC-PCR) is designed to determine the gene-copy number (26). Tandem-competitive PCR is a refinement of competitive PCR and employs a common competitor molecule containing a tandem array of single copies of competitor sequence for several targets. The copy number of the candidate gene can be compared with a gene with a single copy number from the same parasite. It is especially useful for genes such as multidrug resistance genes (e.g. P-glycoprotein, Pgp).
5. A possible new advance is Fluorogenic PCR assays (FCR) (27). They allow a high throughput analysis of mutation hotspots and are particularly useful in polyclonal infections, allowing for both a real genotyping (which alleles are present in a same parasite) and for a quantification of the different genotypes.

While at present most assay endpoints involve the detection of specific size DNA fragments on agarose gels, future applications may use real-time PCR, melting point analysis or hybridisation (e.g. PCR ELISA) to automate endpoints. Molecular beacons and microarrays may find favour in future applications. Molecular tests also offer the advantage that it may be possible to simultaneously detect resistance and differentiate parasite species.

2.3 Sampling

Correct sampling, including sample size and randomisation, is an important consideration in assays for resistance. Sample size requirements will depend on several factors. In herd animals there is generally an aggregated distribution of parasites between hosts. To overcome possible sampling errors, samples from several animals are used to generate mean values. For instance the faecal egg count reduction test (FECRT) is the most common way to detect clinical resistance in helminths in sheep. Calculations are performed by comparisons

of the means derived from faecal egg counts in groups of 10–15 animals each having at least 200 eggs per gram of faeces. If infections are comprised of mixed species then even larger sample sizes are required.

In contrast, in human parasitology we are often concerned with detecting resistance in an individual or a group of individuals. In cases where parasite populations are near clonal fewer samples are required both in terms of parasites and hosts. For example, in malaria sample volumes can be low, ~50 µl (the equivalent of a blood-drop) for molecular analyses such as PCR.

For 'non-clonal' parasite populations the widespread use of molecular diagnosis may be limited by sampling and sample throughput. For example, in order for a molecular assay to be sensitive enough to determine that the prevalence of resistant alleles is less than 1% in the field with 95% confidence, more than 300 alleles have to test negative. With current technology this means more than 150 diploid parasites must be subjected to PCR (28).

A significant dilemma is whether samples should be taken pre-treatment or post-treatment. If samples are pre-treatment they should reflect the true prevalence of resistance. If the samples are post treatment the chances of finding resistant parasites increases and, so too, does the sensitivity of tests.

2.4 Analysis of Data

2.4.1 General

The measurement of resistance is often scored as survival of parasites following a treatment and so parasite numbers (or values that reflect parasite numbers) are commonly recorded. These values are then analysed statistically (e.g. using *t* tests or *F* tests) and comparative efficacies generated. Resistance may also be recognised as a reduction in the period of protection that a persistent treatment provides. This provides different types of data and may be amenable to survival analysis.

Resistance assay outputs are quoted as either the proportion of resistant parasites within a population of parasites (population prevalence) or the proportion of regions where resistance is present (e.g. community, farm or shed prevalence). For example, if 25 out of 100 parasites survived a treatment, population prevalence would be 0.25. On the other hand, if 20 out of 50 farms studied have parasite resistance then there would be a farm prevalence of 0.40.

The latter requires categorical results that can be provided either by a breakpoint (e.g. efficacy of treatment less than 90%) or the presence/absence of a gene known to confer resistance.

By measuring the effect against a representative sample of parasites at each concentration over a range of drug concentrations, such as in an in vitro assay, a plot of response to treatment against dose can be generated. From this the EC_{50} can be calculated. The EC_{50} is the effective concentration or dose of drug that affects 50% of the parasite population. Similar expressions include the ID_{50} (inhibitory dose), LD_{50} (lethal dose), LC_{50} (lethal concentration), and CD_{50} (curative dose). The MIC (minimal inhibitory concentrations) (or MLC, minimum lethal concentration) of a drug is another way to compare isolates. Several of these terms are used in this chapter to describe assay results and are used interchangeably in general descriptions.

The ratio of EC_{50} values between resistant and known susceptible populations of the same species is known as the resistance factor (RF). See Table 1 for known examples of these.

2.4.2 Analysis

Calculations of EC_{50} can be done by converting dose to log dose or ln dose and by plotting these against the response (e.g. % dead) converted to logits or probits. This usually results in a straight line, from which EC_{50} can be read. Modern computer analysis has enabled more accurate curve fitting, as well as automated calculations of EC_{50} and standard errors. Examples include programs such as GraphPad PRISM, and SAS routines. Figure 1 provides an example of a dose–response from an in vitro assay together with calculations of EC_{50} and RF. It also illustrates the shift of the dose–response line to the right in resistant isolates.

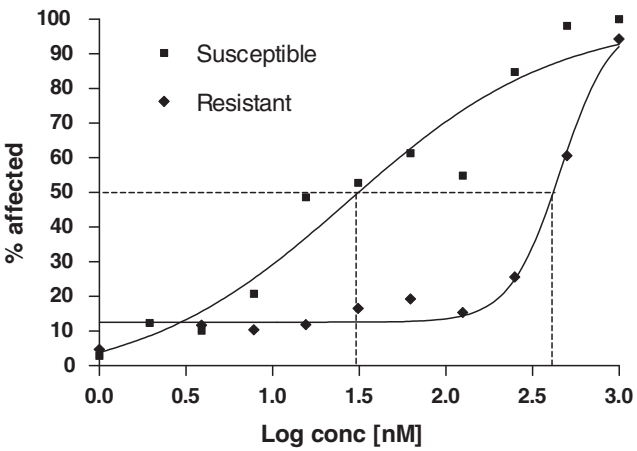

Fig. 1 Concentration/response curves for a larval development assay (LDA) with *Trichostrongylus colubriformis*. Percentage (%) affected refers to failure to develop in the presence of IVM – aglycone. Curves and EC_{50} were plotted and calculated using Graphpad Prism – nonlinear regression (curvefit). EC_{50} for the susceptible isolate was 28 nM (95% confidence interval range of 7–104) and for the resistant isolate was 459 (95% confidence interval range 397–531). The RF is therefore 16.4

2.5 Artefacts

While treatment failure may indicate drug resistance, this is just one cause of treatment failure. Other causes include misdiagnoses of another mimicking aetiological agent, inappropriate drug choice for the causal parasite, rapid reinfection after treatment and recrudescence of the same infection. Under-dosing through underestimates of body weight or reduced concentration of active drug are other possibilities (29). One example is where the faster metabolism in goats results in poorer efficacy of antiparasitic compounds compared with sheep (30).

3 Parasites

3.1 Plasmodium

Infection of humans with *Plasmodium* parasites, especially *P. falciparum* results in the disease malaria. Drug-resistance in *P. falciparum* (and to a lesser extent *Plasmodium vivax* (50)) to the commonly used antimalarial drugs – chloroquine, pyrimethamine/sulphadoxine and mefloquine – is one of the greatest challenges facing the control of malaria (51, 52). We will focus this section on assays developed to measure drug resistance in *P. falciparum*. These assays are unlikely to be useful in determining the outcome of individual treatment, but are required for surveillance of the prevalence of drug resistance and to assist in the shaping of policies to limit drug resistance.

3.1.1 In Vivo

The presence or absence of parasites following treatment is measured by obtaining patient blood samples at the time of treatment, and then on days 7, 14 and 18 following treatment, and by assessing parasitaemia using light microscopy. Guidelines have been drawn up by the WHO to standardise these assays (53). Therapeutic response is graded as either sensitive, or with three degrees of resistance: RI (parasitaemia clears 25% below the original, but returns within 28 days of the treatment), RII (parasitaemia decreases to between 25 and 75% of the original parasitaemia, but then begins to increase by day 7 following treatment) and RIII (parasitaemia does not decrease to less than 75% of the original parasitaemia).

Because reinfections may occur in the study period a modified set of criteria based on clinical outcome has been proposed: (a) adequate clinical response, (b) early and (c) late treatment failure (54). Genetic typing can add a level of sophistication and help to distinguish between true recrudescence and reinfection but this is time-consuming and expensive (55). Other limitations of in vivo testing include the fact that therapeutic failure could be due to inter-individual differences in drug metabolism in the host, the degree of acquired or innate immunity, and variations in the quality of the drug preparation. Further, in many endemic areas there can be huge logistic difficulties associated with following up patients for 28 days.

3.1.2 Survival: In Vitro

A major advantage in the study of *P. falciparum* is the ability to maintain this parasite in short- and long-term cultures. Short-term culture is useful for drug sensitivity assays and continuous culture within the laboratory (56) has permitted both the study of drug-resistance phenotypes as well as the elucidation of the genetic basis of antimalarial resistance.

Typically in vitro assays start with the newly invaded ring stage of the parasite, which is obtained when a peripheral blood sample is drawn from an infected patient. Fresh *P. falciparum* isolates contain only ring-stage parasites, as other mature stages are sequestered to internal tissues. Parasite growth in each sample is measured in the presence of different amounts of the antimalarial drugs. The simplest method to monitor growth is the measurement of schizont maturation through scoring on microscope slides. Nevertheless, this method is laborious and confounded by subjective decisions on the maturity of the parasites.

More common are the methods based on the completion of a complete parasite life-cycle (57). These assays are suitable for all blood-stage drugs irrespective of their stage of action. Typically, serial dilutions of a drug are made in 96-well plates, to which the parasitised cells are added. These plates are incubated at 37°C for between 48 and 72 h (the longer times permit the analysis of slower-acting and cytostatic drugs such as tetracycline and pyrimethamine). Several methods have been devised to measure growth in these assays. The simplest form is the WHO in vitro microtest (58) based on counting Giemsa-stained parasites by light microscopy. Automated endpoints are ones such as incorporation of radiolabelled metabolic precursors (e.g. [^{3}H] hypoxanthine or isoleucine, as a correlate of growth (59)) or immunoquantitation of the parasite lactate dehydrogenase (LDH) (60) or the Histidine-Rich Protein II (HRPII) (61). The more developed assay is known as the double-site enzyme-linked LDH immunodetection (DELI) assay (62) which uses specific mAbs and an ELISA plate reader as an indirect measure of parasite number and hence growth.

3.1.3 Genetic

Much effort has been focussed on elucidating the genetic basis of resistance to antimalarials in *P. falciparum* (reviewed in (63)). The polymorphisms responsible for resistance are either point mutations or gene amplifications and numerous PCR-based methods have been developed to identify the genetic differences. These include AS-PCR (64), PCR-RFLP (65), PCR-SSO probing (25), and TC-PCR (26).

Although the genetic assays are technically demanding, the samples (blood on filter papers) can be processed at a central facility that possesses the required equipment and technical expertise. A prerequisite for the use of resistance alleles in assays, is the knowledge of associations between the specific allele and resistance. For certain alleles conflicting reports of associations have been obtained, which may reflect epidemiological or methodological differences. Another complicating factor is that in regions of higher transmission, isolates often harbour multiple clones and, consequently, genotypes. Once strong universal associations have been obtained, these methods will prove to be very powerful.

3.1.4 Specimen Collection

The conduct of in vivo tests entails considerable logistics and follow-ups. For in vitro tests, blood samples from infected patients are obtained by venipuncture into tubes with anticoagulants. These samples are then washed and placed into short-term culture assays as soon as possible.

Genetic tests are attractive as they require just a pin-prick of blood, collected onto filter papers. DNA in these samples is stable and can be sent to reference laboratories for processing.

3.1.5 Practical Tests, Clinical Significance and Breakpoints

Choosing which of the available tests to use will depend on the expertise available, as well as the required outcome. Arguably, the degree of in vivo resistance is the type of information likely to influence a change in the drug policy. However, as in vivo tests are logistically difficult to conduct, in vitro assays and genetic tests are gaining support. Once the infrastructure is in place they are easier to conduct and the DELI assay obviates the use of radioactivity. Due to the time required to conduct these assays relative to the speed of disease progression, it is unlikely that these assays will aid in the diagnosis of resistance within individuals. Instead, they are essential in determining the level and prevalence of resistance in the population, which may help to influence policy aimed at maximizing the efficacy of the available antimalarial drugs.

3.1.6 Artefacts

A major confounder of in vivo tests is the presence of immunity, acquired and innate, in certain individuals. Immunity will act to clear resistant parasites as well as sensitive ones, hence leading to underestimations of the level of resistance. In contrast, in areas of high transmission, reinfection with new parasites in the course of a drug treatment can be high. This does not reflect resistant parasites but new infections, leading to overestimates of resistance. Additionally, there may be considerable differences in the metabolism of drugs by different individuals and between populations, as well as differences in the stability and purity of the drugs used in the different trials.

Microscopic methods utilised within the assays are not only laborious, but may also lead to erroneous determinations of parasitaemia as a measure of drug response due to the subjectivity involved in assessing parasitaemia microscopically. Additionally, when dealing with field isolates, many do not grow in culture for reasons other than antimalarial toxicity, and in many cases the rate of reinvasion is much lower than that observed for culture-adapted laboratory isolates, increasing the difficulty in counting parasites.

We also noticed previously a severe ‘inoculum effect’ in antimalarial testing in vitro (66), emphasizing the need to control the number of red cells, and the parasitaemia at which the drug tests are being set up.

3.2 *Eimeria*

Coccidiosis caused by several species of the genus *Eimeria* is a major cause of production loss in the chicken industry. Despite resistance being common in several species of *Eimeria* in chickens, drugs such as the ionophores commonly achieve sufficient control in the field.

Diagnosis of resistance is generally performed by infecting groups of birds with suspect resistant *Eimeria* and comparing growth rates and pathology in these birds with those in birds infected with susceptible *Eimeria* in the presence of drug treatment. Resistance in in vivo tests is reported as an anticoccidial index (67) which is calculated using a combination of weight gain, % bird survival, lesion score and oocyst count. Indexes >160 indicate that the isolate is sensitive and those <120 are considered resistant. This approach means diagnosis is delayed to after the point where treatment decisions have already been made.

In order to achieve rapid diagnosis new in vitro tests are essential. The development of such assays would require a significant breakthrough in achieving the in vitro cultivation of the obligate parasitic stages of the life cycle. Understanding the biochemical or genetic mechanisms of resistance to

anti-coccidials would also assist in test development and are high priorities for researchers.

3.3 Trypanosoma (Ruminants)

Trypanosomosis is a serious disease of domestic and some wild ruminants in Africa and has a major impact on cattle production. The major species which cause disease are *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei*. Chemotherapy is the principal form of control and widespread use of the phenanthridinium compound isometamidium (ISMM), the diamidine diminazene aceturate and homidium salts, which have been on the market for more than 40 years, has led to resistance (68).

Drug-susceptible isolates are maintained in laboratories where they are used for comparison with field isolates. Investigations into resistance mechanisms have been assisted by the selection in the laboratory of isogenic lines, resistant and susceptible to drugs but with the same genetic background.

3.3.1 In Vivo

Resistance to ISMM can be assessed under natural *Trypanosoma* challenge in the field using the 'block treatment' approach (69). Two groups of infected cattle, one treated with 1 mg/kg ISMM and one untreated control group (each consisting of 30–80 animals) are exposed to natural challenge and tested for the presence of trypanosomes in blood using the phase contrast buffy coat technique (70) every 2 weeks for 3 months. If more than 25% of ISMM-treated cattle become infected within 8 weeks of exposure, drug resistance is strongly suspected (69).

In what is currently an experimental approach, the use of an ELISA for the detection of ISMM in the serum (32) can be combined with the 'block treatment' or individual treatment of ruminants to detect resistant trypanosomes. The presence of trypanosomes in animals with an ISMM serum concentration >0.4 ng/ml suggests that parasites are resistant (71).

It has been shown that longitudinal parasitological field data can be suitably analysed in order to detect problems of resistance to diminazene aceturate (72, 73).

3.3.2 In Vivo (Experimental Animals)

A standardised test is available for the detection of resistance to ISMM or diminazene in experimentally infected mice or cattle (74). The mouse test (preferably using immunosuppressed mice) can only be used for *T. brucei* or *T. congolense* (mice are refractory to *T. vivax*), whereas cattle are susceptible to all three species of trypanosomes. The single dose 1 mg/kg ISMM or 20 mg/kg diminazene test in mice is useful for screening a large number of trypanosome isolates, say, from regional surveys. Two groups of six mice are infected (100,000 trypanosomes inoculated intraperitoneally) and one group is treated 24 h after infection and the other group is left untreated. The multi-dose test in mice includes one control group and at least five treatment groups given discrete doses over the range 0.01–20 mg/kg ISMM or 1–60 mg/kg diminazene. The mice are checked for parasitaemia twice a week for 2 months. Because mouse tests cannot be used to predict the curative dose for ruminants, standardised tests in cattle (or sheep/goats) are necessary to confirm the efficacy of recommended curative doses of trypanocidal drugs in particular circumstances in the field (74).

3.3.3 Survival: In Vitro

In vitro tests using bloodstream or procyclic trypanosomes can be used to detect resistance to the above-mentioned drugs in *T. brucei* and *T. congolense* (31, 75–77). A major disadvantage of these tests is the slow adaptation of the trypanosomes to the culture conditions (78). An alternative approach is the *Glossina* infectivity test (DIGIT) to measure drug resistance in *T. congolense* (79). The main limiting factor for the latter test is the availability of tsetse flies.

3.3.4 Phenotypic Assay

It has been suggested that variations in the mitochondrial electrical potential (MEP) might be the primary factor determining the rate of ISMM accumulation in the trypanosome kinetoplast (33). Initial studies on a limited number of *T. congolense* populations have shown that an increased or a decreased MEP might be a candidate for being a quantitative marker for ISMM susceptibility or resistance, respectively.

3.3.5 Genetic

Some genetic markers are available for the detection of drug resistance in animal trypanosomes. PCR-RFLP tests allow the detection of *T. congolense* and *T. brucei* isolates resistant to ISMM (80, 81) or to diminazene (82). Apparently, more than one resistance mechanism to ISMM exists in *T. congolense* because the PCR-RFLP is not able to detect all strains which are identified as resistant in in vivo tests (80).

3.3.6 Specimen Collection

Blood of animals infected with *T. congolense* or *T. brucei* can be inoculated intraperitoneally into mice (74). In order to minimise selection of sub-populations from the original isolate only two or three passages should occur in mice before carrying out the drug resistance tests. If drug tests cannot be carried out immediately, the trypanosomes should be cryopreserved in liquid nitrogen for later use.

3.3.7 Practical Tests, Clinical Significance and Breakpoints

The most common tests for the detection of trypanocidal drug resistance are the tests in mice or in ruminants. Mouse and cattle tests are generally well correlated in detecting resistance in isolates, but the curative dose cannot be predicted in mice (81).

The major drawback of in vivo tests is their long time course (100 days). Therefore, the in vivo tests are gradually replaced by the molecular tests, which are able to identify drug resistant isolates within a few days.

3.4 Trypanosoma (Humans)

Drug resistance is a major problem in human African trypanosomiasis (HAT) (8, 84) particularly against melarsoprol where treatment failures have increased to up to 30% (85, 86). In order to measure resistance, suspected resistant isolates (87) need to be compared with known sensitive strains in the laboratory. The success of this is limited by the selective pressure on what could be a mixed population isolated for in vitro culturing and *T. b. gambiense*, for example, adapt poorly to culture conditions and achieve low parasitaemia in primary infections of laboratory rodents.

In vivo assessment of drug sensitivity in mice (see Sect. 3.3.2) is still the accepted standard for assessing drug resistance of African trypanosomes. An in vitro method that is reproducible and sensitive is based on the metabolism of the dye Alamar Blue by live cells to generate both a colorimetric and a fluorescent signal (31). Typically 10^4 trypanosomes in 100 µl medium are added to 100 µl of doubling dilutions of the test drug in 96-well plates. After 24 h, the dye is added and incubated for an additional 48 h to yield reliable IC_{50}s.

3.5 Leishmania

Leishmaniasis is caused by several *Leishmania* species, with *L. amazonensis*, *L. donovani*, *L. infantum* and *L. braziliensis* being the most pathogenic. There are cutaneous and visceral forms of disease. Basic treatment of leishmaniasis consists of a first line of pentavalent antimonials (SbV) such as sodium stibogluconate (SSG) and meglumine antimoniate. Alternative treatments are pentamidine, paromomycin (aminosidine) and amphotericin B. A new oral treatment, miltefosine (alkylphosphocholine) has now also been proven to be an effective treatment for visceral leishmaniasis (88).

Clinical unresponsiveness to SSG is being increasingly reported in endemic areas (89). Although immunological, physiological or pharmacological deficiencies in the host might explain the observed variations in clinical response (90). There is also evidence that the development of resistance of the parasite contributes to treatment failure (35, 91–93). SSG seems to have a dual action mode: (a) SSG is converted to SbIII, which in turn has direct leishmanicidal activity (94), and (b) SSG stimulates infected cells to produce microbicidal compounds such as reactive oxygen species and nitric oxide which kill the intracellular parasites (95).

Several mechanisms have been reported for experimentally induced resistance as reviewed by Croft et al. (96) but their role in natural SbV resistance is not straightforward (97). Furthermore, population genetic approaches on natural *Leishmania* populations have suggested that the parasite has a pleiomorphic response to the pressure exerted by the first-line treatment with SSG in natural conditions (98). Therefore, neither a genetic nor a phenotypic assay is as yet available to determine SbV-susceptibility. In vivo and survival assays are currently the only available tools to assess drug activity on a given isolated *Leishmania* strain and there are benefits in using them sequentially. It is important to make comparisons within the same *Leishmania* species, as the susceptibility to a given drug may vary between species (99).

3.5.1 In Vivo (Experimental Animal)

Hamsters and immunodeficient mice are commonly used as laboratory hosts for infection with *Leishmania*, although non-rodent hosts like dogs may give more accurate information on the likely drug activity in humans (100). In vivo assays provide information on host factors such as absorption, metabolism and pharmacokinetics of the drugs, which are not accounted for in the in vitro screens.

Infections can be induced with either amastigotes or promastigotes, although amastigotes give the highest chance of a successful infection. For viscerotropic species, the animals can be inoculated by intrasplenic, intraperitoneal, intravenous or intracardiac routes. Drug activity following treatment is assessed by evaluation of the parasite burdens either by microscopic counting of amastigotes in the spleen or liver impression smears (101) or by limiting dilution culture of infected tissues, a more sensitive method (102, 103). Recently, a real-time PCR assay was developed to improve the detection and quantification of *Leishmania major* in

mouse tissue (104). The data are statistically analysed to calculate the dose needed to reduce the parasite load of the treated group to 50% (ED_{50}).

Dermotropic species, on the other hand, are initially inoculated intradermally, thereby mimicking the lower temperature of natural skin infections (105). For cutaneous leishmaniasis, the lesion diameter varies with the species (from a few mm in *L. braziliensis* to a few cm in *L. amazonensis*). Periodic measurement and analysis of the mean lesion diameter following treatment allows for evaluation of drug efficacy.

3.5.2 Survival: In Vitro

Promastigotes

Promastigotes, the vector form of *Leishmania*, can be readily cultured in cell-free media (like GLSH (106)) and is therefore the easiest to use in drug resistance assays. Drug sensitivity is assessed by incubating numbers of logarithmic phase promastigotes with discrete dilutions of drugs over a range of concentrations for 48 h at 26°C. Enzymatic activity (ornithine decarboxylase or acid phosphatase) in the resulting culture correlates to the parasite numbers which then allows for EC_{50} to be determined (107, 108). However, this assay has limited applicability because promastigotes are less susceptible to the SbVs than the intracellular amastigote form. Since *Leishmania* exists naturally the vertebrate host as the amastigote it is more suitable to base drug sensitivity assays on an amastigote-macrophage model.

Amastigote-Macrophage Models

Several coculture models have been reported using a range of different types of host cells, including: (1) a Sticker dog sarcoma cell line (fibroblasts), (2) transformed rodent macrophage cell lines like P388D1 and J774, (3) primary isolated mouse peritoneal macrophages, (4) human monocyte-derived macrophages, (5) human cell lines like U937 and THP-1. The requirements of cell cultures mean this type of assay is clearly limited in its use, because it is costly, time-consuming and technically challenging. Typically, amastigote-infected cells are maintained in a medium with serial dilutions of drug or without drug (control) for 4–7 days, at 37°C for viscerotropic species and at 33–35°C for dermotropic species. Drug activity is then assessed by counting the number of amastigotes/100 host cells in drug-exposed infections or by flow cytometry and compared with the control infections (109). The EC_{50}, is then computed by regression analysis (59, 110). Recent studies question the validity of the in vitro survival models for prognosis of in vivo SSG therapy outcome (111, 112) This is likely due to the stimulating effect of SSG on the infected macrophages, which might be different in vitro, in the absence of any immune components, than in vivo. The value of in vitro SSG susceptibility assays might be further enhanced by including some cytokines to mimic the synergistic effect of the immune system in vivo.

Axenic Amastigote Systems

Promastigotes collected from patients can be transformed to axenic amastigotes by reducing the pH of the medium to 5.5–6.5 and elevating the temperature of cultivation (113). So far, axenic amastigote cultures have been reported for *L. mexicana*, *L. donovani*, *L. major*, *L. amazonensis* and *L. braziliensis*, with some being more amenable to culture than others (114, 115). Axenic amastigotes are seeded at an initial concentration equivalent to early log phase (2×10^5 amastigotes/ml) and allowed to multiply over 3–4 days either in the medium alone (controls) or in the presence of serial dilutions of the drug. The number of amastigotes is then determined by counting or enzymatic assays (116–118) and the EC_{50} is calculated.

Measured resistance in amastigote systems is 3–4 times lower than in amastigote/macrophage systems. This difference might be caused by activation or concentration of the drug within the parasitised macrophage. Because the axenic amastigote assay is technically simpler, low cost, faster and uses the clinically relevant stage of the parasite, it promises to be a useful assay for rapid screening (119).

3.6 Giardia, Trichomonas and Entamoeba

Giardia duodenalis, *Trichomonas vaginalis* and *Entamoeba histolytica* are the most clinically important anaerobic protozoan parasites. Because of their anaerobic metabolism they are all susceptible to metronidazole and other related 5-nitroimidazole drugs such as tinidazole that are used to treat giardiasis and trichomoniasis. However, metronidazole is the only drug approved for the treatment of trichomoniasis in some countries, the only safe treatment for invasive amoebiasis, and the favoured treatment for giardiasis. Albendazole is an alternative for the treatment of giardiasis (22).

3.6.1 In Vivo

There are no in vivo tests in human hosts. However, the ability of axenically cultured *G. duodenalis* to infect and complete its life cycle in suckling mice is well documented (120) and this model has been used to assess drug susceptibilities in vivo for metronidazole, azithromycin and erythromycin at 5–100 mg/kg (121). In most cases the infections spontaneously

resolve after about 3 weeks (120). Gerbil and suckling-rat models are also used for giardiasis, the former has the advantage of using adult animals (122). Using isolates from ten infected patients, Lemée et al. (36), infected, treated with metronidazole, and then monitored parasite survival in mice and correlated this with treatment outcomes in the patients. Parasites isolated from three patients in whom treatment with metronidazole failed had ID_{50}s in mice which ranked among the four highest (125–175 mg/kg) of all the ten isolates tested. The ID_{50} in mice of the seven isolates from patients in whom treatment was successful ranged from 31 to 150 mg/kg.

3.6.2 In Vitro

In order to use in vitro tests to estimate the level of susceptibility of *G. duodenalis*, *T. vaginalis* and *E. histolytica* to metronidazole or any other drug, the parasites need to be growing axenically (free of other living organisms). Methods for cultivation of all three parasite species have recently been expertly described (123). However, in spite of their long history of cultivation, fewer than half of *G. duodenalis* samples can be axenised with some taking months to establish before assays can be performed (16), and establishing *E. histolytica* axenically is notoriously difficult (124).

Traditionally two choices for assaying in vitro survival of *Giardia*, *Trichomonas* and *Entamoeba* in the presence of drugs have been available: tube assays and microtitre plate assays. A major consideration for *Giardia* and *Entamoeba* is the generation of an anaerobic environment. Microtitre plates are problematic due to the variability of the environment created in different laboratories and the need to remove the plates from the anaerobic environment to monitor the assays. Tube assays are cumbersome, time-consuming and prone to variability. A variety of end points (thymidine incorporation (37), uptake of [^{14}C]metronidazole (121), inhibition of adherence (125), live/dead (126)) have been used to report susceptibility including IC_{50} values ranging from 0.06 to 6 µM, ID_{50} values of 1.2 and 9 µM, and minimal lethal concentrations (MLC) after 72 h of 50–100 µM for *Giardia*, an MLC value of 11.6 µM for the HM-1 strain of *E. histolytica* and a minimal inhibitory concentration (MIC) of >90 µM after 24 h indicating resistance in anaerobic assays of *T. vaginalis* (see (22) for references). A proposal to standardise these assays, details the use of the 96-well plates with log2 drug dilutions and the Anaerocult systems from Merck. Plates can be monitored throughout the assay without aerobic exposure (127, 128). This system can also be modified to measure IC_{50} and IC_{90} values (129).

MICs for *G. duodenalis* (3 days) ranged from 6.3 µM in metronidazole-sensitive isolates to 50 µM for laboratory metronidazole-resistant lines (RF = 8) (37) with metronidazole resistance as high as 20% reported among *G. duodenalis* isolates (22). MICs (after 2 days) of 3.2 and 25 µM indicated metronidazole-sensitive and highly clinically resistant isolates of *T. vaginalis* in anaerobic assays, respectively (RF = 7–8) (37). *T. vaginalis* can also be maintained aerobically for several days and generally exhibits higher MIC values with metronidazole under these conditions (20). Thus the aerobic MICs were 25 and >100 µM for metronidazole-susceptible and highly resistant *T. vaginalis* (RF = 8). In studies of *T. vaginalis* in two different female populations 1 of 10 isolates and 4 of 24 isolates had aerobic MIC ≥ 100 µM (J. Upcroft, unpublished data). MICs (1 day) of 12.5–25 µM were found for axenic lines of *E. histolytica* but there are no confirmed clinical reports of metronidazole-resistant *E. histolytica*.

3.6.3 Phenotypic Assays

Some effects of anti-giardiasis drugs can be observed microscopically or measured in the laboratory (Table 2). The fluorescent drug quinacrine is visibly excluded from resistant *G. duodenalis* trophozoites (127). Distortion of *Giardia* trophozoite cytoskeleton is associated with albendazole (130) and may not occur in resistant individuals. A decrease in the activity of the key metabolic enzyme pyruvate:ferredoxin oxidoreductase (PFOR) can be measured in the laboratory. Decreased PFOR and ferredoxin mRNA levels occur in highly metronidazole-resistant laboratory lines of *T. vaginalis* (20), increased superoxide dismutase activity occurs in metronidazole-resistant *E. histolytica* (23) and increased levels of mRNA of P-glycoprotein homologues can be detected in emetine-resistant *E. histolytica* (43). The relevance of these observations to clinical resistance is unknown.

3.6.4 Genetic

While correlations between genes involved in drug resistance and decreased drug susceptibilities in the anaerobic protozoa have been reported (e.g. down-regulation of hydrogenosome function in *T. vaginalis* (20), chromosome duplication in *G. duodenalis* (17), increased superoxide dismutase activity in *E. histolytica* (23), a threefold increase in P-glycoprotein expression in emetine-resistant trophozoites (21, 43)), no mutational assay is available (Table 2).

3.6.5 Specimen Collection

G. duodenalis samples can be either cysts in stools (purified) or trophozoites from duodenal biopsies introduced directly into the medium (123). Both can be passaged through

neonatal mice prior to culture to increase the number of parasites and the chance of establishing a culture (131).

Cysts of *E. histolytica* and the closely related non-pathogenic parasite *E. dispar* can be readily established in xenic or monoxenic culture (123) and assays to determine drug susceptibilities in the presence of bacteria provide a compromise between convenience and accuracy.

T. vaginalis trophozoites are obtained from high vaginal swabs and introduced directly into the medium (123). In our experience it is not difficult to establish 100% of positive *T. vaginalis* samples in axenic culture which may be ready to assay in 1–3 weeks.

While the same medium can be used to culture *G. duodenalis*, *E. histolytica* and *T. vaginalis*, the rates of growth vary greatly. Established cultures of *T. vaginalis* generally require a 10- to 50-µl passage into 5 ml of fresh medium every 2 days, *G. duodenalis* requires 100–200 µl for the same volume every 2 days and *E. histolytica* grows more slowly with a 1 in 4-ml passage needed every 3–4 days.

3.6.6 Practical Tests, Clinical Significance and Breakpoints

Using the in vitro assay systems described above (Sect. 3.5.2) (37) the researcher has a realistic common reference assay for the surveillance of the development of drug resistance in the anaerobic protozoa. Results for *T. vaginalis* resistance using this assay have been returned to a patient within a week (J. Upcroft, unpublished data) but information regarding the resistance status of *G. duodenalis* and *E. histolytica* may take too long to be clinically useful.

If 70 µM is used as an estimate of in vivo (serum) metronidazole concentration after usual, recommended doses (levels in the gut and vagina are likely to be less than this) then laboratory-induced metronidazole-resistant *Giardia* lines which grow in 100 µM metronidazole (132) can be expected to demonstrate clinical resistance in a patient. In the assay system described above, a MIC of 50 µM metronidazole was obtained with highly metronidazole-resistant *Giardia* (37) but clinical breakpoints have not been estimated for *Giardia*.

Similarly with *T. vaginalis*, metronidazole-resistant isolates with MICs of >25 µM (37) or >200 µM in aerobic assays (37) are likely to be resistant in vivo.

Resistance to metronidazole or emetine in *E. histolytica* has been demonstrated in the laboratory (37, 133) but no documented clinically resistant *E. histolytica* has been reported.

3.6.7 Artefacts

Artefacts involved in assaying drug susceptibility in the anaerobic protozoa include: variability in anaerobic environments generated; the time taken to establish an isolate in culture may result in the selection of organisms not truly representative of the original sample; the end points of the assays observed under the microscope can give rise to variable results; if motility alone is used as an endpoint, not all *Giardia* and *T. vaginalis* strains are equally motile, and pseudopodia of *Entamoeba* trophozoites may not be readily distinguished in live versus dead, rounded up trophozoites. In comparison with the data of Upcroft and Upcroft (133), the aerobic MLC values obtained by Muller et al. (134) on *T. vaginalis* (with aerobic assays conducted in 24-well plates and anaerobic assays carried out in anaerobic jars over 24 h) were 12–150 µM and 360–1,500 µM for metronidazole-susceptible and metronidazole-resistant isolates, respectively; anaerobic MLCs were 3–6 µM and 12–30 µM, respectively.

3.7 Trichostrongyloids

Resistance in the sheep trichostrongyloids is a serious and widespread problem in sheep industries, especially where treatment failures of *Haemonchus contortus* are involved. Not only has resistance occurred to all major anthelmintic classes in several species of parasite, but several isolates are resistant to all major drug classes. The other major species affected include *Trichostrongylus colubriformis* and *Ostertagia circumcincta*. Resistance of nematodes in cattle, horses and humans is appearing but assays are less well developed for these hosts. Further, correlation between in vivo effects of the drugs and in vitro behaviour in resistance tests will be difficult to confirm.

3.7.1 In Vivo

Treat and Slaughter Trials

The traditional, definitive test for resistance involves artificially infecting worm-free sheep with worms, treating them at the manufacturer's recommended dose rates and counting the number of worms surviving at slaughter. By using various control groups (for example untreated and susceptible worm isolates) dose–response curves can be generated and then the ED_{50} and RF calculated. Disadvantages of this method include the difficulty in finding drug-susceptible reference isolates, the creation of worm-free hosts, the ethics of euthanasia of animals and the expense of the procedure. Accordingly it is now only appropriate to perform slaughter trials on sheep when a novel isolation is made because in many cases the relationship and correlations between in vivo and in vitro assays for anthelmintic resistance in sheep parasites have been established (135).

Faecal Egg Count Reduction Trial

Faecal egg count reduction trial (FECRT) is the most common method of diagnosis of anthelmintic resistance in sheep in the field and is used as a treatment decision tool. Protocols are provided in articles by Lyndal-Murphy (136) and Coles et al. (137). Briefly, the test involves the following steps:

1. Select a flock of sheep, 3–6 months old, not treated in the previous 6 weeks (or longer for persistent drugs), with faecal egg counts of >200 epg.
2. Randomly allocate 10–15 animals to each drug treatment class to be tested and 10–15 to be controls. Use coloured markers to identify animals by group. Typically tests include four drug classes plus drug combinations.
3. Dose with liquid anthelmintic based on the manufacturers recommendations for the heaviest animal.
4. Treat each group with the dose of the particular drug as calculated in (3), leaving the controls untreated. Graze the animals together.
5. Return 10–14 days later, collect faecal samples from each animal (labelled by group) and perform individual faecal egg counts by a standard flotation method. Fourteen days is chosen because it is long enough for drugs to exert their effects and for the eggs to be cleared, but not so long that newly ingested larvae will have developed to patency (egg laying).
6. Calculate % reductions compared with controls, using arithmetic group means, but logarithmic error calculations to calculate confidence limits.
7. Anthelmintic resistance is present if efficacy is <95% AND the lower 95% confidence interval is <90%

The data can be complemented by use of culture from eggs to L3 and attribution of eggs in faeces in the same ratio as the genera identified in the L3 cultures.

The advantages of FECRT assays include their simplicity, that it has been widely validated and that the data provided is relevant to field resistance. On the other hand, FECRT can only detect clinical resistance, often not until the frequencies of resistance alleles reach 25% (138). Two visits to the farm are required unless the farmer conducts the treatments and farmers may be reluctant to participate in these tests, especially if sheep are due for slaughter for meat. To a large extent the interpretation of FECRT relies on a correlation between egg counts and worm counts. Some anthelmintics, particularly the macrocyclic lactones, do not kill resistant worms but, do suppress their egg production. Zero egg counts post-treatment that suggest that the worms are drug susceptible may mask cases where worms resume egg production after more than 14 days of treatment. Sensitivity can be improved by using reduced dose rates of drugs, but this approach requires a good knowledge of resistance phenomena. The protocol can be modified for other hosts and, in the case of horses the calculations can be refined by using individual animal egg counts pre and post treatment (139).

3.7.2 In Vivo (Experimental Animal)

Drug efficacy in guinea pigs (140) and jirds (141) has been used to measure resistance in *T. colubriformis* and *H. contortus*, respectively. Although these techniques are not likely to be used for field detection, they are useful experimental models because several dose rates of drug can be tested more quickly and cheaply than in sheep.

3.7.3 In Vitro

Survival/Development Assays

The best characterised in vitro assay is the larval development assay (LDA) (135). It relies on the development of eggs to L1-L2-L3 in the presence of drugs and has been adapted to a 96-well plate format. Eggs are collected from pooled faecal samples from a farm. Approximately 200 g faeces (collected from the ground after herding the sheep into the corner of a pen for 15 min) are soaked in 200 ml of tap water for at least 30 min and broken up to give a slurry. Eggs are cleaned by sequential sieving, centrifugation on a sucrose gradient and washing. Seventy to one hundred and twenty eggs are pipetted in 10 µl into a pre-prepared plate. The rows of the 96-well plate contain anthelmintics in agar at increasing concentration steps.

Anthelmintic	Approximate concentration range (µM)
Thiabendazole	0.01–10
Levamisole	0.2–20
MBZ plus Levamisole	0.01–10 (MBZ) 0.2–20 (LEV)
IVM – MS (IVM monosaccharide)	0.005–0.5
IVM – AG (IVM aglycone)	0.01–1

After 7 days of incubation at 25°C the stage of development is scored down a microscope. The concentrations on the plate are arranged so that susceptible isolates hatch at low concentrations in wells coloured green and resistant isolates at high concentrations, coloured red. Results can also be analysed by curve fitting and calculating EC_{50}.

The LDA can be applied to resistance in horse cyathostomes (139) and has several advantages over FECRT. Sample collection is simpler and accomplished in one visit. The tests are repeatable, there is no need to test susceptible isolates and the use of dose–response data plus the use of greater than one hundred eggs/well can provide sensitivity of 95–98%.

The LDA simultaneously assays resistance to the major commercial drugs in a single test.

General disadvantages include that the validity of the assay as a measure of resistance must be confirmed under a range of conditions, trained laboratory staff are required and species identification of larvae is needed. For some parasite species, especially *Ostertagia*, detection of ivermectin resistance in the LDA is not sensitive enough for field use.

Phenotypic Assay

Reduced binding of BZ anthelmintics to worm tubulin is a known resistance phenotype and the binding assays can be performed on a pooled sample of L3 following incubation with isotopically labelled BZ (142). The main drawback of this method is that a radiolabelled drug as well as the skills and equipment to use it are required.

3.7.4 Genetic

While there are a number of research-based tests, only one genetic test is validated for anthelmintic resistance. Benzimidazole resistance is linked to the presence of a predominant allele of the β-tubulin isotype 1 gene containing a F200Y transition (143). This amino acid change is due to a single nucleotide difference.

Although other genetic changes, such as F167Y, may contribute to resistance, this allele appears to be almost universally associated with resistance in sheep nematodes and is therefore an ideal diagnostic feature. Tests have been used for pooled worm samples or single worms for the species *H. contortus* (144) and *O. circumcincta* (145, 146).

An elegant single worm method for AS PCR has been described (146). The reaction uses four primers, two are sense and antisense primers either side of, but at different distances from the site of the single nucleotide difference. Within this nest, is a further sense primer with its 3′ end coinciding with the 'susceptible' base pair, plus an antisense primer complementary to the 3′ end coinciding with 'resistant' base pair. Products are separated by gel electrophoresis. The total product, limited by the outside primers, is the amplification control. Two differently sized products indicate that heterozygous alleles are present and either product alone indicates homozygosity, one size of product for the susceptible, and another for the resistance.

Such tests have further potential if species-specific primers can be designed by taking advantage of species-specific sequences in β-tubulin and to provide simultaneous species and resistance information. Further, if the resistance mechanisms are conserved across the parasite species they may indicate diagnostic tools in horses, cattle and human parasites.

Genetic tests have the potential to detect population prevalence below 1%. Despite knowledge of the genetic mechanism of benzimidazole resistance and the usefulness of the test, the logistics of genetic tests preclude their use in the field. Further, the prevalence of benzimidazole resistance in many countries is so high that diagnosis is no longer useful.

Because current genetic tests provide yes/no answers, the design of tests requires careful thought. Assuming that a genetic test is available, and that it detects a single allele (diploid and autosomal) linked to the resistant phenotype in one species of a parasite, it could be applied to measure the prevalence of resistance in several ways. One would be to collect L3 surviving high drug concentrations on in vitro assay plates (e.g. the LDA) and genotype them individually. Another approach would be to test the population of worms (e.g. eggs or larvae) recovered from a farm or an animal. Using PCR to genotype individual worms would be laborious and expensive hence pooling samples of parasites would have to be considered. Real-time (quantitative) PCR may help to simplify the sampling but would require considerable standardisation (28). For example, a pool of 150 worms would have to test negative for the resistance allele to provide 95% confidence that resistance prevalence was <1%. A useful approach would be to perform three PCRs on different pools of DNA. If no resistant alleles were detected in the pooled DNA from 150, 75 and 30 worms, allele prevalences of <1, 1–2 and 2–5%, respectively, (at confidence level of 95%) would be predicted (147). Such tests would require ideal conditions for both extraction of DNA from all of the worms and PCR-based amplification. A perspective on the developments in the molecular diagnosis of anthelmintic resistance appear in von Samson-Himmelstjerna and Blackhall (148).

3.7.5 Specimen Collection

In Vivo

Because sheep vary considerably in the number of worms that they carry, treatment group sizes need to be 10–15 in order to provide sufficient sampling and power for statistical tests. Clinical samples for FECRT are obtained by collecting 3 g of faeces directly from the rectum of individual sheep on the day of treatment (pre-treatment sample) and 10–14 days after treatment (post-treatment sample). Pre-trial samples should exceed 200 epg.

In Vitro

Sampling for LDAs or egg hatch assays are relatively simple as they use pooled faecal samples. Samples can be cooled to

10°C and air excluded to prevent development during transport. Sealed plastic bags are ideal.

3.7.6 Practical Tests, Clinical Significance and Breakpoints

The most widely used test is the FECRT. Arithmetic group mean of egg counts are used because they indicate potential pasture contamination, are simple to calculate and are conservative estimates of resistance (149). The use of the two criteria (breakpoints) (efficacy <95% and lower confidence intervals <90%) provides a 95% confidence of detecting clinical resistance (137).

For in vitro assays such as the LDA, breakpoints can also be visualised on the colour-coded plates. In addition, the EC_{50} can be calculated by curve fitting and comparing with data available for susceptible isolates. Curve fitting also allows subpopulations to be identified and are useful in the early stages of resistance development.

3.7.7 Artefacts

Despite careful test validation and well-validated sampling frames, assaying resistance remains an inexact science. Apart from the limitations of the tests, mixed species infections that are the norm in the field can interfere with these measurements.

Even in well-controlled laboratory situations, results vary. Apart from the day-to-day variation in results other factors are known to influence resistance levels. Resistance varies over the course of infection (150). Cold storage may also influence resistance (151). There are limitations in the practical application of tests as no universal technique (covering all species and all drugs) is available.

3.8 Fasciola

Reports of resistance to triclabendazole and closantel in the liver fluke *Fasciola hepatica* have come from several countries including Australia, Ireland, Scotland and the Netherlands (152). Internationally, fasciolosis is a very significant disease in farm animals and resistance is a serious impediment to control.

Because of the indirect life cycle (sheep/snail hosts), resistance in flukes is difficult to measure. Generally the treat and slaughter assay involves isolating potentially resistant flukes by treating infected sheep several times with a fasciolicide at the recommended dose rate. Eggs from survivors are hatched in the laboratory and passaged through suitable intermediate host snails in the laboratory. Sheep are divided into groups and infected with metacercariae from the suspected resistant strain and the laboratory-based susceptible strain. Eight weeks after infection half of the sheep are treated with the recommended dose rate of the drug. Animals in other groups are used as controls. Sheep are slaughtered 16 weeks after infection, their livers examined and the numbers of flukes counted. The efficacies of treatment against susceptible and resistant isolates are compared.

Egg count reduction tests are not suitable for flukes because resistance is usually apparent against the immature stages of the fluke, prior to egg-laying.

Some in vitro tests have been described but none developed for routine use. There is potential for using motility of miracidia or hatching of eggs from hosts as an assay for resistance in flukes.

3.9 Schistosoma

Schistosomiasis affects more than 200 million people in 76 countries. These infections are most commonly caused by *S. haematobium*, *S. japonicum*, and *S. mansoni* (153). Although the minor drugs hycanthone, metrifonate and oxamniquine have been used, praziquantel (PZQ) is the drug of choice for treatment for all forms of schistosomiasis (154). Recently, reports of decreased efficacy of PZQ in the field and resistance in laboratory strains of the parasite have appeared. Although other possible causes of treatment failure have not been ruled out, these cases strongly suggest that resistance to PZQ is emerging.

In vivo testing is complicated by the fact that PZQ has a triphasic efficacy profile with the earliest stages of schistosomes being susceptible, followed by progressive insensitivity through to 3–4 weeks after infection and then a gradual regaining of susceptibility at weeks 6–7 after infection (155). In cases of endemic exposure to infection even a drug-susceptible parasite population may contain refractory individuals.

In vivo tests in rodent hosts have been used to test for possible drug resistance (156). Eggs are collected from patients still passing eggs after repeated treatments with an antischistomal drug. Laboratory-reared snails are infected and the cercariae used to infect laboratory rodents (mice or hamsters). Experimental chemotherapy at a range of doses is given to the rodents and by comparing worm counts in control and treated groups an ED_{50} is calculated. This is expensive and slow (approximately 12 weeks), and the correlation between drug sensitivity in mice and the patient is unreliable (144).

In vitro tests include muscle contraction studies (47), miracidial morphology and survival analysis in the presence of drug (46).

Leads to the molecular mechanism of resistance are overexpression of messenger RNA coding for subunit 1 of the mitochondrial enzyme cytochrome *c* oxidase (49) and the knowledge that sensitivity of a Ca^{2+} channel to PZQ depends on residues at the interface between the α and β channel subunits (24).

4 Conclusions

Drug resistance is responsible for parasite control failures in a number of diseases. Costs such as death, debility, reduced capacity to work and production losses are significant and on a global scale. Assays for drug resistance have an essential role in future disease management. Simply, we cannot manage resistance if we cannot measure it.

To be most effective, resistance monitoring should be a routine component of parasitic disease management. This requires accurate assays that are simple, rapid, and can simultaneously test against all relevant available control agents often in several parasite species. The ideal would be an assay that could be performed and interpreted by the patient's (or animals') side. These criteria have proven difficult to meet and drug resistance remains difficult to assay. What we have is a collection of assays, most of which are used in monitoring resistance status and, occasionally, measuring resistance so that decisions on therapy in individuals or herds can be made.

Although some in vivo assays are available there are pressures to develop in vitro and genetic tests. While these latter tests need to be validated against in vivo results they offer the potential advantages of improved speed and sensitivity. We also need to base future tests on better knowledge of resistance mechanisms. Further, we need to understand parasite biology and resistance so that we can design sampling frames to match the mechanism-based tests that will arise.

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