Molecular tools for the rapid detection of drug resistance in animal trypanosomes

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Abstract
There are currently 17 African countries in which animal trypanocidal drug resistance has been reported. Large-scale surveys were carried out in only ten of them. The lack of baseline information is mainly due to the fact that the methods currently available for the detection of drug resistance are laborious, expensive and time consuming. In this review the mechanisms involved in resistance to isometamidium and diminazene will be discussed, together with some new molecular detection tools that have been developed recently enabling faster diagnosis of drug resistance than conventional laboratory or field tests.

African animal trypanosomiasis and drug resistance

Tsetse fly-transmitted trypanosomiasis is an important constraint to livestock development in sub-Saharan Africa with estimated annual losses owing to the direct and indirect effects of the disease running into billions of dollars. Approximately 9 million km^2 of sub-Saharan Africa, representing about one-third of the total land, is affected by tsetse flies [1]. Within this region, some 46–62 million head of cattle and other livestock species are at risk of the disease [2]. Trypanosomiasis is controlled either by controlling the vector or by controlling the parasite, or a combination of both. Over the years, a large arsenal of vector-control tools has been developed. Nevertheless, the control of animal trypanosomiasis in often poor rural communities has and will continue to rely heavily on the use of trypanocidal drugs. This is not surprising considering the private nature (i.e. the easy, individual, non-concerted use) of such treatments and the difficulties in maintaining cleared areas in the absence of barriers to re-invasion of tsetse flies. Only a small group of chemoprophylactic and chemotherapeutic trypanocidal compounds are currently in use and new compounds are unlikely to become available in the near future [3]. Geerts and Holmes [4] estimated that in Africa ~35 million doses of veterinary trypanocidal drugs are administered each year with isometamidium chloride (ISM), ethidium bromide (EtBr) and diminazene aceturate (DA) estimated to represent 40%, 26% and 33%, respectively, of the total trypanocidal drug market by value [5]. ISM is mainly used as a prophylactic drug and provides on average 3 months' protection (2–22 weeks) against trypanosome infection. DA has only therapeutic properties and EtBr has limited prophylactic properties and is mainly used as a therapeutic agent [6]. Considering the well known mutagenic properties of EtBr, this drug should ideally be removed from the drug market, but in practice it is still widely used in many countries. Removing this drug from the market would not jeopardize the treatment of animal trypanosomiasis because it can be replaced either by DA for curative purposes or by ISM for prophylactic purposes. When trypanosomes are resistant to ISM, EtBr will be ineffective as cross-resistance is observed between the two drugs [7]. As a result of inappropriate trypanocidal drug-use practices, there is growing concern that the effectiveness of chemotherapy will be severely reduced by the widespread development of resistance in trypanosomes [8] and [9]. The total value of the market for trypanocides for African farmers is estimated at US$30 million [6] but this is considered insufficient by pharmaceutical companies to justify investment in the development of new drugs. Therefore the challenge remains to make optimal use of existing drugs.

Although an increasing number of reports on drug resistance in animal trypanosomes in Africa are published, it is not clear whether this increase is due to a higher incidence of drug-resistant trypanosome strains or to a growing interest of the scientific community in this area of research. The first reports on resistance to ISM and DA date from the 1960s [10] and [11]. However, in the past most information on drug resistance was derived from case reports and in many countries baseline information on the prevalence of drug resistance in trypanosomes is lacking. Currently, there are 17 African countries (Figure 1) in which trypanocidal drug resistance has been reported. In addition to the 13 countries mentioned by Geerts and Holmes [4], drug resistance was reported in Mozambique [12], Mali, Guinea [13] and Cameroon [14]. Moreover, in several African countries trypanocidal drug resistance is suspected to be present but has not been demonstrated using standardized tests. Currently, there are only 10 countries (Burkina Faso, Mali, Cameroon, Ethiopia, Kenya,

Nigeria, Tanzania, Zambia, Uganda and Zimbabwe) in which large-scale surveys were carried out and in which area-wide drug resistance occurred in at least one region of the country [14], [15], [16], [17] and [18]. This lack of baseline information is mainly due to the fact that the current laboratory (Box 1) and field methods (Box 2) for the detection of drug resistance are laborious, expensive and time consuming. Drug resistance to ISM is more widespread than to DA [19], but increasingly there are reports of multiple drug resistance.

Box 1. Conventional laboratory tools for the detection of trypanocidal drug resistance

***In vivo* tests**

A standardized protocol for the assessment of susceptibility and resistance to trypanocidal drugs in mice or in ruminants has been described [47]. The inconvenience of these assays is their long duration: 60 days in mice and up to 100 days in cattle. Although there is a good correlation between the tests in mice and in ruminants, the curative dose that must be used in ruminants cannot be extrapolated from the results in mice [54]. Another disadvantage of the mouse test is the fact that *Trypanosoma vivax* and also some *T. congolense* isolates do not develop in mice.

***In vitro* tests**

In vitro tests using bloodstream or metacyclic trypanosomes can be used to detect resistance in *T. brucei* and *T. congolense* [55] and [56]. A major disadvantage of these tests is the slow adaptation of the trypanosomes to the culture conditions. Furthermore, it is difficult to maintain *T. congolense in vitro* [57]. Two alternative approaches for *T. congolense* have been evaluated in which a short *in vitro* incubation in the presence of various drug concentrations is sufficient. The first approach is the drug incubation *Glossina* infectivity test (DIGIT), the main limiting factor being the availability of tsetse flies [58]. The second approach is the drug incubation infectivity test (DIIT), in which mice are infected with trypanosomes after drug incubation [59].

Other tests

Other tests that are still in the experimental stage or that are not used frequently are the tests based on the mitochondrial electrical potential (MEP) and the ISM-ELISA technique. It has been suggested that variation of the MEP might be the primary factor determining the rate of ISM accumulation in the trypanosome kinetoplast [23]. Initial studies using a limited number of *T. congolense* populations have shown that an increased or decreased MEP might be a candidate quantitative marker for ISM susceptibility or resistance, respectively [23]. The use of an ELISA for the detection of ISM in the serum can be combined with the ‘block treatment’ or individual treatment of ruminants to detect resistant trypanosomes [60]. The presence of trypanosomes in animals with an ISM serum concentration >0.4 ng ml^{-1} suggests that parasites are resistant [61].

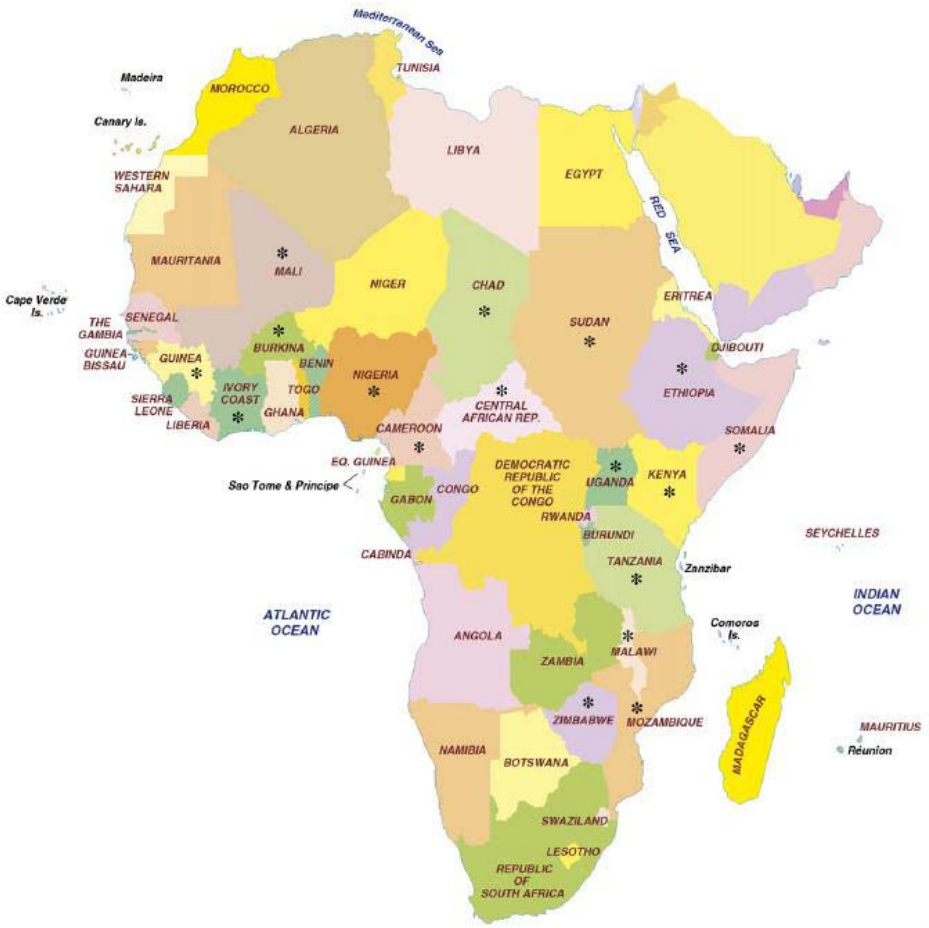

***Figure 1**. African countries with reported resistance to trypanocidal drugs. A star indicates that resistance to trypanocidal drugs has been reported in animal trypanosomes in that country. Figure based on data from [4], [12], [13] and [14].*

Box 2. Conventional field tests for the detection of trypanocidal drug resistance

Resistance to ISM can be assessed under natural *Trypanosoma* challenge in the field using the 'block treatment' approach (Eisler, M.C. *et al.*, 2000, Rapid method for the assessment of trypanocidal drug resistance in the field. *Proceedings of the 9th Symposium of the International Society for veterinary epidemiology and economics, Nairobi, paper 353* 1–3). Two groups of infected cattle, either treated with 1 mg kg^{-1} ISM or untreated (each group consisting of 30 to 80 animals) are exposed to natural challenge and tested for the presence of trypanosomes in the blood using the phase contrast buffy coat technique [62] every two weeks for two to three months. If >25% of ISM-treated cattle become infected within eight weeks of exposure, drug resistance is strongly suspected. This approach can also be used for assessing whether there is suspected resistance to DA by treating the control group at the start of the experiment, and all animals that become infected during the trial, with DA and checking for the presence of parasites two weeks after treatment [14] and [63]. Furthermore, longitudinal parasitological field data can be suitably analysed using appropriate statistical techniques to detect problems of resistance to DA [64].

Mechanisms involved in resistance to isometamidium and diminazene

Resistance to isometamidium chloride

Metamidium was obtained from the coupling reaction between *m*-amidinobenzenediazonium chloride and homidium chloride. This coupling reaction produced a mixture of purple and red compounds that were separated by fractional crystallization and shown to be isomeric. Isometamidium is the red isomer of the mixture [20], [21] and [22]. ISM is accumulated in two compartments of trypanosomes, the cytoplasm and the kinetoplast. No concentration gradient is observed between the cytoplasmic compartment and the outside environment indicating the absence of active transport. A different situation is observed between the cytoplasmic and kinetoplastic compartments where a concentration gradient is observed and where ISM is retained in the mitochondrion. When placed in an ISM-free medium, no difference in ISM diffusion out of the cell was observed between sensitive and resistant strains. Under the same conditions, a large proportion of ISM is retained sequestered within the mitochondrion of sensitive strains [23].

ISM transport within the trypanosome is energy-dependent. ISM seems to cross the cytoplasmic membrane by facilitated diffusion, not requiring expenditure of metabolic energy because diffusion of ISM is down a concentration gradient. From the cytoplasmic compartment, ISM enters the mitochondrion either by the same process (but possibly driven by the mitochondrial electrical potential against a concentration gradient) [23] or through an as yet unidentified energy-consuming transmembrane transporter [24]. Development of resistance could therefore be due to (i) a decrease in diffusion through the mitochondrial membranes (lowered mitochondrial electrical potential); (ii) modification of a possible transporter located in the inner mitochondrial membrane; (iii) increased extrusion of the drug by a transporter located in the cytoplasmic membrane or (iv) a combination of these processes (Figure 2). The direct or indirect ATP consumptions of an extrusion system (primary or secondary transporter) would simultaneously decrease the mitochondrial electrical potential and consequently the accumulation of ISM within the kinetoplast.

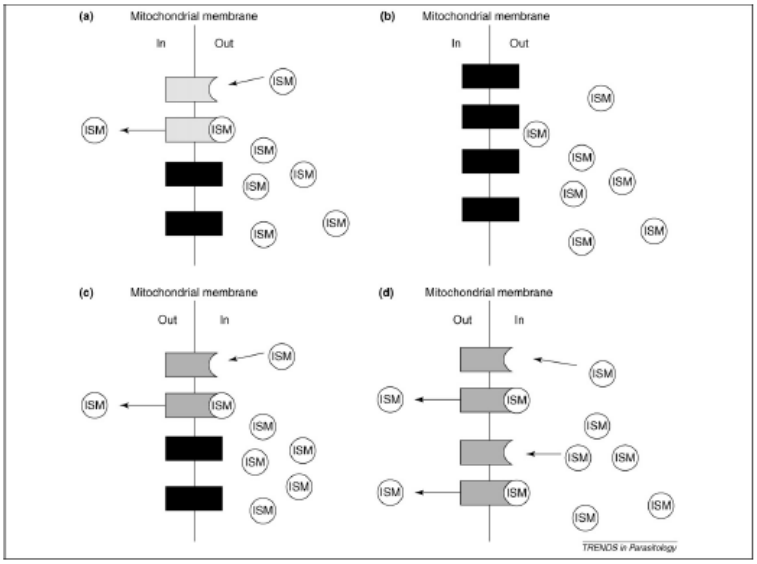

Figure 2*. Model of the uptake of isometamidium (ISM) by T. congolense mitochondria. (**a,b**) Drug importer models. (a) Heterozygous wild-type (light grey) and mutated (black) importers with decreased activity. (b) Homozygous mutated importers with decreased affinity. Only the homozygous mutated importers will be resistant to ISM and this is observed in the PCR-RFLP profiles [24]. (**c,d**) Drug exporter models. (c) Heterozygous wild-type (black) and mutated exporters with increased affinity (dark grey). (d) Homozygous mutated exporters leading to a resistant phenotype. Heterozygous and homozygous mutated exporters would both be resistant to ISM to different degrees, which is not observed from the PCR-RFLP profiles [24]. Reprinted from [23] with permission from Elsevier.*

Mutation in an ATP-binding cassette-like transporter

In a study using amplified fragment length polymorphism (AFLP) to compare two isogenic clones of *Trypanosoma congolense*, the resistant clone withstood ISM doses 94-fold higher than the sensitive parent clone from which it was derived, and showed the presence of a conserved GAA codon insertion (coding for an extra lysine) in a gene coding for a putative ABC (ATP-binding cassette) transporter in the resistant clone [24]. ABC transporters are ubiquitous membrane proteins that use ATP directly to transport a substrate across biomembranes, irrespective of the concentration gradient [25]. However, the fact that some strains characterized as resistant in mouse tests did not show the GAA insertion [24] indicates that more than one resistance mechanism could be involved. Hence, it is likely that the transport mechanism of this compound is not as specific as the P2-type purine transporters for diamidine compounds.

It remains unclear whether the ISM-resistance phenotype is the consequence of reduced uptake or increased efflux of the drug. In one study, sensitive strains were heterozygous for the GAA codon insertion, whereas most resistant strains were homozygous for the same trait [24]. The fact that the sensitive isolates already seem to carry a recessive resistance allele is consistent with the selection of an existing influx transporter expressed at a lower level or with decreased affinity for ISM through loss of heterozygosis. Alternatively, the resistance allele could encode a mitochondrial efflux pump with increased affinity for ISM. However, such an allele would be expected to be dominant, actively clearing the drug from the kinetoplast (Figure 2). To challenge this model, more isolates should be screened to identify an ISM-sensitive phenotype for a strain homozygous for the insertion [26]. A combined mechanism of reduced uptake and increased efflux might also be possible.

Mitochondrial topoisomerases

Alternative pathways of ISM resistance could include the alteration or modification of the targeting site of the drug. It has been suggested that pentamidine, DA, ISM and EtBr have an action within the kinetoplast of *Trypanosoma equiperdum* that is characteristic of type II topoisomerase inhibitors and mimics the effects of the antitumor compound etoposide (a specific inhibitor of mitochondrial topoisomerase enzymes) [27]. The silencing of the mitochondrial topoisomerase gene by RNA interference or by the use of specific topoisomerase II inhibitors induces the progressive shrinking and disappearance of the kinetoplast DNA network [28] and [29]. Other evidence for modification of topoisomerase genes as a drug resistance mechanism is reported in *Leishmania donovani* and in carcinoma cells [30] and [31]. This possible mechanism of resistance to ISM (i.e. modification of the topoisomerase gene) was explored in *T. congolense* [32]. Two distinct topoisomerase II genes were found in *T. brucei*, *T. vivax*, *T. cruzi*, *T. congolense* and in *Leishmania*. The two mitochondrial topoisomerase genes of 10 ISM-sensitive and 14 ISM-resistant strains of *T. congolense* were screened using the single-strand conformation polymorphism (SSCP) technique, sequencing and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and revealed that both genes were highly conserved. No polymorphism related to ISM-resistance could be observed. Thus, contrary to what is observed in *Leishmania donovani* [30] and in some cancer cells [31], changes in topoisomerase II genes do not seem to be a means by which *T. congolense* develops resistance to ISM.

Resistance to diminazene aceturate by a P2-type purine transporter

The role of the P2-type purine transporter in the uptake of arsenical diamidines, pentamidine and DA by *T. brucei*, *T. evansi* and *T. equiperdum*, and the consequences of inhibition, knocking down or silencing this gene have been extensively described and reviewed in the literature [33], [34], [35], [36], [37], [38], [39], [40], [41], [42] and [43]. In addition to this resistance mechanism, a novel gene, *Te*DR40, might be a factor contributing to high DA-resistance in *T. evansi* [44]. A high level of DA-resistance, and significant cross-resistance to pentamidine was induced *in vitro* with a concomitant >1000-fold upregulation of *Te*DR40. However, the high level of resistance in the *in vitro* adapted line, *Te*BR1, was not reproduced by overexpression of *Te*DR40 and neither was the pentamidine cross-resistance. It is likely that such a DA resistance is multi-factorial and that the *Te*DR40 gene might be a contributing factor to the resistance linked to alteration of the gene coding for the P2-type purine transporters. Sequence analysis of the *Tev*AT1 gene from the TeBR1 strain showed differences from the parent strain at the amino acid level [44].

A putative P2-type purine transporter *Tco*AT1 in *T. congolense* was identified by reciprocal blasting of the *Tb*AT1 gene of *T. brucei* and a conserved Val306 to Ile306 permutation in this gene was observed in *T. congolense*

strains that show resistance to DA [45]. This suggests that the purine salvage system based on P2 transport is also involved in the development of resistance to DA in this species. However, contrary to what is observed for the putative ABC transporter involved in ISM resistance [24], heterozygous strains were not observed (N=26 homozygous) [45]. To elucidate the recessive or dominant character of the mutation, the sensitivity to DA of a strain that is heterozygous for the mutation should be characterized.

Molecular tools for the detection of resistance to trypanocides

PCR-RFLP for the detection of ISM resistance

Molecular methods for the diagnosis of ISM resistance were recently developed [24] and [46]. The first method enables discrimination between ISM-sensitive and ISM-resistant strains of *T. congolense* by *Mbo*II-PCR-RFLP [24]. This test is based on the polymorphism observed in a 381 bp fragment (in sensitive strains) or 384 bp fragment (in resistant strains) of a putative gene presenting some homologies with an ABC transporter. The second method has been developed to distinguish ISM-resistant from ISM-sensitive strains of *T. brucei* [46]. This *Sfa*NI-PCR-RFLP test is based on the polymorphism of a 677 bp fragment of the *Tb*AT1 gene. The same set of six point mutations could confer resistance to the melarsenoxyde cysteamine cymelarsan (an arsenical diamidine) and to ISM (diamidine compound) and the detection of one of these six mutations could enable reliable identification of sensitivity or resistance to ISM.

Correlation with in vivo tests

The reference test for the determination of drug resistance remains the standardized single-dose mouse test [47]. The correlation of the *Mbo*II-PCR-RFLP with the mouse test was reported to be 85.7% for *T. congolense* isolates (N=30) originating from different areas throughout the tsetse fly belt [24]. However, the same tool used on 20 *T. congolense* isolates originating from Ethiopia and Burkina Faso and nine isolates from Zambia showed a correlation of only 60% (V.D., unpublished) and 75% [48], respectively. In a recent survey in Cameroon (Adamaoua Plateau), the *Mbo*II-PCR-RFLP identified only four strains as resistant among 12 isolates confirmed to be resistant in the mouse test (33.3%) [49]. This indicates that there might be ISM-resistant strains that have developed alternative pathways of resistance that are not detectable by the *Mbo*II-PCR-RFLP test. This is not surprising considering that Ross and Sutherland [50] previously suggested the existence of more than one mechanism of resistance to ISM.

Data on the correlation of the *Sfa*NI-PCR-RFLP test described by Afework *et al.* [46] and *in vivo* tests are only available for 11 ISM-sensitive strains from Uganda, two ISM-sensitive reference strains from Kenya and two multidrug resistant reference strains from Somalia. The first resistant reference strain, CP547, was resistant to ISM, DA, quinapyramine, melarsoprol, homidium and pentamidine, and the second resistant reference strain, CP2469, was resistant to ISM and DA but not to quinapyramine [46]. It is necessary to further validate the *Sfa*NI-PCR-RFLP test using ISM-resistant reference strain isolates that are sensitive to other drugs. From the study of Afework *et al.* [46] it is difficult to confirm whether or not the *Sfa*NI polymorphism is specifically related to ISM resistance or whether the observed restriction patterns are linked to resistance to other drugs. However, it should be noted that field observations suggest that cross-resistance between ISM and DA does not exist [16]. Conversely, cross-resistance has been observed between homidium (which is not a diamidine compound) and ISM [7].

From available data, it seems that none of the recently developed molecular tools for the detection of ISM resistance are fully satisfactory and that further field validation and investigations are required.

PCR-RFLP and allele-specific PCR for the detection of DA resistance

Recently, the analysis of the P2-type purine transporter *Tco*AT1 of *T. congolense* by SSCP led to a simple *Bcl*I-PCR-RFLP test enabling the rapid identification of DA-resistant stocks [45]. This test is based on a single nucleotide permutation (G to A) observed in the DA-resistant strains that can be easily detected through *Bcl*I restriction of the amplicon. This single point mutation confers a Val306 to Ile306 permutation in the purine transporter. Therefore a single amino acid permutation in *T. congolense* is sufficient to induce resistance to DA. In *T. brucei,* a conserved set of six point mutations was described in the *Tb*AT1 gene of melarsoprol-resistant strains [37]. Evidence that DA is exclusively accumulated by the *Tb*AT1 gene was reviewed in [26]. Although

particularly high levels of resistance to melarsoprol could be due to additional factors or mechanisms, such as action of the high-affinity pentamidine transporter (HAPT1) or ABC transporters [26], [51] and [52], the role of the *Tb*AT1 gene remains crucial. *Sfa*NI polymorphism was used to trace DA resistance in *T. brucei* isolates (V.D., unpublished) together with allele-specific-PCR (AS-PCR) [53]. AS-PCR correlates 100% with *Sfa*NI-PCR-RFLP and is less expensive and quicker than *Sfa*NI-PCR-RFLP.

Correlation with in vivo tests

*Bcl*I-PCR-RFLP was validated using 26 *T. congolense* strains [45]. A correct identification was obtained with 14 strains that were identified as sensitive at 20 mg kg^{-1} of DA, with nine being identified as resistant at 20 mg kg^{-1} in the standardized mouse test. Three strains, sensitive in the mouse test at the commonly accepted discriminatory dose of 20 mg kg^{-1}, were identified as resistant by the *Bcl*I-PCR-RFLP. However, these three strains relapsed at lower doses of DA (5 mg kg^{-1}). Some *T. congolense* strains (N=9) isolated in Cameroon on the Adamaoua Plateau were tested using the *Bcl*I-PCR-RFLP and the mouse test and were found to be resistant by both tests [49].

The excellent correlation between *Bcl*I-PCR-RFLP and the standardized mouse test in both studies (32/35 or 91.4% of correct identification of sensitivity or resistance by both tests) is encouraging but should be confirmed using a larger number of strains. The fact that three strains that were identified as sensitive in the mouse test relapsed at lower doses than the commonly accepted discriminatory dose of 20 mg kg^{-1} suggests a higher sensitivity of the molecular test compared with the mouse test.

Concluding remarks and future directions

The molecular diagnosis of DA resistance in trypanosomes is facilitated by the specificity of the transport mechanism for DA. Further field validation of the *BclI*-PCR-RFLP and AS-PCR for *T. congolense* and *T. brucei,* respectively, is necessary, but good correlation between *in vivo* tests and molecular tools can reasonably be expected. The molecular diagnosis of ISM resistance seems to be more complicated because the transport of this hybrid molecule through biological membranes seems less specific than the transport of DA. Several pathways are probably implicated in the process and several diagnostic tests will have to be performed depending on the number of importers or extruders that are potentially involved. ISM-resistance might be caused by a synergistic combination of reduced uptake and increased efflux. The higher the efflux, the higher the energy (ATP) consumption, which in turn provokes a decrease in the mitochondrial potential and a correlated reduced accumulation of ISM in the kinetoplast. It is also likely that the level of expression of the genes coding for those importers or extruders, as well as a gene dosage (i.e. the number of copies of a gene present in a cell or nucleus) for each of the transporters, will be necessary for a more precise definition of the resistant phenotypes. To achieve this, quantitative real-time PCR will be a powerful tool, but will still require live trypanosomes and therefore will be constrained by the practical implications involved, including the need for intensive labour and the high cost.

Although these newly developed molecular markers need to be validated further, there are good prospects that they will enable faster detection of trypanocidal drug resistance (a few days instead of two to three months). This will provide the opportunity to carry out much-needed area-wide resistance surveys for determining the current prevalence and the spread of resistance genes in trypanosome populations.

Furthermore, having molecular markers for trypanocidal drug resistance will make it possible to determine, for example, the stability of drug-resistance genes after withdrawal of the drug selection pressure or to study the dominance or recessiveness of drug-resistance alleles and these factors will contribute substantially to our understanding of the epidemiology of drug resistance. This kind of research will improve our understanding of the mechanisms contributing to the development of drug resistance and should ultimately lead to the development of better strategies to delay the development of drug resistance in trypanosomes.

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