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Functional Cloning as a Means to Identify *Leishmania* Genes Involved in Drug Resistance

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Abstract: Resistance to anti-leishmanial drugs is a mounting problem in high-endemicity regions of South Asia and, potentially, in the context of HIV-*Leishmania* coinfections in Southern Europe. The molecular basis for clinical drug resistance is still largely unknown. It is important, however, to identify all relevant drug resistance markers for further drug development and for epidemiological surveys. An elegant and powerful method to identify such drug resistance markers without bias is functional cloning, using cosmid-based genomic DNA libraries. This review discusses the merits and caveats of this approach.

Keywords: *Leishmania*, drug resistance, functional cloning, complementation, cosmid.

THE PROBLEM

Resistance to standard antileishmanial therapy is a mounting problem for high endemicity regions, such as Northern India [1,2]. The most widely used chemotheurapeutic agents, pentavalent antimonials, date back to the 1930s. Since then, the dosis/weight ratio has tripled in response to increasing numbers of unresponsive cases. This necessary dosis increase has compounded the problem of severe cardiovascular side effects of antimony treatment but could not prevent the spread of therapy resistance in the highly endemic foci in Northern India.

Several factors contribute to the emergence of therapy resistance.

- Reliance on a single standard treatment. This has been true for leishmaniasis since antimonials have been the drug of choice for several decades due to their low cost and the lack of affordable alternatives.
- Ineffective treatment. The cost pressure on the public health services in developing countries and regions often precludes necessary hospitalisation of patients and the use of effective drugs from choice manufacturers. This causes a lack of therapy compliance and a high relapse rate, both furthering the emergence of resistant parasite strains. This problem is compounded by the frequent need to abort chemotherapy due to severe side effects.
- An anthroponotic transmission cycle. Therapy resistance in *Leishmania spp.* has been restricted, until recently, to *Leishmania donovani* which can spread *via* an anthroponotic infection cycle. Other viscerotropic leishmaniae have not been associated with widespread resistance to date.
- The exclusion of vector and reservoir hosts in cases of syringe-mediated transmission, e.g. of *L. infantum* in Southern Europe, prevents the selection processes encountered by the parasite inside the sand fly vector and the animal hosts and shifts the total selective pressure towards drug resistance. This is aided, in part, by partial immune suppression caused by other pathogens [3, 4]. This problem is now becoming evident in Southern European regions where frequent therapy resistance had not been observed before [5].

Although new drugs have been introduced, the main stay of anti-leishmanial therapy in high endemicity regions are still the formulations of pentavalent antimony. Table **1** lists the most widely used therapeutics and the status of resistance *in vitro* and in the field. The use of alternative drugs is hampered mostly by economic factors. Although clinical resistance to amphotericine B has not yet been observed, resistant strains could be obtained *in vitro* [6, 7]. Note also that development of clinical resistance against miltefosine cannot yet be expected due to the fact that the drug was introduced only recently, however spontaneous development of resistance *in vitro* has been observed [8].

SPECIAL FEATURES OF THE LEISHMANIAE

Over the past 15 years, *Leishmania spp.* have emerged as model eukaryotic pathogens that are accessible to a broad range of genetic analyses [9-13]. The leishmaniae share some, but not all genetic properties of Trypanosomes.

Common to all kinetoplastid protozoa is their overall lack of transcription regulation. Large portions of chromosomes form unidirectional, polycistronic transcription units [14]. The resulting pre-mRNA molecules are processed co-transcriptionally by a coupled process of poly-adenylation and trans-splicing, whereby a common leader RNA of 35nt (*Trypanosoma brucei*) to 39nt (*Leishmania major*) is spliced onto the 5' ends of the monocistronic mRNAs [15, 16]. While this process creates the monocistronic, capped mRNA required for translation by eukaryotic ribosomes, it does not allow for individual control of gene transcription. Consequently, no homologues to transcription factor genes have been found in the course of the *Leishmania* and *Trypanosoma* genome projects.

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Table 1. Anti-Leishmanial Drugs, their Structures (Taken from http://pubchem.ncbi.nlm.nih.gov), and the Occurrence of Resistance *in vitro* and in the Field, According to [1, 6-8, 18, 19, 49, 52-54]

Name	Structure	Resistance *in vitro*	Clinical resistance
Sodium stibogluconate		yes [19]	yes [1]
Pentamidine		yes [52]	? [1]
Amphotericine B		yes [6,7]	n.o.
Miltefosine		yes [8]	n.o.
Allopurinol		varying sensitivity	
Paromomycin		yes [49]	yes [54]

(Table 1) contd…..

Name	Structure	Resistance *in vitro*	Clinical resistance
Ketoconazol		varying sensitivity	n.o.

The expression of individual genes depends on parameters such as gene copy number, RNA stability and translation efficiency, and the parameters determining the latter two reside in the mRNA. The exact nature of cis-regulatory determinants of gene expression is largely in the dark, and systems for targeted expression of transgenes are unreliable.

THE MECHANISMS UNDERLYING DRUG RESISTANCE

The mechanisms underlying clinical drug resistance are mostly in the dark and must be separated into host factors and inherent resistance of the parasite. However, no true separation between host and parasite factors is possible when one looks at clinical therapy failure. Therefore, isolates from therapy resistant cases must first be tested for inherent resistance to exclude host specific reasons for treatment failure. Such host-specific factors may range from variations in the innate immune response, over co-infections with other pathogens, immune-suppressive conditions, acquired or pharmacologically induced, to socio-economic factors.

On the parasite's side, several types of mechanisms may cause drug resistance and are listed below.

- Amplification of genes that encode detoxifying proteins such as the members of the multi drug resistance loci. This is the best studied mechanism, and it is employed in *Leishmania spp. in vitro* [17-20]. The clinical impact of this mechanism is yet unclear.
- Amplification and/or increased expression of genes that encode a drug target or a protein that binds the drug. Such increased expression will lead to a titration of the drug. Example for this mechanism acting in an *in vitro* selection situation are a spontaneous *L. donovani* variant that has acquired resistance against the ansamycin antibiotic geldanamycin through massive overexpression of the target, HSP90, and arsenite resistance associated with topoisomerase overexpression [21, 22].
- Overexpression of a protein that constitutes or contributes to a functional bypass to a function that is abolished by the drug's action.

These mechanisms involve a massive overexpression of genes above the natural levels and thus constitute a metabolic burden and are bound to raise problems for the parasite with regard to functional pathways, but also in the context of the host's immune defense. Consequently, gene amplification so far was mostly observed under *in vitro* growth conditions. However, recent reports indicate a role for gene amplification in clinical drug resistance [23, 24].

Loss of function mutations are the alternative to gene amplifications. Mutations can effect therapeutic effects at several levels.

- Transporter mutations. If a drug is dependent on transmembrane transporters, then a mutation in the transporter's active sites can prohibit the import of the compound. This has been shown for *in vitro* induced resistance against tubercidin and miltefosine [25-27].
- An alteration of a ligand binding domain in the drug target may render the protein less affine and thus less sensitive to the compound. However, alterations in active sites of a protein may well impair its function or regulation, rendering the parasite less viable or virulent.

MODEL SYSTEMS TO STUDY EMERGENCE OF DRUG RESISTANCE

In Vitro Promastigote Culture

The most widely used system is the culture of promastigotes *in vitro* under the selective pressure of a drug [28]. The advantages are obvious, as promastigotes have fast growth rates *in vitro*, can be cloned or subjected to reverse and forward genetic approaches.

There are severe drawbacks to this system. Firstly and most importantly, the promastigote is not the pathogenic form, and therapies are not directed against this life cycle stage. For instance, promastigotes are far less susceptible to pentavalent antimonials than the amastigote [29]. Also, culture conditions may have a considerable impact on the toxicity of a compound [30]. One way to work around this is to perform the initial studies in promastigote cultures and evaluate the findings in an amastigote system. The second

drawback is the loss of virulence usually associated with prolonged *in vitro* culture.

Axenic Amastigote Culture

In recent years *in vitro* differentiation and cultivation of amastigotes has been established for several *L. donovani* strains. Differentiation is verified by morphological (reduction of flagella, cell length reduction) [31] and biochemical criteria (expression of A2 protein family [32]). Key triggers for axenic amastigote differentiation in *L. donovani* is the increase from ambient temperature to 37°C and a subsequent pH shift into the acidic range. These axenic amastigotes display gene expression patterns that closely resemble those of true, tissue-derived amastigotes [32-37]. Although this system has already been employed in drug resistance studies [38, 39], there are important caveats to this strategy. Firstly, axenic amastigotes are cultivated in essentially the same growth media as promastigotes, although natural promastigotes and amastigotes are exposed to widely different milieus and nutrient sources and supplies. Secondly, survival within the macrophages appears to depend on factors that are not required for cultured, axenic amastigotes [40]. Thirdly, the effect of drugs on intracellular parasites that are separated from the extracellular environment by two additional membranes, plasma membrane and phagolysosome membrane of the host cells, is largely dependent on effective transport over these membranes, adding modification of host cell properties to the list of resistance mechanisms.

In Vitro Infection of Macrophages

Leishmania promastigotes can efficiently infect macrophages from peritoneal exudates, bone marrow-derived macrophages and other primary antigen presenting cells. In addition, macrophage-like cell lines have been established that also serve as host cells to *Leishmania in vitro*. Infected macrophages can then be placed under drug selection to test therapy resistance of a parasite isolate [30]. The availability of fluorescent life stains allows for flow-cytometric assessment of parasite survival and/or proliferation.

Selection *In Vivo*

The use of laboratory animals for the selection of drug-resistant parasites is as close to the natural situation as possible. The main drawback to this approach is the long incubation period required for *L. donovani* and the lack of an immunologically well defined infection model for *L. donovani*. Since the progression of VL cannot be assessed from live animals, large numbers of animals are required to monitor the disease, and clearance for such experiments will be hard to obtain in most settings. Therefore, in most cases, *in vivo* tests will be restricted to the verification stage following screening for and identification of resistance marker genes.

Natural Drug Resistance

Parasites from cases of clinical therapy failure are the most relevant model to study the mechanisms underlying drug resistance. There are caveats with this approach, too. Firstly, the inherent drug resistance of an isolate has to be established by *in vitro* assays. Secondly, clonal variations within a clinical isolate cannot be excluded and even have to be expected. The subsequent *in vitro* cultivation may select for variants that do not carry the resistance trait. Such *in vitro* selection artefacts can be reduced by limiting dilution cloning directly from clinical samples, allowing each clone within the isolate to proliferate at its own pace.

Do We Need *In Vitro* Screening?

In many instances, drug resistance *in vitro* was linked to the amplification of genes that encode detoxifying proteins such as members of the p-glycoprotein family, a.k.a. multi drug resistance complex. So far, there have been no reports showing amplification of these genes in clinical isolates from cases of therapy failure. The impact of MDR locus on clinical resistance therefore needs to be ascertained.

There are reasons, however, to continue with the analysis of drug resistance mechanisms using promastigotes and axenic amastigotes *in vitro*. It is highly desirable to identify potential drug resistance risks attached to novel therapeutic compounds for which there is not enough clinical data yet. It should be ascertained that novel compounds are not affected by the resistance mechanisms directed against existing drugs. Such proactive research can, by definition, not be conducted with parasite isolates from therapy resistant cases. Here, *in vitro* selection screens complemented by studies in animal models should prove helpful.

FUNCTIONAL CLONING

Once a drug resistant parasite isolate or clone has been obtained, it is necessary to identify the molecular basis or at least the gene or genetic variation responsible for the observed loss of sensitivity. With spontaneous, resistant clones, this usually involves the search for changes of molecular parameters that correlate with the appearance of resistance. This will include Pulsed Field Gel Electrophoresis to monitor karyotype changes, RNA microarray screening to identify increases of mRNA abundance, or 2D-SDS-PAGE to search for protein abundance and/or post-translational modifications. Candidate genes must be overexpressed specifically to verify a role in drug resistance. Such strategies are time-consuming, and the outcome is influenced by factors not always in the control of the experimentator.

Quantitative mutations have been linked to drug resistance *in vitro*. However, they were rarely observed, to date, in drug resistant isolates from the wild. Non-quantitative genetic alterations, i.e. point mutations, frame shift mutations or small deletions will only show in 2D-PAGE, and only if the protein in question is expressed to significant quantities. Also, 2D-PAGE requires rather large quantities of cell lysates, necessitating prolonged *in vitro* cultivation.

A promising escape out of this quandary is the use of functional cloning, also known as genetic complementation. The preferred approach is the generation of a genomic DNA cosmid library from a donor clone that possesses the desired trait. DNA prepared from the amplified library is used to transfect a parasite clone lacking that trait. The recombinant parasite population is then placed under a stringent selection for the trait.

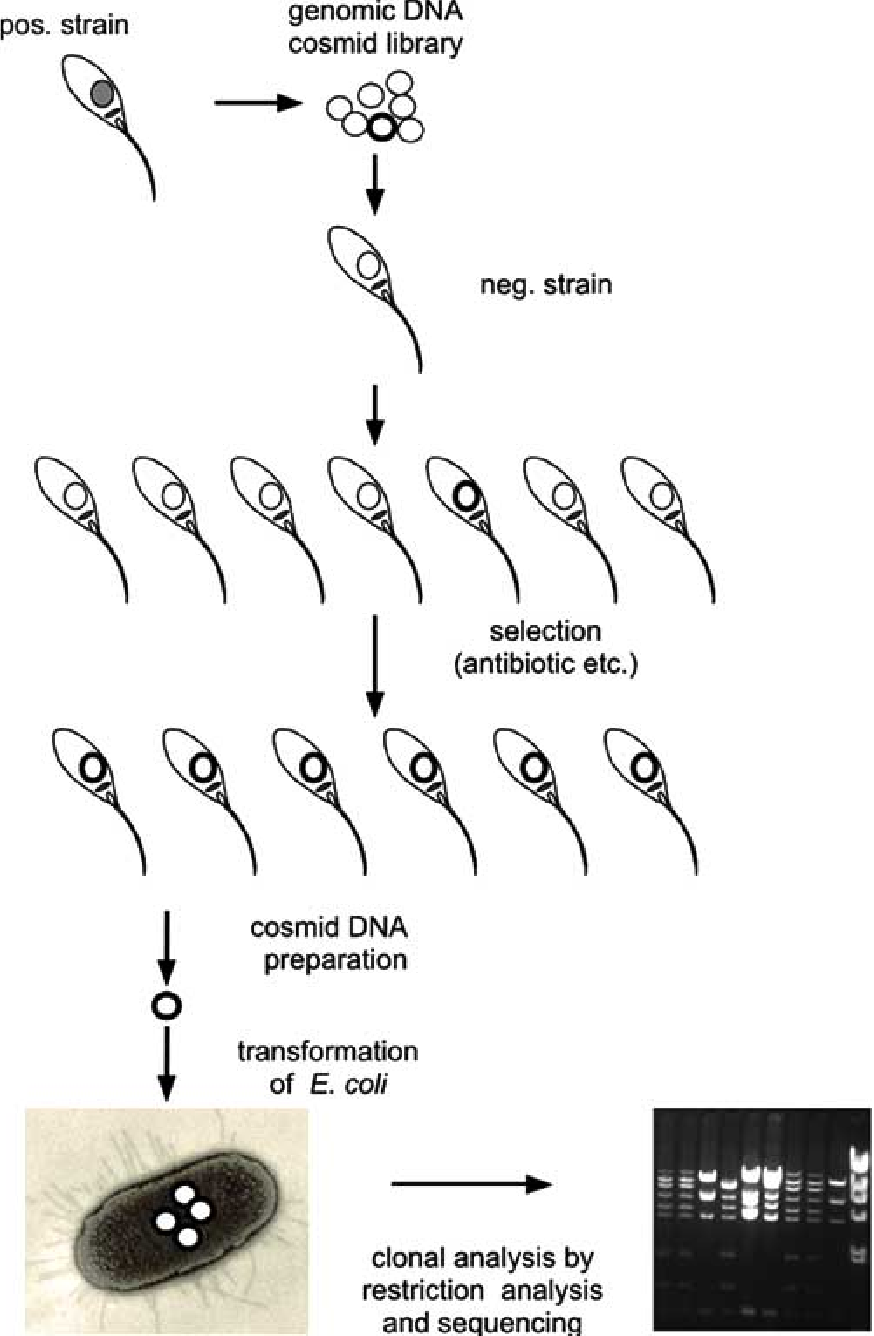

Fig. (1). Schematic representation of cosmid library-based functional cloning. A cosmid library is produced of the genomic DNA from a strain that is positive for the desired trait. Cosmid DNA of this library is used to transfect a strain negative for the trait. Selective pressure, e.g. by drug selection, is applied. Cosmid DNA from the surviving population is used to transform competent *E. coli.* Cosmids from bacterial clones are characterised according to restriction fragment pattern and partial sequence analysis.

The obvious advantage of this approach is the easy recovery and identification of the gene(s) responsible for the selected trait. Plasmid or cosmid episomes can be isolated from a *Leishmania* population by standard alkaline lysis, very similar to plasmid and cosmid preparation from *E. coli*. The isolated DNA can then be used to transform *E. coli* laboratory strains (Fig. **1**).

Characterisation of cosmids is performed by analytical restriction enzyme digest and field inversion gel electrophoresis. According to the fragment pattern, cosmid clones are grouped into prototypes, and the relative prevalence of the cosmid types is recorded. Representatives of the prototypes are subjected to partial sequence analysis from either end of the genomic DNA insert. By alignment with the *Leishmania major* and *L. infantum* DNA databases, the gene loci included in the cosmids are mapped.

Cosmid libraries are preferable to the standard cDNA plasmid libraries used in other eukaryotic models for several reasons:

- The high G/C content of *Leishmania* genes hampers reverse transcriptase and is an obstacle to generating full-length cDNA molecules.
- *Leishmania* genes lack introns and are compact enough for cloning as genomic DNA.
- The presence of long stretches of genomic DNA in a cosmid allows to study the function of a gene in its natural genomic context, including regulatory flanking sequences.
- In cosmids, the entire *Leishmania* genome can be represented in as little a 2000 clones with no bias based on mRNA abundance.

Examples for Functional Cloning in *Leishmania*

The strategy was pioneered by S. Beverley and coworkers in a series of complementation screens aimed at the identification of factors involved in the synthesis of *L. donovani* lipophosphoglycans [41, 42], leading to the discovery of *LPG1* and *LPG2* genes. Mutants defective in LPG synthesis were transfected with cosmid DNA libraries from wild type *L. major*. The recombinant populations were then subjected to ricin and anti-LPG antibody panning to isolate parasites with restored LPG synthesis. Isolation and characterisation of cosmids from these re-isolates facilitated the identification of genes involved in LPG biosynthesis, namely *LPG1* and *LPG2*.

Nucleoside transporters with specificity for adenine and pyrimidines were identified in another complementation screen in which a mutant line incapable of nucleoside import and thus resistant to tubercidin, a toxic adenin analog, were complemented with DNA from a *L. donovani* genomic DNA cosmid library. In this case, the transfected population was subjected to cloning by limiting dilution, and individual clones were tested for tubercidin sensitivity. Cosmids from the sensitive clones were then analysed to identify two related nucleoside transporters, LdNT1.1 and LdNT1.2 [43].

By virtue of the unbiased nature of this approach, the involvement of unknown or unexpected genes in biological processes can be shown. For instance, the identification of a GP94 homolog, a chaperone of the endoplasmatic reticulum, as a factor in LPG expression [44] was not anticipated. This is a clear advantage over biased, reverse genetic strategies, e.g. gene replacement, that can only serve to test for predicted functions of previously identified candidate genes. The proper use of functional cloning will therefore contribute to the annotation of many genes that are currently listed as hypothetical genes without known function.

Using a trans-species complementation screening approach, genes involved in LPG side chain synthesis were identified from a cosmid library of *L. major* genomic DNA using *L. donovani* which does not have side chains on its LPG surface molecules as host [45]. This cross-species complementation approach has a great potential in future studies aimed at the molecular basis of species-specific traits, for instance in the contexts of organ tropism [46] or virulence [47]. In the latter reference, genes encoding the *L. donovani* miniexon RNA were selected as virulence markers in *L. major*.

In another, more recent example, a protein which has a strong impact on the cell cycle of *L. donovani* and *L. major* was identified [48]. The gene, tentatively termed *Leishmania donovani Growth Factor 1* (*LdGF1*), was selected under long-term *in vitro* cultivation. Upon overexpression, it promotes the release of promastigotes from a stationary phase growth arrest. Interestingly, its overexpression renders *L. major* less virulent in BALB/c mice. The protein is listed as a sequence orphan in the LeishDB database and has no known homologues outside the leishmaniae.

Application to Drug Resistance

The ability of cosmid-based overexpression/selection strategies to identify drug resistance gene loci was confirmed by Cotrim and colleagues [49] who isolated several genes involved in resistance to drugs that affect nucleoside metabolism.

The benzoquinoic ansamycin geldanamycin (GA) is a specific inhibitor of the HSP90 family of chaperones due to its specific binding to the ATP binding domain [50]. Inhibition of HSP90 prevents the structural maturation of various regulatory factors [51] and causes a growth arrest. In *Leishmania*, GA treatment induces a heat shock response and differentiation at low doses and a G2/M cell cycle phase arrest at high doses [22]. A functional complementation screening was employed to identify genes that, upon extrachromosomal amplification, allowed for overexpression of proteins that would bind GA and thus titrate the drug. All the cosmids selected, however, represented the HSP90 gene repeat locus, suggesting that HSP90 is the sole target for GA in *L. donovani*.

Another excellent example for the *de novo* identification of resistance mediating genes was reported recently [52]. A population of *L. major* was transfected with a genomic DNA cosmid library. After challenge with pentamidine, a real world anti-leishmanial drug, the cosmids of the survivor population were analysed. Two cosmids were recovered, and the resistance gene was identified by deletion/selection analysis. The open reading frame identified encodes a probable ABC transporter protein remotely related to p-glycoproteins.

A reverse approach was taken in a recent study by Perez-Victoria *et al.*. A spontaneous *L. donovani* mutant was found to be resistant against miltefosine owing to an impaired inward translocation of the drug [26]. Using functional complementation to restore miltefosine sensitivity of the mutant, a miltefosine transporter gene, *LdMT* was identified [27]. This study elegantly links traditional studies relying on spontaneous drug resistance with a functional cloning strategy and may serve as model for future efforts.

The Caveats

Using complementation screening with cosmid libraries, there are several caveats to observe. Firstly, to establish an amplified recombinant population from which to select, a selection marker has to be employed, usually an antibiotic. Expression of selection marker genes and the exposure to antibiotics constitute a metabolic burden and cellular stress.

Both may interfere with the selection procedure and taint the results. If possible, care should be taken not to place the population under antibiotics during the functional selection. Secondly, the necessary period of *in vitro* amplification of the selected sub-population following the functional selection will favor cells with fast *in vitro* growth rates. Under these conditions, a considerable selection within the survivor population can happen, favouring or eliminating previously selected cosmids [48]. This can be avoided, however, by early division of the survivor populations into clones, e.g. by plating or limiting dilution under drug selection. A third limitation of the approach is the strong bias on dominant traits. Since wild type alleles are usually still in place, a recessive trait conferred by an episome may not allow for a successful complementation.

The Future

Several lines of research will have to be pursued. Firstly, the molecular mechanisms leading to resistance in the field against approved drugs must be identified. Secondly, the potential of *Leishmania* to develop resistance against novel therapeutic compounds should be explored before such compounds go into clinical application. Only if resistance mechanisms for drugs are known, suitable, complementary compounds can be identified for future multidrug treatments.

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