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REVIEW

Susceptibility Testing of Medically Important Parasites

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SUMMARY In the last 2 decades, renewed attention to neglected tropical diseases (NTDs) has spurred the development of antiparasitic agents, especially in light of emerging drug resistance. The need for new drugs has required *in vitro* screening methods using parasite culture. Furthermore, clinical laboratories sought to correlate *in vitro* susceptibility methods with treatment outcomes, most notably with malaria. Parasites with their various life cycles present greater complexity than bacteria, for which standardized susceptibility methods exist. This review catalogs the state-of-the-art methodologies used to evaluate the effects of drugs on key human parasites from the point of view of drug discovery as well as the need for laboratory methods that correlate with clinical outcomes.

KEYWORDS parasites, resistance, susceptibility tests

INTRODUCTION

The methods employed to test the activity of either established or novel medicines against parasites remain largely within the research domain. However, the development of public-private partnership consortia to align biological and parasitological testing platforms against the various parasite stages has started to pay off by strengthening and rationalizing the drug discovery and development pipeline. To give an

example, with malaria now, there is a renewed effort to identify drugs that target liver stages and gametocytes in efforts not only to treat patients with acute infection but also to kill dormant liver stages (hypnozoites) and prevent transmission (gametocytes). Therefore, different assays have emerged in the research arena to address these pressing needs to identify compounds that serve as chemical starting points for drug development. Ultimately, from a clinical point of view, interpretive breakpoints (drug concentrations above which infectious agents are killed or growth is inhibited in patients) would be of immense use for treating patients. Here again, difficulty arises because parasites are difficult to culture from human specimens (so-called *ex vivo* testing), and in some cases, polyclonal infections may occur; thus, testing may select for certain strains and not others present in that human infection. Further confounding attempts to standardize testing is the fact that certain drugs act at a particular stage of the parasite life cycle, thus affecting the results of the assay. Standardized methods are also essential to drug discovery efforts so that promising leads do not fall by the wayside in preclinical testing and that "false-positive" leads do not progress too far in the development pipeline.

This review attempts to conduct an impartial environmental scan of the laboratory testing methods available to perform susceptibility testing against medically important parasites and to provide a critical appraisal from a clinical perspective (Table 1). Organisms covered in this review include *Plasmodium*, *Leishmania*, *Trypanosoma*, enteric protozoa (*Giardia*, *Entamoeba*, and *Cryptosporidium*), *Schistosoma*, and filariae. In the realm of bacteria and viruses, organizations like the Clinical and Laboratory Standards Institute (CLSI) (http://clsi.org/) in North America and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/) in Europe deal directly with the standardization of phenotypic methods and also the interpretation of clinical breakpoints based on *in vitro* testing methods. While the CLSI and EUCAST do not always agree on breakpoints for certain bug-drug combinations, the decision of clinical breakpoints is based on evidence in the literature. This includes primarily pharmacokinetic and pharmacodynamic data from clinical trials, supported by *in vitro* growth and resistance data based on specified testing methods. In certain instances, there is a paucity of clinical evidence to guide the interpretation of *in vitro* data, and here epidemiological cutoffs (ECOFFs) are used to give a sense of the wild-type distribution of inhibitory concentrations in the population. The EUCAST also guides clinical interpretation of breakpoints for regulatory agencies such as the European Medicines Agency, whereas in the United States, it is not uncommon to have the Food and Drug Administration espouse different breakpoints to the CLSI (http://www.fda.gov/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDER/ucm275763.htm). However, for patient management, most clinical laboratories that test patient samples adhere to CLSI guidelines on testing methods for bacteria, viruses, and fungi and participate in rigorous quality assurance programs such as the College of American Pathology (CAP) in the United States (http://www.cap.org/).

In this vein, nascent groups such as the Worldwide Antimalarial Resistance Network (http://www.wwarn.org/) have made a similar attempt to provide standardization of testing methods for malaria, quality control of reagents used, interpretation of data (graphing 50% inhibitory concentration [IC_{50}] data), and epidemiologically relevant updates on molecular markers of resistance emerging throughout the endemic world (1). From a microbiological and clinical perspective, the science and medicine of antimalarial susceptibility testing lag far behind those for bacteria, fungi, and viruses. This review attempts to catalog the current state of affairs of testing methods, clinical validation studies, and molecular data available for susceptibility testing of parasites. Additionally, this review considers further developments, driven in large part by the stimulus to the new antimalarial clinical trial funding space, that may allow clinical interpretation of laboratory testing to guide patient management.

TABLE 1 Summary of susceptibility testing assays of medically important human parasites

Parasite	Parasite stages	Type(s) of assay[a]	Measure(s)	Reference(s)
Plasmodium	Liver stages (sporozoites, schizonts, hypnozoites)	High-content imaging	*P. berghei*/*P. yoelii* liver schizont development	161
		Bioluminescence imaging	*P. berghei*/*P. yoelii* liver schizont development	27, 162
		Immunofluorescence	Development of *P. chabaudi* liver small (hypnozoites) and large (schizonts) forms	33
		Immunofluorescence	*P. falciparum*/*P. vivax* sporozoite invasion and development	28, 29
	Asexual blood stages (rings, trophozoites, schizonts)	Microscopic	*P. falciparum*/*P. vivax* schizont maturation	163
			P. falciparum ring-stage survival	164
		Isotopic	*P. falciparum* intraerythrocytic growth	4
			P. falciparum intraerythrocytic growth	5
		Colorimetric	*P. falciparum* intraerythrocytic growth	7
		ELISA	*P. falciparum* intraerythrocytic growth	6
		Fluorescent DNA dye based	*P. falciparum* intraerythrocytic growth	9, 165, 166
	Transmission stages (gametocytes, gametes, zygotes, ookinetes, oocysts, sporozoites)	ATP bioluminescence	*P. falciparum* gametocyte viability	167
		Flow cytometry	*P. falciparum* gametocyte viability	168
		Microscopic-immunofluorescence	*P. falciparum* male (exflagellation) and female (fluorescence, shape, size) gamete formation	23
		Microscopic	*P. falciparum* presence/no. of oocysts	169
Leishmania	Amastigotes, promastigotes	Macrophage infection model	Increase in no. of amastigotes	40, 41
		Colorimetric	Promastigote viability	39
		Flow cytometry	No. of parasites	42
Trypanosoma cruzi	Epimastigotes, trypomastigotes, amastigotes	Murine models of Chagas disease	Parasite burden measured using PCR, immunofluorescence, bioluminescence	43, 44, 46, 47, 56
Trypanosoma brucei	Trypomastigotes, epimastigotes	PCR-RFLP with SfaNI	Detection of mutated parasite transporter and drug-resistant parasites	69
Giardia lamblia	Trophozoites, cysts	Parasite culture	No. of parasites	78
		Colorimetric	Color change due to parasite growth	76
		Imaging	Parasite viability	78
		Bioluminescence	Luminescence signal	80
Entamoeba histolytica	Trophozoites, cysts	Bioluminescence	ATP-dependent luciferase bioluminescence signal	86
Cryptosporidium	Oocysts, gametocytes	Microscopy, qRT-PCR	Parasite invasion and growth	100, 102–105
Schistosoma	Eggs, miracidiae, cercariae, schistosomulae, adult worms	Real-time parasite mobility	Worm viability and motility	100, 101
		Isothermal microcalorimetry	Motility	102
		Image-based high-content screening	Enzymatic and metabolic activity	103
		Fluorescence	Viability and cytotoxicity	104, 105
		Luminescence	Viability	106
Filaria	Microfilariae, L3 larvae, adult worms	Microscopy	Motility	132–135
		Colorimetry	Viability	154, 155
		Worm fecundity	Microfilaria release	134, 138
		Embryogenesis	No. of embryos and stage	134, 153, 157
		Trypan blue exclusion	Viability	151, 152
		Third larval stage (L3) to fourth (L4)	Molting	137, 141
		Histology and electron microscopy	Anatomical changes	142, 143
		WormAssay (software)	Motility scoring	156

[a]qRT-PCR, quantitative reverse transcription-PCR; ELISA, enzyme-linked immunosorbent assay; PCR-RFLP, PCR-restriction fragment length polymorphism.

MALARIA

Malaria is one of the major causes of death and illness by an infectious disease. The World Health Organization (WHO) estimates that 3.2 billion people are at risk of acquiring malaria, with 214 million cases occurring worldwide, resulting in 438,000

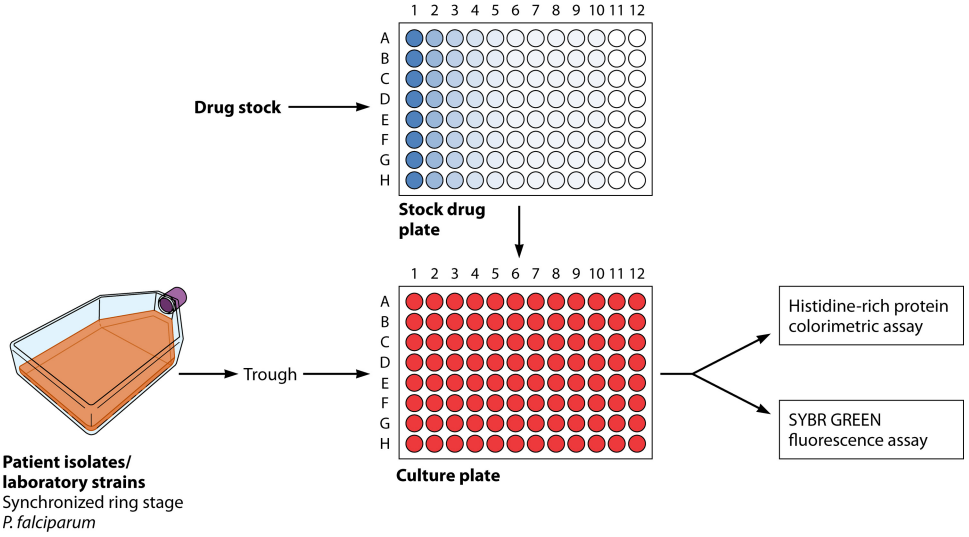

FIG 1 Typical *in vitro* susceptibility testing workflow for *Plasmodium falciparum*. Synchronization of ring-stage intraerythrocytic parasites is required, followed by the addition of drugs to a microtiter plate. There are assays for culture-adapted laboratory strains as well as direct testing from patient blood. Parasitemia can be assessed in several ways, including by an ELISA or fluorescence via flow cytometry. There are several modifications of this basic format, including the RSA.

deaths. Ninety percent of the deaths occurred in the WHO African region. *Plasmodium falciparum* and *Plasmodium vivax* are the main etiological agents. One area of concern is the rise in the incidence of artemisinin-resistant *P. falciparum* infection in Southeast Asian countries, with the consequent risk of further spread to sub-Saharan Africa (2).

Plasmodium falciparum Susceptibility Testing Assays

Trager and Jensen were the first to culture the intraerythrocytic stage of *P. falciparum* in human erythrocytes supplemented with human serum and other nutrients essential for parasite survival (3). The advent of this culture method permitted the laboratory investigation of this stage of the parasite life cycle. Soon after, in 1979, a susceptibility testing method was developed by Desjardins et al. using radioactive hypoxanthine incorporation into nucleic acids as the assay readout in the presence or absence of drugs (4). Others then developed a method using ethanolamine incorporation into membrane lipids (5). With both approaches, the presence of radioactivity was directly related to parasitemia, and thus, microscopy-based counting of Giemsa-stained parasites was not required. This enabled testing to be done in a microwell format with serial dilutions of the drug. Typically, parasites are exposed for a full 48-h life cycle in standard assays using unsynchronized cultures. More recently, enzyme-linked immunosorbent assays (ELISAs) relying on antibodies to detect malaria antigens have been used. These antigens include histidine-rich protein 2 (HRP2) (6) and lactate dehydrogenase (LDH) (7). Both proteins are expressed proportionally to the amount of viable parasites left after drug treatment (Fig. 1). Unlike LDH, HRP2 is secreted by the parasite and very stable. A fortunate consequence of intraerythrocytic survival is that the majority of DNA present in infected red blood cells is parasite derived. Thus, others developed an assay relying on a nucleic acid stain (SYBR green) to determine parasite levels (8, 9). This stain can be applied to flow cytometry-based susceptibility tests that monitor the drug effect by gating the red blood cell fraction after incubation with the drug. Taken together, these assays enable researchers to establish the IC_{50} or IC_{90}, at which one-half or 90% of the parasites are killed by the drug being tested, respectively. However, Wein et al. showed that these tests are not all equal and in fact may be influenced by the mechanism of action of the drug (10). For example, when pyrimethamine was tested by the LDH and SYBR green methods, the parasites appeared resistant to the drug, whereas by radiolabeled hypoxanthine release and HRP2 meth-

ods, the parasites were sensitive, with IC_{50}s below 10 nM. It has been proposed that hemoglobin may affect fluorescence-based assays relying on SYBR green (11). These experiments were conducted with exposure to the parasites to one full life cycle of 48 h. When the investigators repeated the assays with 72 h of exposure, all test methods displayed similar results (susceptible at IC_{50}s of less than 10 nM). The question arises as to whether DNA replication, which is a function of the metabolic activity of the parasite, needs more than 48 h to reflect true parasite survival after drug exposure. The mechanism of action of the compound may also be stage specific, such as in the ring stage (e.g., artemisinins [12, 13]) or in the mature stages (e.g., mefloquine [10]), and thus, a 48- or 72-h exposure may mask the effects of the drug.

In the context of antimicrobial susceptibility for bacteria or viruses, there are no obvious analogies to find. In general, testing methods for viruses or bacteria may change by antimicrobial, not because of the mechanism of action *per se* but because drugs may interact with certain media or incubation conditions specific to the organism. Typically, bacterial testing is done by broth microdilution or by use of strips impregnated with an antibiotic. Bacterial proliferation is usually exponential, and thus, retardation of growth is easy to identify either on agar plates or in broth by the use of turbidity as a readout. Certain antibiotics are cidal, causing permanent effects on viability, whereas others may be static, implying that if the drug is removed, the microbe may recover. On the other hand, malaria may be similar in that dormant but not dead forms of the parasite have been noted by several investigators upon drug exposure, most notably with the artemisinins. Dormancy may in fact be analogous to the static effect of certain antibiotics on clinically relevant bacteria.

The discordance between *in vitro* testing results (IC_{50}) and clinical treatment outcomes confounded researchers and prompted clinicians to use an *in vivo* surrogate. Here parasitemia is monitored on an hourly and then a daily basis to determine if there is a delay in parasite clearance. This led to the development of drug-specific assays such as the "ring-stage survival assay" (RSA) for artemisinin (14). The RSA is the first assay to correlate strongly with antimalarial clinical treatment outcomes when conducted on isolates derived from patients who failed artemisinin therapy (14, 15). An interesting feature of the RSA is that the parasites have to be very tightly synchronous in the phase of growth, and drug exposure is focused on one part of the life cycle stages (very early rings).

The significance of this is that researchers applied the drug at the stage of the life cycle where the drug is purported to act (14). This raises the question of whether all susceptibility testing should be stage specific in such a way. Unfortunately, the mechanism of action and stage specificity of all antimalarials are not known. Furthermore, genetic mutations were identified in a protein (kelch 13), which presaged the resistance phenotype in both cultured laboratory strains and patient isolates (16). While there are no clinical breakpoints, clinicians began to use *in vivo* data such as day 3-positive parasitemia or delayed parasite clearance in patients as an indicator of resistance (16, 17). The assay readout is therefore relative to parasites not exposed to the drug. Researchers have suggested that parasite survival of $>1\%$, when exposed to 700 nM dihydroartemisinin for 6 h at the ring stage, correlates with *in vivo* treatment failure (14). The association is arbitrary but seems to hold true, especially in Cambodia, where artemisinin resistance is on the rise. One cannot rule out that the RSA is a measure of dormancy of the ring stage in response to artemisinins and thus a propensity for clinical treatment failure in patients who cannot clear the dormant stages.

A further confounding issue is the testing of parasites either after *in vitro* culture adaptation or directly from the patient (so-called *ex vivo* testing). The former approach requires a long-term culture system to be set up and for the isolate to adapt to the artificial system. The risk here is that culture may select for a certain genotype when an infection is often polygenic (so-called multiplicity of infection), especially in regions of hyperendemicity such as Africa (18). Selection may occur for the fitter clone or genotype, which may not reflect the situation in the patient. Direct testing of patient blood via the *ex vivo* method may also be problematic in the situation where there are

multiple clones. In general, *ex vivo* testing is more difficult to conduct in resource-limited settings and is time sensitive.

Transmission-Blocking Drug Tests

An important stage in the life cycle of malaria in humans is the production of gametocytes, which are taken up in a blood meal by anopheline mosquitoes. Therefore, drugs that are able to kill the gametocyte stage may prevent transmission. Culturing of gametocytes at the exclusion of asexual stages requires special culture conditions. In 1986, Ponnudurai et al. were able to demonstrate that culture of gametocytes alone was possible (19). The addition of *N*-acetylglucosamine to the culture medium likely blocks the entry of merozoites into uninfected red blood cells, which ultimately depletes the asexual stages that require this invasion step. Other methods, such as the addition of heparin and depletion of nutrients in parasite-oversaturated medium, can result in gametocyte differentiation. Gametocytogenesis, whose triggers are poorly understood, occurs at a low rate of 0.2 to 1% from sexually committed merozoites (20, 21). Mature gametocytes form over a period of 12 to 14 days and have five distinct stages (stages I to V), with the latter two stages being insensitive to many current antimalarials (22, 23). Mature gametocytes are relatively quiescent from a metabolic perspective and remain so until gamete formation is triggered upon mosquito feeding and the subsequent change of the milieu. It is the most mature gametocyte stage (the so-called stage V gametocyte) that is responsible for transmission once taken up in a mosquito blood meal.

Therefore, researchers have developed assays to factor in transmission-blocking properties in drug discovery programs. A sex bias ratio has been cited in the literature, whereby more female than male gametocytes (female-over-male gametocyte ratio of up to 4:1) are produced, and concerns that drugs may affect each sex differentially have been raised, although it is worth noting that both male and female gametocytes are required in a mosquito blood meal for propagation (22). This emphasized the need for assays with sex-specific readouts to account for differences in both drug sensitivity and thresholds of detection. The process by which male gametocytes emerge from the red blood cell is called exflagellation. Certain groups have used the percentage or rate of exflagellation as a measure of drug activity, whereas others may use metabolic readouts or reporter-driven transgenic parasites. The relative clinical merits of these assays are not well established, specifically whether they translate into better patient outcomes. The theoretical benefit of preventing gametocytogenesis is clear from a transmission-blocking perspective and, at the population level, may help promote disease control and eradication. Ultimately, the value of transmission-blocking activity will have to be assessed from clinical studies and correlated back to these *in vitro* assays to determine if there is an association between gametocytocidal concentrations *in vitro*, drug exposure at the patient level (or cohort of patients), and benefit at the population level.

Antihypnozoite Drug Tests

In addition to blocking transmission, another major pursuit in the drug development arena has been to target the liver stages for the use of new antimalarials for both prophylaxis and treatment. Currently, atovaquone and primaquine (and related 8-aminoquinolines) are the only approved drugs that retain activity against the liver stage of malaria. The 8-aminoquinolines but not atovaquone are able to kill the hypnozoite stage of *P. vivax* and *Plasmodium ovale*. The approved drug here is primaquine, which requires 14 days of treatment and carries a risk of hemolysis in patients with severe glucose-6-phosphate dehydrogenase (G6PD) deficiency (24, 25). Other drugs in this class in clinical development include tafenoquine, which is completing phase III studies and kills hypnozoites effectively after a single dose, but there are concerns about G6PD deficiency, and an accompanying diagnostic test may be required to rule out this potential toxicity (26). More drugs with activity against liver-stage parasites through new modes of action and lacking G6PD liability are

needed. Recently, this gap in the portfolio of new antimalarials encouraged the malaria community to develop assays to measure drug activity at this specific stage of the life cycle.

Significant progress has been made in developing stable *in vitro* human hepatocyte assays using specific spatial control in three-dimensional arrays or with the specifically selected HC04 cell line (27–29). Certain groups have used the *P. berghei* liver-stage assay in murine models to good effect (30). Others took advantage of the fact that the primate malaria agent *Plasmodium cynomolgi* forms liver-stage hypnozoites, thus potentially making it a useful surrogate for *P. vivax* or *P. ovale*. However, early attempts relied on *in vivo* inoculation of rhesus macaques (31). An *in vitro* liver-stage model has remained elusive because primary hepatocytes dedifferentiate after 1 week of culture, making them refractory to hypnozoite formation, and also, infection of hepatocytes by sporozoites results in host cell demise. Recent laboratory studies were able to show the persistence of hypnozoite stages of *P. cynomolgi* in *Macaca fascicularis* hepatocytes up to 40 days under modified growth conditions (32, 33). Mikolajczak and colleagues successfully used a humanized mouse model to accommodate *P. vivax* infection, which shows promise (34). Persistence beyond 8 to 15 days in hepatocytes is essential for the study of the hypnozoite stage, as it forms only after this point. From a translational perspective, there are no direct diagnostic tests for the identification of latent malaria in the liver stage to enroll patients in a radical-cure trial. Detection of *P. vivax* relapse, as opposed to reinfection, is also difficult to assess in areas of endemicity, where both phenomena may occur.

KINETOPLASTIDS

Leishmania

Leishmania is a digenetic parasite that alternates between a flagellated promastigote form in the gastrointestinal tract of female phlebotomine sandflies and an intracellular amastigote form in mammalian host macrophages. Macrophages engulf promastigotes into the phagosome, where differentiation to the amastigote form occurs. Fibroblasts and dendritic cells can also serve as host cells for amastigotes in an infected patient. Disease manifestations of leishmania infection vary by species type and the geographic location where these species reside. Immune responses by the host may also affect clinical manifestations, which range from cutaneous disease to mucosal and even visceral (organ) infection.

Antimonial drugs and miltefosine treatment failure were reported for both visceral and cutaneous leishmaniases (35–38). Assessment of susceptibilities of different clinical strains of *Leishmania* to drugs has been limited by a lack of consensus on testing methodology, especially as it relates to the different life cycle stages. The intracellular amastigote stage is the target of treatment for leishmaniasis. Drug susceptibility testing against amastigotes is considered the "gold standard" for antimonial drugs (Fig. 2). Susceptibility testing against promastigotes shows results different from those for amastigotes (39). However, several factors influence the testing of clinical strains against amastigotes, making it a low-throughput assay (40). Traditional *Leishmania* drug susceptibility testing relies on the determination of the 50% effective dose (ED_{50}) using either mouse peritoneal macrophages or other macrophage cell lines. These tests are conducted under diverse conditions with different concentrations of drugs, drug exposure times, and readout specifications. Since these variables affect the final results (41), drug susceptibility assay outcomes have not always been uniform. Fernández et al. (40) found that the determination of the reduction of the parasite load at a single predefined drug concentration at 34°C provided a better method for susceptibility assessment. However, this measurement depended on microscopic reading. Combining this approach with the detection of parasite RNA may provide a method to be routinely used to monitor clinical strains of *Leishmania* (40).

A simplified colorimetric resazurin assay was developed for measuring *Leishmania donovani* susceptibility to miltefosine using promastigotes. Miltefosine susceptibility for clinical isolates correlated strongly by using the assay with intracellular amastigotes and

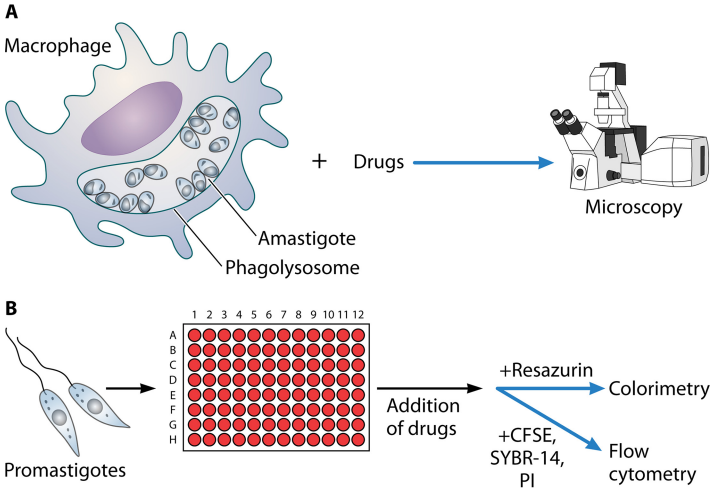

FIG 2 *Leishmania* susceptibility testing can be performed by using either amastigotes within macrophages (A) or promastigotes (B). Endpoints can include microscopy or measurement of promastigote survival using colorimetric or fluorescent readouts.

the resazurin-based assay for promastigotes (39). However, further study is necessary to examine the usefulness of this assay as a surveillance tool for susceptibility testing with other drugs in clinical isolates.

Flow cytometry has been used to assess viability and cellular changes in *Leishmania* and is a promising tool for examining the sensitivity and resistance of *Leishmania* promastigotes to different drugs. The effect of the drug on the proliferation of promastigotes at different time intervals was determined by using 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE). The combination of SYBR-14 and propidium iodide (PI) stains was an efficient way to concurrently visualize both live and dead promastigotes of *Leishmania*. This staining method has the advantage of being rapid, without requiring extra processing before staining. Since promastigotes are easy to cultivate *in vitro*, this assay was performed with promastigotes to measure the activity of drugs on this parasitic form (42). Future research should be directed toward understanding the applicability of similar techniques in clinical isolates and the amastigote stage of the parasite and increasing the throughput of testing modalities to screen a large number of compounds.

Trypanosoma

Chagas disease. Chagas disease, caused by an intracellular protozoan parasite, *Trypanosoma cruzi*, is the leading form of infectious heart disease in Central and South America. There are distinct stages of the clinical forms of Chagas disease (43). The acute phase remains asymptomatic in several cases, but this can be severe, typically characterized by high parasitemia fever and lymphadenopathy, causing death in up to 5% of diagnosed cases. Chronic infections result cardiomyopathy in 20 to 30% of cases and digestive megasyndromes in about 10% of cases (44, 45). Treatment is indicated for patients in the acute phase and in the early chronic phase, immunosuppressed patients with reactivation of infection, and children with congenital infection. Treatment of patients in the acute phase and in the indeterminate or asymptomatic period relies on nifurtimox and benznidazole. Variation in drug susceptibilities of *T. cruzi* was observed in a study by Filardi and Brener (46), and about 30% of the parasite populations led to infections that were resistant to both or either drug. A simple drug susceptibility assay can provide a routine, reproducible surveillance tool for measuring the worldwide development of drug resistance. Drug susceptibility testing in *T. cruzi* is particularly challenging because most Chagas disease patients are diagnosed only in the chronic stage or when tests are performed during blood donation or surgery (47). Drug susceptibility of *T. cruzi* has been evaluated in animal models of acute and chronic

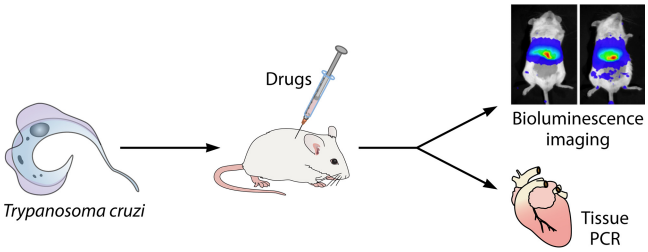

FIG 3 Drug susceptibility in *T. cruzi* evaluated in animal models of acute and chronic Chagas disease. The mouse model for *Trypanosoma cruzi* depicted here is used for testing the preclinical efficacy of candidate drugs, followed by evaluation of tissue by molecular or imaging methods.

Chagas disease. Information on the effect of drugs on the dissemination of parasites in specific tissues after infection required the sacrifice of animals and enumeration of the parasite burden in organs and whole animals. Techniques such as PCR amplification or *in situ* hybridization of parasite-specific genes (48–51), parasite antigen-specific immunofluorescence (52–54), and quantification of amastigotes or trypomastigotes present in tissue sections or blood (55–58) have been cumbersome and inconsistent and suffered from significant limitations. There are no definitive cure criteria available for Chagas disease, and in the absence of a predictive treatment model, several diagnostic methods are frequently used, including fresh blood cultures and examination, parasite-specific gene PCR from blood and tissue DNA, and serological assays. Tissue PCR is arguably the most sensitive method and can identify several noncured mice (59). The inconsistency of PCR-based methodologies was due to the lack of knowledge of the locations of parasite persistence. This makes real-time assessment of the parasite burden difficult (47). In a recent clinical trial study with a new drug, posaconazole, identification of *T. cruzi* DNA was used in a PCR assay. Instead of using it as a marker of cure, those researchers used it to measure treatment failure. A negative PCR result, therefore, may indicate the absence of circulating DNA in fresh blood drawn for testing (60). A new, more sensitive, bioluminescence-based mouse model has been developed to monitor the progress of *T. cruzi* infection and to interrogate the activities of different drugs. This imaging method allowed the quantification of the parasite burden in specific tissue and minimized bias in tissue sampling (61) (Fig. 3). This shows the limitation of tissue PCR, especially the misclassification of treatment failures as cures, because of the localized distribution of parasites. An immunosuppressive drug, cyclophosphamide, has been used to increase the sensitivity of detection of parasites after drug treatment (62–64). Integrating bioluminescence imaging and posttreatment immunosuppression methods may provide a maximally sensitive way to estimate the true dynamics of *T. cruzi* reactivation and cure (65). Whether these methods can be utilized in human clinical specimens remains to be seen.

Human African trypanosomiasis. "Sleeping sickness," or human African trypanosomiasis (HAT), causes morbidity and mortality in sub-Saharan Africa. Two protozoan parasites of the genus *Trypanosoma*, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, are responsible for HAT, and both parasites are transmitted by tsetse flies. According to the World Health Organization, in 2014, 3,796 actual cases were reported, with <15,000 estimated cases of HAT. The first stage of the disease is treated with pentamidine, whereas the second neurological stage can be treated with melarsoprol, eflornithine, or nifurtimox-eflornithine combination therapy (NECT). A promising compound in the pipeline at the Drugs for Neglected Diseases Initiative (DNDi) (Geneva, Switzerland) is fexinidazole. Fexinidazole is an oral agent used primarily for stage 2 of HAT but potentially stage 1 in children.

There has been an increasing incidence of melarsoprol treatment failure because of drug resistance. For example, 20 to 30% treatment failure rates were reported in Uganda, the Democratic Republic of Congo, and the Sudan (66). This rise in drug resistance underscores the need for the development of drug susceptibility testing

against HAT. Fortunately, in 2009, the WHO Essential Medicines List (EML) added NECT to treat the neurological stage of HAT caused by *T. brucei gambiense*. NECT is efficacious, relatively safe, and easy to administer (67).

In a previous study to analyze the melarsoprol treatment failure rate among late-stage HAT patients in Aura, northern Uganda, a glandular puncture and a lumbar puncture were performed, and the number of trypanosomes was counted in cerebrospinal fluid (CSF) or lymph node fluid (68). The invasive nature and some false-positive diagnoses associated with testing emphasize the need for the development of new techniques accessible for routine use.

To determine the susceptibility of *T. brucei gambiense* in northwestern Uganda to drugs, PCR and SfaNI restriction digestion of the adenosine transporter gene were employed (69). It should be mentioned that out of 11 mutations described by Mäser et al. (70), SfaNI restriction fragment length polymorphism (RFLP) analysis examined only 1. It is also possible that other factors, such as ABC transporters, may play a role in reduced drug susceptibility in African trypanosomes (71). Moreover, *T. brucei gambiense* parasites isolated from patients adapt poorly to culture conditions, and the parasites underwent infection in rodent and adaptation in culture medium before they were ready for propagation. It is unclear if the adapted parasites retain characteristics similar to those of parasites originally isolated from patients and if rodent infection and culture conditions imposed selection pressures on the parasites before susceptibility to drugs could be ascertained (69).

Recently, genome-scale RNA interference (RNAi) screening linked two closely related aquaglyceroporins, AQP2 and AQP3, to melarsoprol-pentamidine cross-resistance (MPXR) (72), suggesting that aquaglyceroporins have an important role in influencing susceptibility to these drugs. In a follow-up paper, Baker et al. (73) showed that the loss of function of one of these proteins, AQP2, renders MPXR in a laboratory-selected MPXR strain. It will be important to understand the status of *AQP2* in drug-resistant clinical isolates and to check if there is a relation between the clinical outcome and the status of AQP2 (73, 74).

STOOL PROTOZOA

Diarrheal infections are considered one of the top four contributors to the global burden of disease. Intestinal parasites are leading causes of morbidity and mortality associated with diarrheal diseases in both the developed and developing worlds. Infections by two anaerobic protozoan parasites, *Entamoeba histolytica* and *Giardia lamblia*, result in more than 300 million cases annually. In the absence of vaccines or prophylactic drugs, treatment of amebiasis and giardiasis depends on nitroimidazoles, with metronidazole being the most commonly used drug worldwide. Despite the efficacy of nitroimidazole drugs, treatment failures in giardiasis happen in up to 20% of cases (75). The clinical resistance of *G. lamblia* to metronidazole is evident, and cross-resistance to commonly used antigiardial drugs is a major concern (76). The potential resistance of *E. histolytica* to metronidazole is also an increasing concern, as *in vitro*, *E. histolytica* trophozoites adapt to therapeutically relevant levels of metronidazole (77). Global surveillance of drug resistance in these organisms requires a simple, reproducible drug susceptibility testing method for these anaerobic protozoa.

Giardia

Traditionally, susceptibility testing in *Giardia* has been performed with closed tubes, which requires more compounds and depends on tedious and labor-intensive manual counting of parasites, with few replicates available. The development of an assay in multiwell microtiter plates has been hindered due to the sensitivity of *Giardia* to oxygen. Several methods have been developed to form a low-oxygen intestinal atmosphere. These methods include a 24-h assay using 2-ml cultures in 24-well plates in a CO_2 atmosphere; 96-well plates incubated at 35°C for 48 h in the presence of 3% O_2, 4% CO_2, and 93% N_2; 96-well plates flushed with nitrogen; 96-well airtight plates incubated for 72 h; and 384-well plates under 5% CO_2 in air for 24 h. However, all these

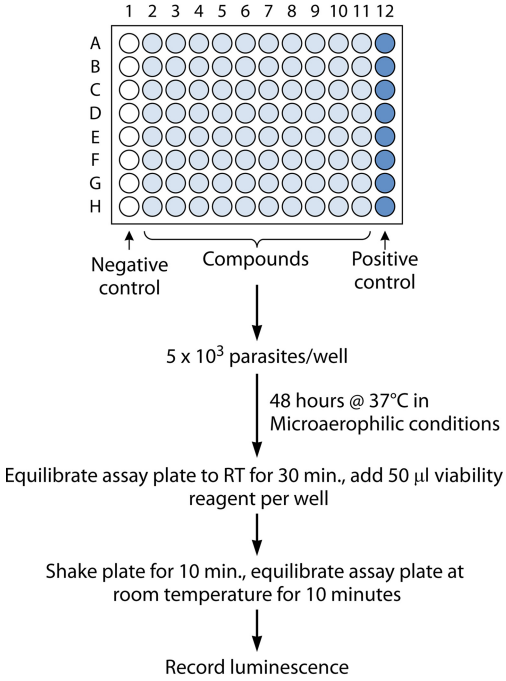

FIG 4 *In vitro* susceptibility testing workflow using a luminescence assay of cultured *Giardia* or *Entamoeba* parasites under microaerophilic conditions in a 96-well-microtiter-plate format. RT, room temperature.

methods resulted in unequal growth of parasites in different wells of the microtiter plates. To overcome these challenges, several changes of culture conditions were made by Gut et al. (78) and led to better growth of *G. lamblia*. Those researchers removed nonadherent and dead *G. lamblia* parasites from cultures and limited the presence of O_2 to less than 1% in special incubation bags or 3% O_2–5% CO_2 without the use of incubation bags.

Giardia drug susceptibility testing was performed by microscopy, which included parasite counting and evaluation of motility, morphology, or adherence. Radioactive incorporation assays have also been used, but these assays require associated equipment and radioactive waste disposal. The accessibility and higher cost of radioactive compounds make these assays less user friendly. Both fluorescence-based assays using SYBR green and colorimetric tests with formazan dyes {e.g., MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assays} and synthetic substrates of purine salvage pathway enzymes (79) were used, but the assays required an ELISA reader and multiple washes, which affected signal intensity and reproducibility.

Recently, Gut et al. (78) developed an image-based high-throughput screen using 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain and successfully identified 12 compounds that inhibited the growth of *G. lamblia* at low-micromolar or submicromolar concentrations. The disadvantages of this assay included the requirement for expensive automated cell imaging equipment and the storage of large electronic files.

A bioluminescence-based high-throughput screen was also developed to identify compounds that kill *G. lamblia* trophozoites (80). This assay monitors the viability of the trophozoites based on the ATP content of *G. lamblia* trophozoites.

The metabolic activity of trophozoites is inhibited due to the effect of a compound, and the corresponding ATP-dependent luciferase bioluminescence signal is also diminished (Fig. 4). These organisms may remain viable and resume growth after the completion of treatment. Moreover, the morphological changes of trophozoites due to treatment with compounds cannot be detected by this method. Therefore, a follow-up microscopic study and proliferation assay would be a reliable procedure for examining the effect of compounds on *G. lamblia* trophozoite viability. A target-based screen was developed by using *Giardia* carbamate kinase, and this luminescence-based enzyme

assay can be a useful tool for drug screening (81). Recently, a microfluidic device was developed to culture *G. lamblia*, and two currently used drugs were tested with an on-chip drug susceptibility assay using an imaging method (82). This assay holds the possibility of using the microfluidic platform for future high-throughput drug susceptibility testing.

Entamoeba histolytica

Drug susceptibility testing in *E. histolytica* has been challenged by the absence of competent screening methods, with available assays being exhaustive, depending mostly on microscopy (83), radioisotopes (84), and a complex staining method (85). Like *Giardia*, *E. histolytica* is an anaerobe and cannot survive in an oxygen-rich environment present in the wells of microtiter plates, and this posed a challenge for developing a testing method with microtiter plates, and above all, there was no rapid readout assay available. Debnath et al. (86) developed a microtiter plate-based screen that could be performed under anaerobic conditions, imitating the ameba's natural growth environment. This robotic-driven assay used the CellTiter-Glo luminescent cell viability assay (Promega) technology that measures ATP bioluminescence and represents a speedy, sensitive, and labor-saving assay to identify potent compounds against *E. histolytica* (86) (Fig. 4). The assay was developed by using an inoculum of *E. histolytica* in the logarithmic phase of growth, which led to confluence but not excessive growth. This high-throughput screen is performed with 96-well and 384-well microtiter plates with 50,000 parasites/ml and 30,000 parasites/ml, respectively (87). Similarly to *Giardia*, the effect of compounds on trophozoite morphology cannot be determined by this assay. Combining a microscopic study with this method would provide a better testing method.

Cryptosporidium

Cryptosporidium species are sporozoans that infect small intestinal epithelial cells, within which they replicate asexually for several rounds prior to differentiation into male and female gametocytes, fertilization, and the production of oocysts. The oocysts sporulate rapidly, thus becoming infectious and enabling both spread into the environment and autoinfection characterized by additional rounds of asexual replication within the same host. Cryptosporidiosis was discovered in the 1970s but first came to attention as a major human pathogen in immunocompromised patients, especially those with AIDS, in whom it is a prominent cause of chronic diarrhea (88, 89). However, young children suffer the preponderance of morbidity due to cryptosporidiosis. The recently reported Global Enterics Multicenter Study (GEMS), a large molecular epidemiological study of life-threatening childhood diarrhea conducted at sites in Africa and the Indian subcontinent, highlighted the importance of *Cryptosporidium*, which ranked second to rotavirus as an etiological agent among children <1 year of age (90). In the MAL-ED study, which investigated causes of less severe diarrhea, cryptosporidium ranked lower on the list of causative agents (91). Nitazoxanide, which is effective in immunocompetent adults, is the only drug known to have any efficacy. Unfortunately, nitazoxanide is not effective in immunocompromised people and is unreliable for the treatment of malnourished infants with cryptosporidiosis (92–94).

In the absence of reliably efficacious drugs for the treatment of human cryptosporidiosis, correlations of the results of *in vitro* drug susceptibility testing and clinical efficacy are not possible. Furthermore, it is not yet clear if *Cryptosporidium* spp. will become resistant to drugs under drug pressure, as drugs have not been used in large-enough groups of humans or livestock, and standardized methods to evaluate drug sensitivity have yet to be developed. Nevertheless, a number of investigators working on drug development have optimized methods to measure *in vitro* drug susceptibility. All of the approaches to date rely upon inducing oocyst excystation by simulating entry into the small intestine (95–98). The freed sporozoites then invade mammalian tissue culture cells, within which the asexual forms of the parasite replicate for several rounds. The efficiency of host cell infection is highly dependent on the cell type used, with the human colonic carcinoma cell line HCT-8 being among the most efficient (98, 99). This

method does not enable continual parasite culture, but replication and expansion of the asexual forms can be quantified by using a number of methods. Cai et al. (100) developed a quantitative real-time reverse transcription-PCR method to quantify parasite mRNA levels, using the abundance of host mRNA for normalization. This method takes advantage of the short half-life of mRNA following parasite death and thus provides a reliable means of measuring parasite elimination. However, this method is limited by its relatively low throughput and high cost. By using commercially available reagents, this assay was made amenable to high-throughput screening (101). Gut and Nelson first noted that *Vicia villosa* lectin specifically labels *Cryptosporidium* parasites, providing a simple method for microscopy-based assays (102). Bessoff et al. (103, 104) subsequently used this approach to develop a high-content imaging assay that has proven useful to screen small-molecule libraries. Recently, Vinayak et al. (105) succeeded at modifying the genome of *Cryptosporidium parvum* using CRISPR-Cas9 technology, which lays the groundwork for simpler screening assays using parasite lines modified to express reporter genes (105). Several groups are using luciferase-tagged parasites to measure the expansion of *C. parvum*. Another method of note is the use of multiplexed recombinase polymerase amplification (RPA) to detect *Cryptosporidium*, which may have useful applications for susceptibility testing (106). Each of these systems provides an *in vitro* method to assess the effects of drugs and drug-like compounds on *Cryptosporidium* invasion of and growth within host cells. At present, however, there are no methods to assess drug effects on the sexual forms of *Cryptosporidium* parasites and on *Cryptosporidium* transmission. Developing such methods is an active area of investigation.

SCHISTOSOMA

Schistosomiasis is an important neglected tropical disease and causes 3.3 million disability-adjusted life years (DALYs) (107). For over 30 years, praziquantel has been the only drug available to treat and control this disease. There are several advantages of praziquantel, such as good safety, tolerability, efficacy, and low cost, although the latter may no longer be the case in the United States. However, its limitations include its inability to protect individuals from reinfection and its inactivity against the schistosomula, preadult, and juvenile-adult stages of the worm (108, 109). Moreover, the emergence of praziquantel-resistant strains of *Schistosoma* is looming, since praziquantel is estimated to cover 235 million people by 2018 (110, 111). There are variations among individual *Schistosoma mansoni* isolates in susceptibility to praziquantel in mice. Protocols have been developed to quantify the praziquantel ED_{50}s of *S. mansoni* isolates to monitor the susceptibility of parasite isolates obtained from areas of endemicity to drugs. This will help to determine if variation in susceptibility to praziquantel in *S. mansoni* is a natural phenomenon or due to the effect of mass treatment (112). It has been noted that mass drug administration can reduce the efficacy of drugs like praziquantel (113).

Previously, drug susceptibility testing was performed with adult worms after incubating them with different drugs for 72 h, and parasite viability was monitored by using a microscope (114, 115). Viability testing and determination of worm motility by microscopic readout are slow, laborious, and subjective. Recently, a range of automated technologies has emerged, and different readouts are available. These methods include the impedance-based real-time measurement of parasite mobility (116, 117), isothermal microcalorimetry (118), image-based high-content screening (119), and fluorescence-based (120, 121) and luminescence-based (122) assays (Fig. 5).

Dye-based assays have several advantages, including being cheap, simple, and able to be read by an automated plate reader (123). The alamarBlue viability assay (resazurin), the fluorescein diacetate-propidium iodide bioassay, and a fluorometric L-lactate assay have been used as fluorescence assays. alamarBlue was able to discriminate only live and dead newly transformed schistosomulae (NTS) after a week of incubation with reference drugs but not at earlier time points, and this assay could not measure the dose-response of drugs (124). The fluorescein diacetate-propidium iodide

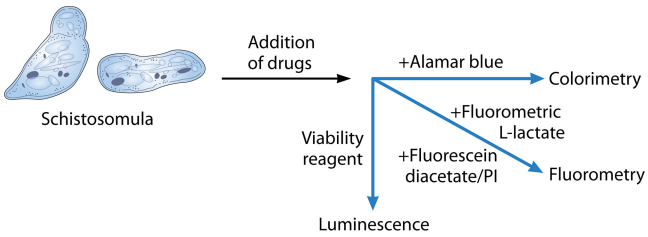

FIG 5 *Schistosoma in vitro* testing relies on the use of culture-adapted schistosomula-stage parasites followed by the addition of drugs. Readouts include luminescence, colorimetry, and fluorometry.

fluorescence-based bioassay can detect viability and differentiate live and dead *S. mansoni* adults and NTS. With this method, fluorescein diacetate stains live NTS, and propidium iodide stains dead NTS (120). Practical issues, such as a low signal to discriminate between live and dead NTS, the requirement for a high number of NTS, the involvement of a rinsing step, and the potential to calculate the dose-response for all standard drugs, are major concerns for this assay (123). The fluorescence-based lactate assay was used to measure the viability of NTS and adult *S. mansoni* worms, and dose-dependent effects could be obtained for some but not all standard drugs (121). The limitation of this assay is that it requires a specific number of worms, and the supernatant needs to be removed to obtain high and consistent signals (123). The search for a simple, cheap, and specific dye without the requirement for additional equipment or analysis is still continuing.

Recently, a luminescence assay using CellTiter-Glo has been conducted on *S. mansoni* NTS and adult worms (122). This assay required a multidrop sorter to distribute a precise number of worms into each well. Panic et al. (123) tested and compared 11 fluorescence or luminescence viability and cytotoxicity marker assays and dyes to standardize assay conditions and to examine if they correlated with *S. mansoni* NTS viability. This study demonstrated that it was not easy to develop a simple, inexpensive, "just-add" colorimetric marker-based drug screening assay for the larval stage of *S. mansoni*. Those authors, however, confirmed that CellTiter-Glo could be used for differentiating live and dead NTS in a test with a single drug concentration and potentially also in dose-response studies (123). An important computational tool developed recently is the quantal dose-response calculator (QDREC), which can calculate the dose-response characteristics for helminths and possibly other parasites (125).

FILARIAE

Filariae are tissue-invasive roundworms transmitted by arthropods. Pathogenic human filariae include *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, which cause lymphatic filariasis, and *Onchocerca volvulus*, the cause of river blindness. Other filarial pathogens of humans include *Loa loa*, the cause of African eyeworm, and several species of *Mansonella*. Filarial parasites cause substantial morbidity worldwide. Lymphatic filariasis infects over 67 million people and causes hydrocele in 20 million and lymphedema in 17 million people (126). *Onchocerca volvulus* infects 16 million to 37 million people and is a leading cause of skin disease and blindness (127, 128).

Filariae pass through several life cycle stages in the human host, and susceptibility of one stage to a medication does not guarantee susceptibility of the other stages. For example, ivermectin has potent activity against microfilarial stages but does not readily kill adult filarial worms (129). Thus, *in vitro* drug testing is often performed on several stages of the worm, including microfilariae, adult worms, and third-larval-stage (L3) larvae.

The infective form of the parasite that is transmitted from the vector to humans is the L3 stage. Once in their host, L3 larvae migrate over a period of a few days to their preferred niche in the human host. These sites include the lymphatic vessels for *W. bancrofti* and *Brugia* species, subcutaneous tissues and skin nodules for *O. volvulus*, subcutaneous tissues for *L. loa*, and the peritoneal cavity for *Mansonella perstans* (130). Over a period of several weeks to months, depending on the species of filarial

nematode (reviewed in reference 131), L3 larvae molt twice and become mature adult worms, which mate and release microfilariae. Microfilariae are L1-stage larvae, which are acquired by the arthropod vector. Microfilariae of most species circulate in the bloodstream, although those of *O. volvulus* and *Mansonella streptocerca* reside in the skin (130). L3 larvae, adult worms, and microfilariae have all been used for *in vitro* susceptibility testing.

Because the *Brugia malayi* life cycle can be maintained in small mammals, this species is the one used most often for *in vitro* drug screening studies (132–143). Other filariae used for *in vitro* drug testing include *Brugia pahangi* (153), *O. volvulus* and other *Onchocerca* species (141, 144), *L. loa* (145), *Litomosoides sigmodontis* and *Acanthocheilonema viteae* (filarial nematodes of rodents) (146–148), and *Setaria* species (filarial nematodes of sheep, cattle, and other ruminant mammals) (149–152). Even though it is the most common filarial pathogen of humans, *Wuchereria bancrofti* is rarely used in screening assays because it cannot be maintained in animal models (147).

Traditionally, the primary endpoint used to determine filarial susceptibility during *in vitro* testing has been changes in motility as determined by observation by microscopy (132). For most studies evaluating the effects of potential antifilarial agents, microfilariae are observed on a regular basis for a period of 1 to 7 days, and adult filarial worms are observed for a period of days to weeks (132–136, 138, 140–144, 148, 153). While worms can be identified as simply motile or nonmotile (136, 139, 140, 142, 145), in many studies, scoring systems have been applied to characterize the degree of motility observed at different time points (132–135, 138, 143, 144, 148, 151–153).

A complementary approach to determine the viability of microfilariae is MTT-formazan colorimetry (154, 155). In this assay, microfilariae are incubated for 1 to 4 h with 0.5 mg/ml of the tetrazolium salt MTT. Viable filarial worms take up the colorless salt and reduce it to formazan, which is blue. Filariae can then be transferred manually into microtiter wells containing dimethyl sulfoxide (DMSO). Next, usually after 1 h of gentle agitation, absorbance is measured to obtain a semiquantitative measurement of filarial viability (155). This approach has been used successfully to evaluate compounds for activity against adult worms, L3 larvae, and microfilariae (132, 134, 135, 140, 144, 147–149, 151, 153).

When monitoring adult worm cultures, some studies also evaluate effects of potential antifilarial agents on worm fecundity by measuring the number of microfilariae released by adult female worms on a daily basis (134, 138, 146, 148, 153). Adult worms can also be dissected at the study endpoint to enable microscopic assessment of effects on embryogenesis with regard to both numbers of embryos and stages of embryo development (134, 153, 157).

Other tests that are done for drug susceptibility studies include trypan blue exclusion with microfilariae (151, 152), assessment of L3 to L4 molting (137, 141), and histology and electron microscopy to detect anatomical changes (134, 141–143, 147).

A major drawback of most filaria susceptibility assays is that they are highly labor-intensive, greatly limiting their potential use in high-throughput screening systems. To overcome this, Marcellino and colleagues developed a software program (WormAssay) that can be used with a video camera to measure movements of adult filarial worms in 24-well culture plates (156). Visualization and motility scoring can be automated and integrated with a robotic plate manipulation system (156). Recently, this system was used successfully to identify auranofin as having macrofilaricidal activity (141). An adaptation of the WormAssay software has been made, which enables its use for screening microfilaria and L3 stages using microscopy and multiple worms per well (158).

Another limitation of current screening assays is that *in vitro* culture puts substantial physiological stress on filariae. Microfilariae fail to develop substantially *in vitro*, and adult worms neither mate nor produce new oocytes *in vitro* (131). However, it is noteworthy that microfilariae do not develop substantially in the mammalian host either. Indeed, a recent transcriptomics study showed that adult *B. malayi* female worms exhibit marked changes in gene expression upon transfer into cell culture

medium (159). Coculture with insect or mammalian cell lines often improves microfilaria and adult worm survival *in vitro* but does not overcome these developmental blocks (131).

Thus, the development of *in vitro* systems that better model the usual environment of filarial worms is an ongoing research goal. To date, the most advanced *in vitro* system developed is likely that of Kassis and colleagues (160). Using a motorized microscope and a polydimethylsiloxane microchannel lined with dermal lymphatic endothelial cells and human dermal fibroblasts, those researchers developed a highly advanced *in vitro* imaging platform that can continuously measure adult filarial worm speed, thrashing, and migration patterns (160). While it has not yet been used for drug screening, it has the ability to evaluate subtle changes in worm activity and behavior.

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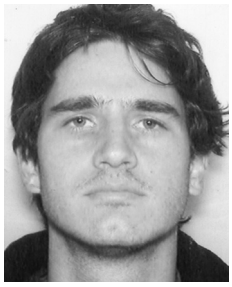

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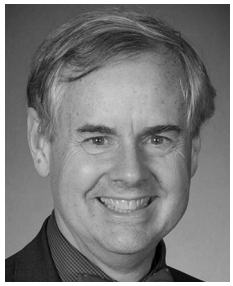

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