

Novel Approach to *In Vitro* Drug Susceptibility Assessment of Clinical Strains of *Leishmania* spp.

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Resistance to antimonial drugs has been documented in *Leishmania* isolates transmitted in South America, Europe, and Asia. The frequency and distribution of resistance to these and other antileishmanial drugs are unknown. Technical constraints have limited the assessment of drug susceptibility of clinical strains of *Leishmania*. Susceptibility of experimentally selected lines and 130 clinical strains of *Leishmania panamensis*, *L. braziliensis*, and *L. guyanensis* to meglumine antimoniate and miltefosine was determined on the basis of parasite burden and percentage of infected U-937 human macrophages. Reductions of infection at single predefined concentrations of meglumine antimoniate and miltefosine and 50% effective doses (ED₅₀s) were measured and correlated. The effects of 34°C and 37°C incubation temperatures and different parasite-to-host cell ratios on drug susceptibility were evaluated at 5, 10, and 20 parasites/cell. Reduction of the intracellular burden of *Leishmania* amastigotes in U-937 cells exposed to the predefined concentrations of meglumine antimoniate or miltefosine discriminated sensitive and experimentally derived resistant *Leishmania* populations and was significantly correlated with ED₅₀ values of clinical strains (for meglumine antimoniate, $\rho = -0.926$ and $P < 0.001$; for miltefosine, $\rho = -0.906$ and $P < 0.001$). Incubation at 37°C significantly inhibited parasite growth compared to that at 34°C in the absence of antileishmanial drugs and resulted in a significantly lower ED₅₀ in the presence of drugs. Susceptibility assessment was not altered by the parasite-to-cell ratio over the range evaluated. In conclusion, measurement of the reduction of parasite burden at a single predetermined drug concentration under standardized conditions provides an efficient and reliable strategy for susceptibility evaluation and monitoring of clinical strains of *Leishmania*.

Long-term use of antimonial drugs as monotherapy for leishmaniasis and nonadherence to treatment have contributed to the increase in treatment failures (3, 5). In addition to intrinsic differences in the susceptibility of different *Leishmania* populations (18, 23), acquired resistance has also been reported (12, 15). Consequent variations in drug efficacy impact clinical management and leishmaniasis control programs, yet little is known about the prevalence of tolerant and/or resistant populations (11) and the impact of treatment policies on drug susceptibility.

Evaluation of drug susceptibility of clinical strains of *Leishmania* has been constrained by the unavailability of an efficient method that yields consistent results. Intracellular amastigotes are the target of treatment, are strikingly more susceptible to antimony than promastigotes (2, 11, 21), and importantly, are killed *in vitro* at clinically achievable drug concentrations. However, drug susceptibility evaluation of clinical strains using intracellular amastigotes is a slow, low-throughput biological assay that is influenced by numerous poorly understood variables. Individual clinical strains are heterogeneous; even strains having a low 50% effective dose (ED₅₀) often contain subpopulations that are effectively resistant (15). Hence, although cloned parasites transfected with reporter genes have proven useful for drug screening (17), the protracted, intensive *in vitro* antibiotic selection required to recover and maintain the transfected population may alter the original composition of clinical strains and, consequently, the susceptibility phenotype.

Conventional susceptibility assays are based on the determination of the ED₅₀ using murine peritoneal macrophages or macrophage cell lines under diverse conditions of infection with different numbers and intervals of drug concentrations, periods of drug exposure, and readout parameters. Since these factors influence

the outcome of the evaluation (20), results of drug susceptibility testing have been neither consistent nor generalizable.

We report a novel approach to drug susceptibility assessment of clinical strains of *Leishmania* based on quantification of parasite burden after exposure to single discriminatory concentrations of two representative antileishmanial drugs, meglumine antimoniate (Sb^V) and miltefosine (HePC).

MATERIALS AND METHODS

Experimental strategy. Susceptibility of clinical strains of *Leishmania* (*Viannia*) species to Sb^V and HePC was determined in parallel by conventional dose-response assay and reduction of intracellular parasite burden at a single drug concentration. Discriminatory drug concentrations for susceptibility were defined as follows: the antimony concentration of 32 $\mu\text{g Sb}^{\text{V}}/\text{ml}$ was based on the estimated maximum concentration (C_{max}) in plasma (6), and the miltefosine concentration of 16 μM was chosen because it clearly and consistently distinguished the susceptible wild-type (WT) control strain and an experimentally selected resistant line without cytotoxicity for host macrophages. Parasite burden based on enumeration of intracellular amastigotes and the percentage of cells infected was determined microscopically and by luminometry (Lucy 1; Anthos-Labtec Instruments, Lagerhausstrasse, Austria) using *luc*-transfected WT and drug-resistant control populations.

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Leishmania strains and lines. One hundred thirty clinical strains originating from patients diagnosed with dermal leishmaniasis were obtained from institutional biobanks, and their use for this study was approved by the Ethical Review Committees of the Centro Internacional de Entrenamiento en Investigaciones Médicas (CIDEIM) and the Centro Dermatológico Federico Lleras Acosta (CDFLLA). The control WT strain of *L. (V.) panamensis* (MHOM/COL/86/1166LUC) is sensitive to both antimony and miltefosine (14, 22). Drug-resistant lines derived from this strain were employed as controls: MHOM/COL/86/1166-1000.1, resistant to antimony (10, 22), and MHOM/COL/86/1166-LUC056, resistant to miltefosine. The latter line was selected from MHOM/COL/86/1166LUC by culturing of promastigotes in the presence of increasing concentrations of miltefosine of from 20 to 60 μM . The promastigote form of this line is propagated in 60 μM miltefosine, and the amastigote form has an ED_{50} exceeding 32 μM , the maximum concentration of miltefosine used for *in vitro* intracellular assays. The WT line has an ED_{50} approximating 4 μM (see Fig. 1A and B).

Susceptibility assessment. Susceptibility assays were conducted with human promonocytic U-937 cells (ATCC CRL-159.3) as previously described (15) but using 1.2×10^5 cells/well of 24-well cell culture plates containing sterile round 12-mm glass coverslips (Fisher Scientific) for microscopic evaluation. Phorbol 12-myristate 13-acetate (PMA; 100 ng/ml; Sigma) was added together with the cell suspension to induce differentiation of U-937 cells during 5 days of culture (9, 13). To evaluate the effect of temperature on susceptibility assessment, promastigotes opsonized for 1 h with 10% heat-inactivated type AB-positive human serum in RPMI medium were used to infect macrophages at a ratio of 5:1, 10:1, or 20:1 (parasite/cell) for 2 h at 34°C or 37°C in 5% CO_2 . Infected cells were washed twice with phosphate-buffered saline to remove free parasites and then incubated for 24 h at 34°C or 37°C in 5% CO_2 in RPMI 1640 medium (R6504; Sigma) containing 10% heat-inactivated fetal bovine serum (FBS; 10082; Gibco). For evaluation of susceptibility at the defined discriminatory drug concentration, medium was replaced with complete RPMI medium containing 32 μg Sb^{V} /ml as additive-free meglumine antimoniate (Glucantime; Walter Reed 214975AK; lot no. BLO918690-278-1A1W601; antimony analysis, 25% to 26.5%, by weight) or 16 μM hexadecylphosphocholine (miltefosine; Cayman Chemical Co., Ann Arbor, MI). ED_{50} assays were performed with 2, 4, 8, 16, 32, 64, and 128 Sb^{V} /ml and 0.5, 1, 2, 4, 8, 16, and 32 μM HePC. Complete RPMI medium containing the discriminatory drug concentration or serial dilutions of meglumine antimoniate was replenished 48 h later, and incubation was continued for 24 h. Incubation with miltefosine was conducted over 48 h without replenishment. Infected macrophages in complete medium served as controls.

Glass coverslips with infected cells were fixed with methanol, stained with 3% Giemsa (Sigma), and then evaluated in a blinded manner by two experienced microscopists. One hundred macrophages per well were examined microscopically to determine the percentage of cells infected and the number of morphologically intact amastigotes in 100 cells. Four replicate wells were evaluated for susceptibility at the predefined discriminatory drug concentration, and three replicate wells were evaluated for each drug concentration in ED_{50} assays. For assays using the single discriminatory drug concentration, drug susceptibility was expressed as percent reduction of infection in cells exposed to the drug versus cells cultured without drug. Luciferase activity of WT and drug-resistant lines was expressed as relative light units (RLU).

Statistical methods. The ED_{50} value was determined using the probit procedure of the SPSS program (version 7.5 for Windows, 1996; SPSS). The receiver operating characteristic (ROC) curve was constructed to define the cutoff to classify clinical strains as sensitive or of low susceptibility on the basis of assessment at the discriminatory drug concentration. ED_{50} values of ≤ 32 μg Sb^{V} /ml and ≤ 16 μM HePC were adopted as reference standards to define sensitive strains, and ED_{50} s of > 32 μg Sb^{V} /ml and > 16 μM HePC were adopted as reference standards to define strains of low susceptibility. Comparison of groups was conducted using

the Mann-Whitney *U* test. Correlation analysis between ED_{50} and percent reduction of parasite burden was performed using the Spearman test (nonparametric methods allow the inclusion of assays in which an actual value of the ED_{50} was not found because it exceeded the highest drug concentration evaluated), and concordance analysis of ED_{50} was performed using different readouts (percentage of cells infected and number of amastigotes in 100 cells) and the intraclass correlation coefficient. A two-sided significance level of 5% was used.

RESULTS

In vitro drug susceptibility testing of clinical strains of *Leishmania* has been hindered by the inconsistency of results obtained using diverse experimental conditions. The results of this study substantiated the greater accuracy of a readout based on reduction of intracellular parasite burden than an evaluation based on the percentage of cells infected. Assessment of parasite burden by microscopy clearly distinguished experimentally selected resistant and susceptible populations with a resolution comparable to that of luminometry (Fig. 1A and B), whereas readouts based on the percentage of cells infected did not consistently discern the susceptible WT but did distinguish experimentally selected resistant populations (Fig. 1C). Concordance between the ED_{50} for miltefosine determined microscopically as parasite burden and the ED_{50} based on luciferase activity was 99.2%. In contrast, concordance between the ED_{50} based on microscopic determination of the percentage of cells infected and luciferase activity was 81% (data not shown), reflecting the inability of the readout based on the percentage of infected cells to discriminate between cells with one or a few parasites and cells harboring abundant parasites. This limitation leads to significantly higher ED_{50} values (Fig. 1D and E) and overestimation of low susceptibility to meglumine antimoniate and miltefosine compared to those from the readout as the parasite burden for clinical strains (Fig. 2A and B). Reduction of infection by exposure to discriminatory concentrations of miltefosine ($n = 125$) and meglumine antimoniate ($n = 130$) was significantly lower ($P < 0.001$) for readout as the percentage of cells infected (median reductions, 69% for Sb^{V} and 27% for HePC) than for readout as the parasite burden (median reductions, 86% for Sb^{V} and 60% for HePC). Hence, evaluation of the reduction of infection by a single drug concentration corroborated the overestimate of low drug susceptibility when evaluation was based on the percentage of cells infected.

A high and significant correlation (for meglumine antimoniate, $\rho = -0.926$ and $P < 0.001$; for miltefosine, $\rho = -0.906$ and $P < 0.001$) between susceptibility, expressed as percent reduction of parasite burden by a predefined single drug concentration, and ED_{50} values was shown for both meglumine antimoniate and miltefosine (Fig. 3A and B). Strains with high ED_{50} values displayed a low percent reduction of parasite burden at the discriminatory concentration of each drug. Conversely, strains with low ED_{50} values showed a high percent reduction of parasite burden. Low susceptibility to discriminatory concentrations of meglumine antimoniate and miltefosine was predicted using the ROC curve at cutoffs of a $< 41\%$ (having 100% sensitivity and 100% specificity) and a $< 50\%$ (having 93% sensitivity and 100% specificity) reduction of parasite burden, respectively (Fig. 3C and D).

Standardization of the susceptibility evaluation included evaluation of the effects of the parasite-to-host cell ratio and incubation temperature on the outcome of susceptibility assessment. The infective dose of parasites and resulting level of *in vitro* infection of

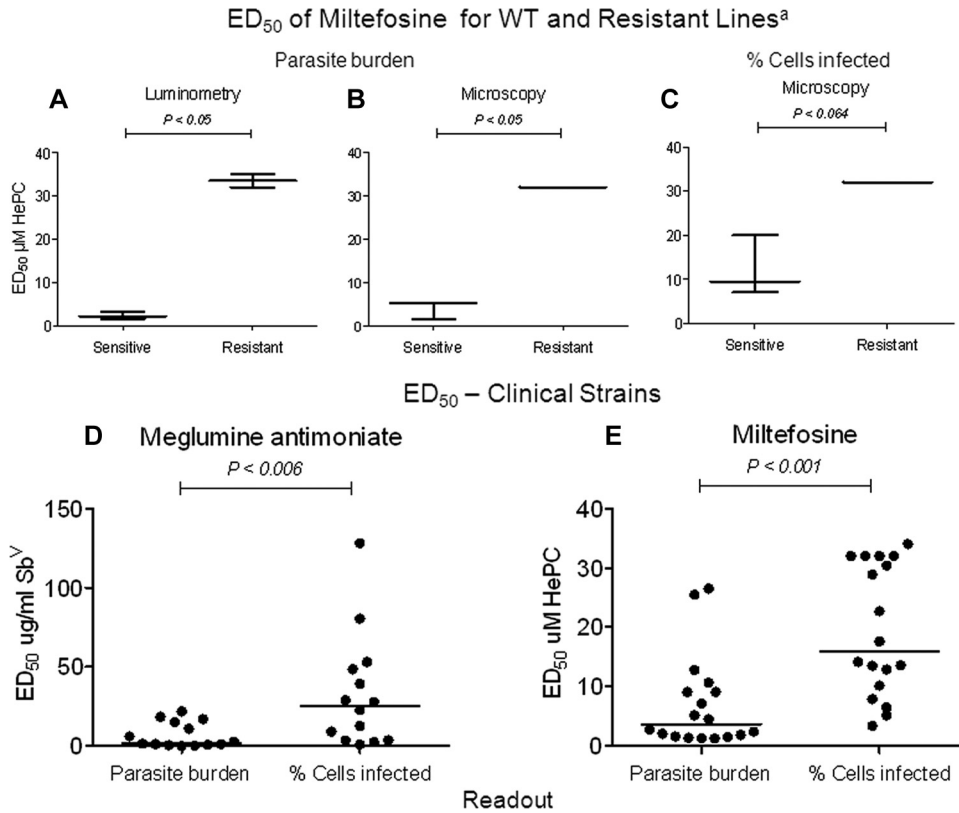


FIG 1 Comparison of readout as parasite burden and percentage of cells infected in evaluation of *Leishmania* susceptibility to meglumine antimoniate and miltefosine. The ED₅₀ of miltefosine determined as parasite burden using luciferase activity (A) and microscopy (B) consistently distinguished sensitive and experimentally selected resistant populations. Statistically significant differences substantiate discrimination of sensitive and resistant populations based on parasite burden but not by readout as the percentage of cells infected (C). The ED₅₀s of meglumine antimoniate ($n = 14$) (D) and miltefosine ($n = 18$) (E) for clinical strains of *Leishmania* were significantly higher when determined as the percentage of cells infected than as parasite burden. ED₅₀ values are presented as medians. ^a, based on three independent assays. Significant differences were determined by the Mann-Whitney *U* test.

host macrophages did not significantly alter the outcome of susceptibility assessment based on parasite burden. Infection with 5, 10, and 20 *luc*-transfected parasites per cell resulted in proportionately higher parasite burdens of 5,869, 10,497, and 30,144 RLU, respectively; however, ED₅₀ values for miltefosine were similar (medians, 1.7, 4.2, and 3.3 μ M, respectively). Likewise, reduction of parasite burden at the discriminatory drug concentration yielded comparable results (medians, 96, 89, and 91%, respec-

tively; $n = 3$), irrespective of the parasite-to-host cell ratio. The susceptibility readout was not affected by parasite ratios of between 5 and 20 parasites per cell. Nevertheless, the lower ratio facilitates microscopic evaluation. An incubation temperature above 34°C during infection with *L. (V.) panamensis* resulted in lower rates and burdens of infection and altered host and amastigote morphology, impairing microscopic evaluation. Furthermore, the percent reduction of parasites after incubation at 37°C

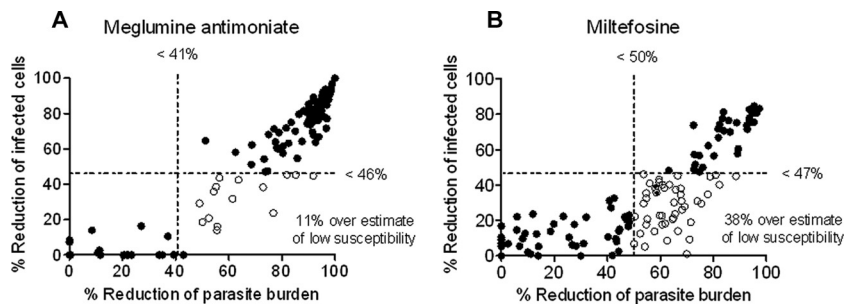


FIG 2 Comparison of the susceptibility of clinical strains of *Leishmania* (*Viannia*) to meglumine antimoniate and miltefosine evaluated as the percentage of cells infected and parasite burden. Using thresholds of susceptibility defined by ROC curve analyses, low susceptibility (tolerance/resistance) was overestimated in clinical strains by readout as the percentage of cells infected compared to readout as the parasite burden at discriminatory concentrations of meglumine antimoniate ($n = 130$) (A) and miltefosine ($n = 125$) (B). Discontinuous lines correspond to cutoff thresholds. Empty circles represent strains erroneously classified as having low drug susceptibility by readout as the percentage of cells infected.

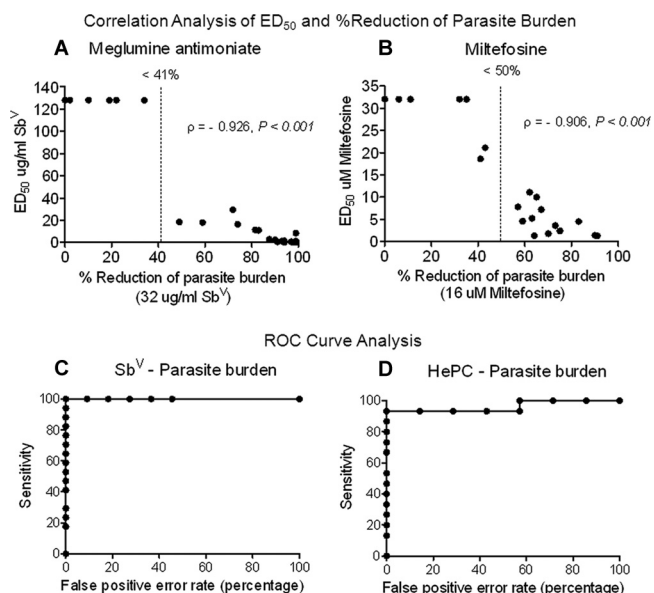


FIG 3 Correlation of drug susceptibility evaluation based on discriminatory drug concentration and ED_{50} values of clinical strains of *Leishmania* (*Viannia*) and determination of cutoff threshold for screening test. Susceptibility determination using the discriminatory drug concentration was significantly correlated (using the Spearman coefficient) with the ED_{50} of meglumine antimoniate (A) and miltefosine (B). Both the ED_{50} and susceptibility to discriminatory drug concentrations were determined by readout as parasite burden. The cutoff thresholds for low susceptibility defined by ROC curve analysis was a <41% reduction of parasite burden by meglumine antimoniate ($n = 28$) (C) and a <50% reduction of parasite burden by miltefosine ($n = 20$) (D).

in the presence or absence of antimony was significantly higher than that obtained at 34°C with the same strains (Table 1).

DISCUSSION

In vitro assessment of drug susceptibility of clinical strains of *Leishmania* based on reduction of parasite burden was feasible. Determination of the reduction of parasite burden at drug concentrations that distinguish experimentally selected resistant lines from the corresponding WT population allowed classification of clinical strains as sensitive or of low susceptibility. The wide spectrum of the proportional reduction of parasite burden in clinical strains (Fig. 3A and B) demonstrates the resolution of evaluation based on the discriminatory drug concentrations in discerning populations with different levels of susceptibility to the test drugs.

Definition of cutoffs based on ROC curve analyses of ED_{50} and percent reduction of parasite burden at the discriminatory drug concentration provided a reference threshold for emerging insusceptibility whether it is due to acquired resistance or expansion of populations having low susceptibility to specific drugs. The strains classified as having low susceptibility to meglumine antimoniate in this study presented ED_{50} values above the reported C_{max} in plasma (32 $\mu\text{g}/\text{ml}$) (6) that could signal an increased risk of treatment failure. Although the C_{max} of miltefosine in plasma is well above the concentration used for *in vitro* evaluation, the recognized toxicity of miltefosine for host macrophages *in vitro* limits the maximum concentration that can be evaluated. However, the interaction of miltefosine with blood components, which bind approximately 95% of the drug (7), effectively reduces the amount of unbound drug to which intracellular parasites may be exposed

in vivo. This fact and the ability of 16 μM to discriminate between an experimentally selected line and corresponding WT support the biological relevance of insusceptibility to this concentration and its use in surveillance for early detection of tolerance or resistance to miltefosine.

The results of this study document the impact of the incubation temperature on the interpretation of susceptibility assessment. The temperature sensitivity of intracellular amastigotes has long been recognized. Though not systematically examined, evidence has generally shown that dermatotropic species have significantly lower growth at temperatures above 35°C, while vicero-tropic species grow equally well at 37°C and 35°C (1, 19). We found that parasite burden was significantly lower after 4 days of culture at 37°C than at 34°C in the absence or presence of antileishmanial drug (Table 1). Hence, assessment of drug susceptibility at 34°C avoids the potentially confounding effect of temperature on the reduction of infection and accommodates the temperature-dependent growth of all *Leishmania* species.

Microscopic readout is influenced by the level of infection: a very high level of infection encumbers the visual enumeration of parasites to determine burden. Furthermore, drug-mediated reduction of the number of parasites may not comparably diminish the proportion of cells that harbor one or a few parasites, leading to a perception of lower susceptibility when readout is based on the percentage of cells infected. Infectivity varies among *Leishmania* strains. Consequently, a fixed parasite-to-host cell ratio results in an intensity of infection that varies among different clinical strains. Over the range of 5 to 20 parasites per cell, we found that the ratio did not alter the outcome of susceptibility assessment, although high ratios led to levels of infection that can be difficult to visually evaluate.

In this study, significantly higher ED_{50} values were obtained when drug-mediated reduction of infection was based on the percentage of cells infected than on reduction of parasite burden. Hence readout based on the percentage of cells infected is vulnerable to overestimation of low susceptibility (drug tolerance or resistance). These findings and ROC curve analyses substantiate the greater sensitivity and specificity of quantitative evaluation of the parasite burden compared to those achieved by estimation of the percentage of cells infected. Notably, most published reports of drug susceptibility evaluation of intracellular *Leishmania* have been based on the assessment

TABLE 1 Effect of incubation temperature above 34°C on susceptibility assessment of clinical strains of *L. (V.) panamensis*^a

Experimental condition	No. of parasites in 100 cells at incubation temp during infection		% reduction of parasite burden at 37°C	P value ^b
	34°C	37°C		
Without drug	371	83	91	0.016
With Sb ^V	264	3	98	0.008
% reduction of parasite burden by addition of drug	21	91		0.021

^a Data correspond to the median ($n = 5$).

^b Mann-Whitney *U* test.

of the percentage of cells infected (4, 8, 23, 24). Evaluation of percentage of cells infected is more rapid and convenient yet may underestimate drug cytotoxicity for intracellular amastigotes, since one or a few intracellular parasites are not discriminated from large numbers of parasites in a single cell, which effectively leads to overestimation of low susceptibility.

The accuracy of ED₅₀ values and reliability of their interpretation depend upon the number and range of drug concentrations evaluated, since informative drug concentration intervals vary in relation to the susceptibility of the population being tested. Therefore, use of a standard range of drug concentrations, such as is generally used for ED₅₀ determination, is not uniformly suited to the spectrum of susceptibility phenotypes found among clinical strains. Moreover, strains are often heterogeneous with respect to drug sensitivity; isolates of a strain presenting a low ED₅₀ may nevertheless include a substantial population that is tolerant or resistant to high drug concentrations. The assessment of drug susceptibility based on the absolute reduction of parasites at a drug concentration defined in relation to *in vivo* pharmacokinetics and tolerance thresholds of experimentally selected lines obviates these important and underappreciated sources of error in ED₅₀ determination and interpretation.

The reduced complexity and reduced cost of evaluation based on a single discriminatory drug concentration facilitate the monitoring of drug susceptibility. This strategy is applicable to any *Leishmania* species/strain and can be standardized for other drugs, just as the conventional ED₅₀ assay. However, dependence on microscopic reading remains a limitation. The development of methods amenable to medium throughput for the determination of viable parasites is needed. Methods based on detection of parasite RNA combined with this novel approach to susceptibility evaluation may allow monitoring of drug susceptibility to be routinely used in defining treatment policy (16).

In summary, evaluation of susceptibility using a single drug concentration under standardized conditions provides an efficient and reliable strategy for susceptibility evaluation and monitoring of clinical strains of *Leishmania*.

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