High-throughput screening of amastigotes of *Leishmania donovani* clinical isolates against drugs using a colorimetric β-lactamase assay

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Abstract

A simple colorimetric β-lactamase assay for quantifying *Leishmania* amastigotes in macrophages grown in microtiter plates has been reported. The β-lactamase gene was integrated into the rRNA region of the genome, thereby allowing for high-level stable expression of the enzyme. Both visceral leishmaniasis (VL) and post-kala azar dermal leishmaniasis (PKDL) isolates were transfected with β-Lactamase gene. These β-lactamase-expressing promastigotes were used for infecting intracellular J774A.1 macrophages *in vitro*. Quantification was done by a colorimetric readout with CENTA™ β-lactamase as substrate and with an optical density plate reader. The assay was carried out in 96-well plates. Results obtained demonstrate that this methodology could be a valuable high-throughput screening assay for checking efficacy of anti-leishmanial drugs in the clinical isolates.

Keywords

Amastigotes; β-lactamase assay; Drug screening; Indian clinical isolates; *Leishmania donovani*

Visceral leishmaniasis (VL) is a disease caused by a protozoan parasite of the *Leishmania donovani* complex and is often fatal if left untreated. Estimated worldwide annual incidence of VL is 500,000, of which 90% occur in the Indian subcontinent, Sudan and Brazil[1]. More than 100,000 cases of VL occur in India alone each year and the state of Bihar accounts for more than 90% of these cases[2, 3].

Kala-azar transmission in India is thought to be anthroponotic and post-kala azar dermal leismaniasis (PKDL) patients are considered to serve as a source for new outbreaks[4]. Post-kala azar dermal leismaniasis (PKDL) is a sequel to VL in India and Sudan; the disease develops months to years after the patient recovery from VL[5]. *Leishmania donovani*, a flagellated protozoan parasite, is the causative agent of VL. Current increase, to epidemic proportions, in leishmaniasis throughout the world and emergence of VL as an important opportunistic infection among people with human immunodeficiency virus-1 (HIV -1) infection[6,7] has increased the need for new treatments for this intracellular infection.

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Alarming emergence of resistance to currently used drugs exacerbates the need for new drugs.

Leishmania genome project has identified new genes at a rapid rate. The haploid genome of *Leishmania major* (Friedlin strain) is sequenced with around 8272 protein-coding genes, of which 36% can be ascribed a putative function[8]. Several new molecular drug targets have been identified lately. As a result, it is expected that several new lead compounds would be identified in near future. Hence, there is an urgent need for establishing high-throughput screening assays against the clinical isolates of *Leishmania*.

Generally, intracellular amastigote-macrophage model that use catalytic reporter gene technology like luciferase, β-galactosidase, β-lactamase are more sensitive than methods based on the fluorescent proteins[9,10]. A useful method employing luciferase reporter gene has been practiced since long time, but it has certain drawbacks. First, the luminescent readout is transient and mixing of sample and reagent needs to be timed with entering the samples into luminometer. Second, reagents for running luciferase assay and plate luminometers are expensive. *Leishmania* parasites have also been engineered to express β-galactosidase, but high background activity from host macrophages prevents use of this reporter system in amastigote-macrophage drug screening assays (Buckner FS, unpublished data).

In the present study, the use of modified β-lactamase assay for quantifying *Leishmania* amastigotes in macrophages grown in microtiter plates has been reported. The β-lactamase gene was integrated into the rRNA region of the genome[12], thereby allowing for high-level stable expression of the enzyme. Both VL and PKDL clinical isolates were transfected with β-lactamase (pIR1SAT-βLA) construct for this assay. A colorimetric readout using the CENTA™ β-lactamase substrate was quantified by enzyme-linked immunosorbent assay (ELISA) plate reader. Results obtained demonstrate that this methodology may be valuable for high-throughput screening against antileishmanial drugs.

Materials and Methods

Materials

CENTA™ β-lactamase substrate was purchased from Calbiochem (La Jolla, CA). Sodium stibogluconate was from IP Albert David Ltd. Kolkata, India. AmB-deoxycholate (AmB-Doc, Mycol®) was obtained from VHB Life Sciences Inc., India. Miltefosine was procured from Zentaris (Frankfurt, Germany). Paromomycin sulphate, pentamidine, penicillin, streptomycin and nourseothricin were purchased from Sigma (St. Louis, MO).

Parasite and culture condition

Promastigotes of *Leishmania donovani* VL strains AG83 (MHOM/IN/80/AG83), untyped 2001- S and Indian PKDL clinical isolates, RK1, MS2, NR3A, RMP8 (HM/IN/RMP-8), RMP19 (HM/IN/RMP-19), RMP142 (HM/IN/RMP-142), RMP155 (HM/IN/RMP-155) and RMP240 (HM/IN/RMP-240) were kindly provided by Dr. Mitali Chatterjee, Institute of Post Graduate Medical Education and Research, Kolkata, India; and Dr Sarman Singh, Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, India. These isolates were routinely cultured at 22°C in modified M-199 medium (Sigma, USA) with 100 U/mL penicillin (Sigma, USA), 100 μg/mL streptomycin (Sigma, USA) supplemented with 10% heatinactivated fetal bovine serum (FBS; Gibco/BRL, Life Technologies Scotland, UK).

DNA construct and transfection in Leishmania

β-lactamase expression vector (pIR1SAT-βLA) was kindly gifted by Dr Frederick S Buckner (Washington University, Seattle). The plasmid was linearized by digesting with *Swa*I restriction enzyme (MBI, Fermentas). Linearized plasmid (10 μg) having the β-lactamase gene was transfected into *L. donovani* promastigotes by electroporation. This linearized vector is designed in such a manner that it integrates into the *Leishmania* genome by replacing one copy of the SSU rRNA gene by homologus recombination. For transfection, 4×10^7 log phase promastigotes were harvested. The cells were centrifuged at $5,200 \times g$ for 12 min. The pellet was first washed with PBSG (10 mM $Na_2 HPO_4$, 10 mM $NaH_2 PO_4$, 145 mM NaCl, 2% glucose, filter sterilized) and then with EP buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0,7 mM NaH_2, PO_4, 6 mM glucose, filter sterilized) followed by centrifugation at $5,200 \times g$ for 12 min. The cells were resuspended in 400 μL EP buffer and 10 μg of the construct was added and then transferred to 2 mm gap electroporation cuvette. Electroporation was done with a single pulse with the following parameters 450 V, 500 μF, (Bio-RAD). Cells were incubated on ice for 10 min. The transfectants were then transferred to a T25 flask in 5 ml of M199 medium with 20% FBS. Nourseothricin (50 μg/mL) was added after 48 hr to select for parasites expressing the streptothricin acetyltransferase (SAT) gene. Robust cultures of nourseothricin resistant cells were selected after 10-14 days. Clonal selection of the parasites containing β-lactamase reporter gene was undertaken before using them for infection.

Intracellular amastigote-macrophage cultures

J774A.1 macrophages were maintained in RPMI-1640 medium supplemented with 10% FBS at 37°C in the CO_2 incubator (5% CO_2). Macrophages (5×105 cells/mL) were plated in a volume of 100 μL of RPMI-1640 medium plus 10% heat-inactivated fetal calf serum/well in 96-well tissue culture plates as reported earlier[12]. Macrophages were allowed to adhere overnight and were infected with stationary-phase promastigotes (β-lactamase transfectants) in a volume of 100 μL at a ratio of 10 parasites per macrophage. After 12 hr of infection, the unattached parasites and non adherent macrophages were washed off with RPMI medium. Infected macrophages were further incubated for 96 hr in 200 μL of the above mentioned RPMI-1640 medium containing different dilutions of drugs (inhibitors) for screening assays.

β-lactamase assay

Intracellular amastigotes grown in macrophages were quantified for β-lactamase activity by first removing the medium by gentle pipetting. Subsequently, 50 μL of 50 μM substrate (CENTA) in PBS and 0.1% Nonidet P-40 were added. The plates were incubated at 37°C for 4 hr and then read at an OD of 405 nm. The 50% inhibitory concentration (IC_{50}) was determined from the graph representing different concentrations of inhibitors plotted against cell proliferation (% of growth control).

Data analysis

All the experiments were repeated 3 times with triplicates in each set. Data was fitted to appropriate equations using GraphPad Prism® Version 4.0 software package.

Results

Expression of β-lactamase gene in Leishmania

The linearized pIR1SAT-βla plasmid was electroporated into VL and PKDL promastigotes. Tranfectants were selected with nourseothricin and used for β-lactamase assay. Transfected VL and PKDL isolates were maintained in liquid medium for 24 hr before plating on a semi-

solid medium containing drug. After selection and stabilization of the liquid culture, transfected parasites were spread on the plates containing 1% Bacto™ Agar (DIFCO) in M199 media (with 10% FCS) with nourseothricin (50 μg/mL) to select for single colonies. The single targeted clones were maintained in M199 liquid medium to have homogeneous transfected parasite population. These β-lactamase-expressing *Leishmania* promastigotes showed a near linear relationship between the number of parasites and β-lactamase activity (data not shown).

Screening of inhibitors

Transfected promastigotes were allowed to enter stationary-growth phase and were infected onto monolayers of monocyte macrophage cell line J774A.1 macrophages. The parasites efficiently infected the macrophages and converted to amastigotes. In the present study *L. donovani* isolates from Indian patients with either VL or PKDL, from zones of varying leishmaniasis endemicity and who had different responses to treatment with SAG were used. Utility of β-lactamase assay was demonstrated by screening 5 conventional anti-leishmanial drugs namely sodium stibogluconate, amphotericin B, miltefosine, paromomycin sulphate and pentamidine. Each drug was tested in 3 independent assays against the VL and PKDL isolates. Within individual assays, dilutions were performed in triplicate with well-to-well variability consistently < 10%. Infected macrophages J774A.1 were cultured over a range of increasing concentrations of each drug. Effective concentration that caused 50% inhibition of growth (IC_{50}) after 96 hr of drug addition has been determined from a graph representing different concentrations of drug plotted against optical density (OD).

Data in Table 1 indicates that the susceptibility to these antileishmanial conventional drugs is specific. The IC_{50} of the conventional drug like sodium stibogluconate was comparable to published values for amastigotes in VL strains, AG83 and 2001-S[13]. PKDL isolates, RK1 and MS2 were also found to be sensitive to antimonial drug (Table 1). The PKDL strains, NR3A, RMP8, RMP19, RMP142, RMP155, RMP240 were 7.8, 5.5, 7.0, >10.5, 4.4 and 10.5 fold resistant respectively to the antimony-sensitive isolate AG83. The antimony-resistant isolates were not cross-resistant to other anti-leishmanial drugs, namely, paromomycin sulphate, pentamidine, amphotericin B and miltefosine (Table 1). Antimony-susceptibility and resistance of these isolates was similar to that obtained by luciferase assay thereby further confirming the feasibility of the assay system (data not shown). Results demonstrate that β-lactamase assay is an appropriate colorimetric viability indicator for drug-screening assays with amastigotes of *L. donovani*.

Discussion

The β-lactamase reporter gene system used in the present study was designed to have characteristics that optimize its utility in amastigote-macrophage drug screening assays. The reporter gene used in the present construct integrates into the rRNA region of the genome to ensure its stable expression even without antibiotic (nourseothricin) selection pressure[12]. This is important because the parasites are necessarily cultured without antibiotic selection pressure when cultured in macrophages. Integration into rRNA region results in its being transcribed by RNA polymerase 1, thereby resulting in high levels of expression which is necessary to generate a detectable readout with the numbers of parasites present in a microtiter plate. In addition, the rRNA gene is highly conserved among *Leishmania* species, allowing a single vector to be used in different species. Moreover, the presence of a β-lactamase gene does not alter the sensitivity of the strains to inhibitors[12].

Other advantage of this method is that it is a simple colorimetric assay and does not involve any sophisticated equipment. A large number of drugs could be screened using 96-well microtitre plates. β-lactamase substrate used in this assay system, CENTA is a β-lactamase-

labile, chromogenic cephalosporin reagent which changes color from light yellow (lambda maximum ca. 340 nm) to chrome yellow (lambda maximum ca. 405 nm) concomitant with hydrolysis of the β-lactam ring. It has been reported earlier that CENTA is relatively unaffected by commonly used human serum[14] for converting CENTA to its chrome yellow coloured product. Other β-lactamase substrates like nitrocefin convert to red coloured product in the presence of fetal bovine serum[12]. In order to overcome this problem, it is important that the medium should be completely removed from the plate followed by addition of the substrate in a serum-free buffer. These experiments demonstrate that CENTA can be conveniently used for detecting β-lactamase.

In this study the use of monocyte macrophage cell line J774A.1 instead of murine peritoneal macrophages has been reported. This modification has made the assay economically viable and a highthroughput screening assay. Unlike other cytotoxicity assays, subsequent manipulation steps are not required[10], thus increasing the reproducibility and reliability of this assay and the speed at which data can be collected. This colorimetric assay may be helpful for laboratories involved in screening large numbers of compounds for anti-leishmanial activity.

High-throughput screening of 5 conventional anti-leishmanial drugs by this modified β-lactamase assay indicated that antimony-resistant field isolates are sensitive to paromomycin, amphotericin B and miltefosine. These drugs have been shown to be effective against visceral leishmaniasis at least in the Indian subcontinent[15-19]. Many published reports have shown the effective concentration of antileishmanial drugs on the basis of the experiment done in insect stage (promastigotes) of the parasites. The strong correlation with clinical response can only be achieved when the effective doses of the crucial drugs are recommended on the basis of assays carried out in human stage (amastigotes). In conclusion, modified β- lactamase assay is comparatively cost effective and high-throughput screening assay. This method can be recommended for continuous monitoring of the effective doses of drugs being used in the field and to assess the potential of new drugs which are presently in the clinical trials using the amastigote model.

Acknowledgments

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Table 1

High-throughput screening of compounds tested against amastigote form of clinical isolates by β-lactamase assay [Values are mean ±SD of at least 3 independent determinations with triplicates in each set]

Strain ID	Sodium stibogluconate (Sb V)	IC$_{50}$ (μM)				
		Amphotericin B	Miltefosine	Paromomycin sulphate	Pentamidine isethionate
VL isolates						
HM/IN/AG83	9±0.5	0.01 ± 0.01	0.95 ± 0.03	8.00 ± 3.2	1.00± 0.002
2001-S	15 ±4.9	0.01 ± 0.001	0.60 ± 0.07	4.50 ± 1.06	0.10±0.02
PKDL isolates						
RKI	9±0.7	0.01 ± 0.01	0.80 ± 0.07	12.50 ± 2.47	0.20 ± 0.03
MS2	6 ± 1.06	0.03±0.02	0.87 ± 0.04	11.50 ± 1.7	0.06± 0.01
NR3A	70±1.41(7.8)*	0.01 ± 0.007	0.82 ± 0.04	36.00 ± 0.7	0.08 ± 0.01
HM/IN/RMP-8	50 ±3.5(5.5)*	0.01 ± 0.002	0.67±0.007	9.00± 0.7	0.06± 0.001
HM/IN/RMP-19	62.5±4.24(7.0)*	0.02 ± 0.003	0.43 ± 0.05	12.50 ± 0.35	0.05 ± 0.01
HM/IN/RMP-142	>100.00(>10.0)*	0.02 ± 0.007	0.35 ± 0.03	4.90±0.14	0.21 ± 0.05
HM/IN/RMP-155	40 ± 8.4 (4.4)*	0.01 ± 0.001	0.85 ± 0.03	3.60 ± 0.7	0.14 ± 0.05
HM/IN/RMP-240	95 ± 3.5 (10.5)*	0.01 ± 0.007	0.80 ± 0.07	32.00 ± 0.07	0.34 ± 0.2

IC$_{50}$s were determined in intracellular amastigotes in macrophages of VL and PKDL clinical isolates after 96 hr of drug addition The fold differences in IC$_{50}$s with respect to HM/IN/AG83 are shown within bracket

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Statistically different at *P*<0.0001 compared to corresponding values obtained for HM/IN/AG83