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A Simple Technique for Simultaneously Quantifying Cell Death Index and Infection Index on *in vitro* Cell- Based Susceptibility Assays

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ABBREVIATIONS
AO/EB Staining: Acridine Orange/ Ethidium Bromide staining
MiR-155 CP: miR-155 Chitosan Polyplex
L.major: Leishmania Major
eGFP: enhanced Green Fluorescent Protein
PBS: Phosphate- buffered saline
PCR: Polymerase Chain Reaction

ABSTRACT

Background and Aim: It is a common practice for biomedical researchers to evaluate *Leishmania* infection indexes on *in vitro* cell cultures to address biological questions or test the efficacy of novel anti- parasitic compounds. In *Leishmania* infections that parasitize macrophages, infection indexes are usually determined by visual inspection of the cells directly using Giemsa staining. However, the mechanism of action of treatment on *Leishmania* parasites in this method is not evaluated, therefore motivating the finding of analysis approaches that allow simultaneously quantifying *Leishmania* infection index and cell death on *in vitro* cell cultures.

Methods: We proposed a method for simultaneously evaluating parasite infection indexes and cell death indexes using fluorescent microscopy. To perform such an analysis, we used a fluorescent dye mix of ethidium bromide and acridine orange (EB/AO), which binds nucleic acids.

Results: EB/AO achieved simultaneous quantification of infection index and death index under normal growth and treatment conditions that were comparable to the conventional Giemsa staining method while overcoming the drawbacks of the current method.

Conclusion: The cell death index and *Leishmania* infection index can be simultaneously quantified using our method, which is less user- skill- dependent and faster than the general test.

INTRODUCTION

Leishmania infects and grows in macrophages (1). *In vitro* cultures of macrophages infected with the parasite are used to study the mammalian stage of *Leishmania*. Macrophages can be infected *in vitro* on the cover slip, which allows the average intracellular parasite burden to be determined by visual inspection. In this setting, macrophage infection indexes are used as a measure of parasite growth and/ or survival (2). The infection index is determined by multiplying the percentage of infected macrophages by the average number of *Leishmania* per cell (3). Both of these parameters are determined by visual inspection of cells and counting under the microscope with common Giemsa staining. It is got one drawback: it only detects infected cells and cannot quantify the proportion of living and dead ones.

In this work, we have used EB/AO which is based on DNA staining that infection index and cell membrane integrity can be checked at the same time and there is no cell fixation step. The use of dual acridine orange/ ethidium bromide (AO/EB) fluorescent staining, observed with a fluorescent microscope,

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allows for the detection of apoptosis- related alterations in cell membranes throughout the apoptotic process (4). AO permeates all cells and makes the nuclei appear green. EB is only taken up by cells when cytoplasmic membrane integrity is lost and stains the nucleus red. Thus, live cells have a normal green nucleus, while dead cells display a red cytoplasm and nucleus (5-7). So cell and parasite status can be distinguished based on the type of dye adsorbed by them.

MATERIALS and METHODS

General Reagents and Equipment

Sterile phosphate- buffered saline (PBS), Penicillin/ Streptomycin (Invitrogen) stored at -20°C, FBS (heat inactivated) stored at -20°C, RPMI with l-glutamine (Invitrogen) stored at 4°C, and Trypan blue (Sigma) diluted at 0.4% in PBS stored at RT, Giemsa and Giemsa buffer pH=7.2 (Merk), RAW 264.7 cell lines (ATCC® TIB-71™), *Leishmania major* (MRHO/IR/75/ER) and miR-155 chitosan polyplex (miR-155 CP) used for a treatment.

The dye mix prepared for the EB/AO staining was 100 µg/ml acridine orange (Sigma) and 100 µg/ml ethidium bromide (Sigma) in PBS (8). Cells were viewed and counted using a Nikon eclipse microscope at 400× magnification with an excitation filter of 480/30 nm, a dichromatic mirror cut- on 505 nm LP, and a barrier filter of 535/40 nm (Melville, NY, USA).

Enumeration of Macrophage Parasite Burden and cell death index

In vitro infection of RAW 264.7 cells on a cover slip allows the average intracellular parasite burden to be determined by microscopy. Macrophages are seeded on the coverslip, placed into the wells, and allowed to adhere for 2 hours at 37 °C in a CO2 incubator. The supernatant, comprising medium and floating cells, was either flicked or sucked away, and the cells were washed three times with PBS prior to being infected. Although cell lines are known for being more stable and standardizable than primary cells, it is important to consider the importance of subculture, as simple things as cell density can influence the response of a cell line.

After that, adherent cells were exposed to *Leishmania* at a parasite- to- macrophage ratio of five *L. major* promastigotes to one macrophage for four hours at 37°C. This results in an intermediate level of infection, roughly 60-70 percent. The multiplicity and duration of infection can be adjusted to attain the desired level of initial infection, as drug studies necessitate a near 100% infection rate to accurately measure parasite killing. The choice of parasite species and strain influences the outcome of the infection. For the used parental *Leishmania* strain, *Leishmania major* (MRHO/IR/75/ER), the timing of promastigotes loss of overtime virulence is well established. Therefore, promastigote cultures should only be used for less than 7 passages after recovery from infected animals.

Extracellular parasites are washed away with several rounds of PBS with a multichannel pipette before the infected cells are incubated for the required time in the presence of the treatment agent (miR-155 CP). The infection ratio must be established case- by- case, depending on the objective. For miR-155 CP screening, we did not want to overwhelm the macrophages. The infected cells should remain infected but functional.

Finally, EB/AO dye mix (5 µl) was added to each cover slip, and cells were viewed and counted using a Nikon Eclipse microscope at 400× magnification with an excitation filter of 480/30 nm, a dichromatic mirror cut-on of 505 nm, and a barrier filter of 535/40 nm (Melville, NY, USA). The pictures were taken with a digital camera. Tests were done in triplicate, counting a minimum of 100 total cells each. EB/AO induces cell death at high concentrations. Hence, the EB/AO dilution should be selected as the optimum concentration for each specific cell type, cell number, and incubation time.

The average intracellular parasite load was determined by counting a minimum of 100 total cells from random fields (performed in duplicate), the number of infected macrophages was determined in 100 macrophages in each area. The average number of parasites per macrophage was determined by counting the total number of intercellular amastigotes in 100 cells. Infection index, which is the percentage of infected macrophages multiplied by the average number of amastigotes per macrophage, was estimated using the formula: (parasites/infected cells) X (infected cells/total cells) X 100.

Statistical analysis

To test the hypothesis that frequencies of infection index were independent of the staining method used, we employed T-test analysis using GraphPad Prism. For each assay, triplicate counts of the infection index for both the traditional Giemsa staining and modified staining methods (EB/AO dye mix) were averaged.

Ethical consideration:

The protocol has been confirmed by institutional ethical committee (Ethical code: IR.SBMU.MSP.REC.1401.399).

RESULTS

We employed the EB/AO technique to obtain simultaneous quantifications of the infection index and death index under normal growth and treatment conditions, which are comparable to the conventional Giemsa staining method. Figure 1 illustrated the cell morphology obtained using the EB/AO method. The morphology of live and dead cells and the parasite have been shown in Figure 1. In contrast to the

Giemsa method, in the new method, it is possible to distinguish between dead cells (apoptotic cell or necrotic cells) and living cells, in addition to the fact that counting the parasite inside the cell will be easier.

We were able to observe more features for distinguishing live cells from dead cells using the EB/AO method. In contrast, all the cells stained from the Giemsa method were died since it requires fixation the cells. Furthermore, by eliminating the fixation steps, EB/AO method drastically reduces the time needed to perform the test.

Figure 2 compared the results from the EB/AO method and the Giemsa method for both control (untreated) and treated cells during treatment efficacy assessment. Treatment effectiveness can be detected by determining the reduction in the average percentage of infected cells or counting, and calculating the percentage reduction of the average number of amastigotes per macrophage (infection index). The former is simpler and less time-consuming, while the latter is more precise. Results indicated the percent of the infected cell (2A) and infection index (2B) in treated and untreated groups using both methods are not statistically different. It means that cell and parasite counts were independent of the process. But the new technique has the advantage of showing whether the change in parasite burden is due to cell death or immunostimulatory effects (2C).

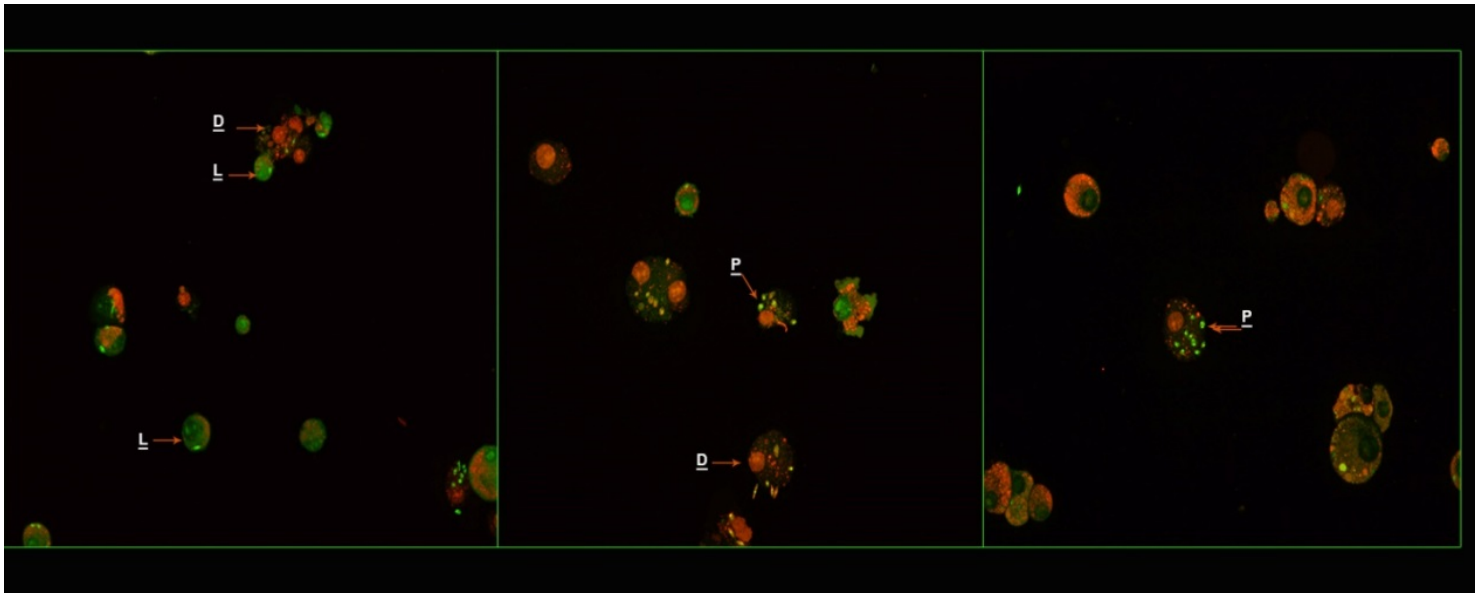

FIGURE 1. EB/AO method. Status of the infected cells in the EB/AO staining method "L" points to live cells; "D" indicates dead cells; and "P" points to parasites.

DISCUSSION

The determination of infection rate is fundamental in vaccine and new drug studies of *leishmaniasis*, and monitoring the drug susceptibility of *Leishmania* isolates still largely relies on *in vitro* cell- based susceptibility assays (9). Various methods, including PCR method and non- DNA- based methods, microscopic examination (Giemsa- stain and Leishman stain) and immunohistochemistry for tissue samples, can provide *Leishmania* parasite quantification (10-13). Some studies also use *L. major* expressing eGFP (14); however none of these methods can detect cell death simultaneously. An improved staining method is described in the present work to overcome the shortcomings of Giemsa staining methods on *in vitro* cell- based susceptibility assays. We proposed an approach for evaluating the *L. major* parasite infection index on an *in vitro* macrophage cell culture, based on fluorescence microscopy. This approach provides the conditions to investigate the effect of treatment on cell death that could not be obtained by Giemsa stain. We found that our treatment, miR-155 CP, in addition to reducing the contamination of macrophages, also causes the death of infected cells. Cell death is the mechanism that reduces parasite load in macrophages, not the immunomodulation effects of treatment.

There is no one approach to fit all questions however, our method can be used in the primary screening assay to identify the mechanism of action of an anti- parasitic drug, and it can also be used to determine the ratio of necrotic death to apoptosis after treatment.

We use this protocol routinely for our experiments. Despite the original technique, this method has flexibility for researchers according to their study needs. In previous study, we have utilized ED/AO staining to assess NETosis (15). Recently, Datta and colleagues employed confocal

microscopy as a multi-color immunofluorescence assay to distinguish intracellular from external *Leishmania* parasites (16). Our protocol, however, makes it easier to visualize live and dead intracellular parasites and external parasites. It is possible to quantify the number of intracellular parasites, external parasites, and macrophages, providing a more comprehensive understanding of drug toxicity studies and host-pathogen interactions. The EB/AO staining could be adjusted to screen for drug toxicity, invasion, and proliferation capacities in the cell- based anti- infective assay. The ED/AO technique is less labor-intensive and quicker than the general infection index test. This method requires only small quantities of inexpensive EB and AO for each experiment. This technique is straightforward to execute, time- efficient, and particularly suited for adherent cells.

The current study had limitations because we didn't use standard anti- *Leishmania* drugs as a treatment. However, the miR-155 CP demonstrated anti- *Leishmania* properties. Another problem with our protocol was that we had to watch and look at our AO/EB stained cells immediately after adding stain because AO/EB is toxic and causes cell death quickly.

As discussed above, a variety of methods are available to measure infection index *in vitro*. However, the high-throughput screening system is limited. The AO/EB staining may be performed entirely on a 96-well plate using inverted fluorescence microscopes (8). This protocol may be adapted for high- throughput screening and analysis for drug discovery. Further studies are needed to apply an image-based approach and develop a computer- assisted algorithm to automate analysis and interpret data.

CONCLUSION

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CONFLICT OF INTEREST

All authors certify that this manuscript has not been published in whole or in part nor is it being considered for publication elsewhere. The authors have no conflicts of interest to declare.

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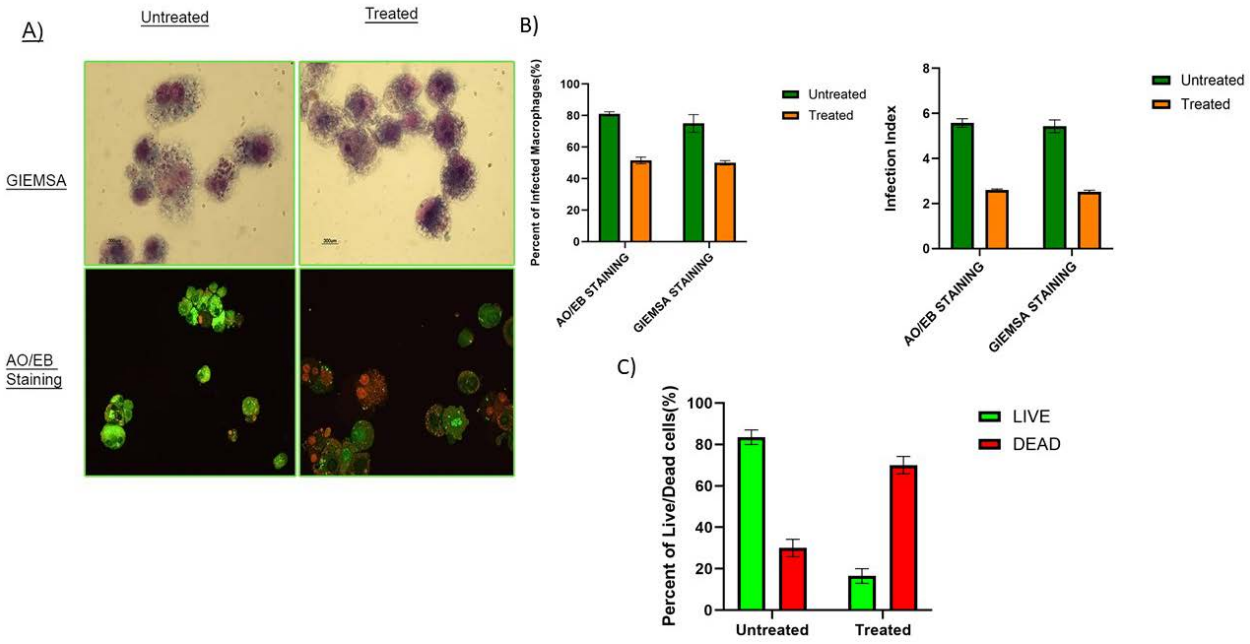

FIGURE 2. Comparison of the ED/AO- based and conventional methods. Representative image of *Leishmania*- infected RAW264.7 macrophages (A). Overall, quantification of infected macrophages and infection index were comparable using both methods (B). The AO/EB staining showed the change in parasite burden is due to cell death in treated groups but not immunomodulatory effects (C). There was no significant difference between the results of two different staining methods. T-Test was used to compare between groups. Data are shown as mean ± SD The data were represented of three independent experiments (n = 3).

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