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Simple Resazurin-Based Microplate Assay for Measuring *Chlamydia* Infections

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The conventional method for quantification of *Chlamydia* infection using fluorescence microscopy typically involves time- and labor-intensive manual enumeration, which is not applicable for a large-scale analysis required for an inhibitory compound screen. In this study, an alamarBlue (resazurin) assay was adopted to measure *Chlamydia* infection by measuring the redox capability of infected host cells in a 96-well format. The assay provided measurements comparable to those of the conventional microscopy method while drastically reducing the time required for analysis.

The development of new classes of antimicrobial agents is imperative due to the increasing resistance of pathogenic bacteria to existing antibiotics and its growing threat to public health. Among these organisms, *Chlamydia trachomatis* is the causative agent for the most common bacterial sexually transmitted infection in the United States (1). The unique biology of *Chlamydia* as an obligate intracellular organism presents challenges to identifying novel compounds against the organism. The standard technique for quantification of *Chlamydia* infection is the immunofluorescence assay (IFA), which relies on visual detection of fluorescently stained bacteria and host cells. This process is labor-intensive and not suitable for large-scale screening required for antimicrobial compound development. Effective methods for large-scale, image-based quantification have been reported for *Chlamydia* infection (2–4). However, automation of image acquisition typically requires specialized equipment and extensive computational resources. Therefore, development of a facile and accurate method would be beneficial for screening potential antichlamydial compounds.

alamarBlue (resazurin) is a colorimetric cell viability indicator widely used to monitor eukaryotic cell proliferative activity (5). The redox dye resazurin enters the cytosol in the oxidized form (blue) and is converted to the reduced form, resorufin (red). The reduced and oxidized forms of alamarBlue can be separately measured by a spectrophotometer and used to determine the reduction capability of cells, which reflects the status of mitochondrial function and cell viability. One significant advantage of alamarBlue over other metabolic indicators is that the compound allows continuous monitoring of cells (6). This is due to the fact that the compound does not interfere with the activity of the respiratory chain and, therefore, is nontoxic to the cells. alamarBlue has found applications in quantitative analysis of cell viability (7), proliferation (8), cytotoxicity (9), and drug susceptibility (10, 11) in both bacterial and eukaryotic systems as well as high-throughput screening of antimicrobial compounds in microorganisms such as *Mycobacterium tuberculosis* (12) and *Staphylococcus aureus* (13). In this study, the alamarBlue assay was adapted for enumeration of *C. trachomatis* infection by measuring the lysis of infected host cells as indicated by a decrease in cell viability. To demonstrate the ability of this assay to measure anti-*Chlamydia* activity, infections in the presence of the well-established anti-*Chlamydia* compounds tetracycline and polymyxin B were enumerated.

A productive developmental cycle of *C. trachomatis* strain L2 typically takes 48 to 72 h and is completed with the host cell lysis or extrusion (14). In order to correlate the infection level with the host cell viability, we first determined a time point where the lysis of infected host cells results in a significant decrease of the overall host cell viability among the samples. In addition, to validate this method as an effective enumeration tool for *Chlamydia* infection, sensitivity of the assay was examined by analyzing samples infected with a 2-fold dilution of *Chlamydia* elementary body (EB). The parallel samples were also prepared and analyzed by the conventional IFA for comparison. L929 mouse fibroblast cells in a 96-well tissue culture plate (BD Bioscience, Billerica, MA) were infected with *C. trachomatis* lymphogranuloma venereum (LGV) L2/434/Bu elementary bodies diluted in Hanks' balanced salt solution (HBSS; Mediatech, Inc., Manassas, VA). Following a 2-h inoculation at room temperature, the inoculum was removed, RPMI 1640 tissue culture medium (Mediatech, Inc.) was supplemented with 5% fetal bovine serum (Thermo Fisher Scientific, Liverpool, NY), and 10 μg/ml gentamicin (MP Biomedicals, Santa Ana, CA) was added. At 24, 48, and 72 h postinfection (hpi), alamarBlue (Invitrogen, Grand Island, NY) was diluted in tissue culture medium without phenol red to a final concentration of 10% and added to the cells. The reducing capability of the infected cells was monitored by measuring absorbance at 570 nm (reduced) and 600 nm (oxidized) using a PowerWave microplate spectrophotometer equipped with KC[4] data collection/analysis software (BioTek Instruments, Inc., Winooski, VT) and reported as a percentage relative to the mock-infected sample, using the following formula provided by the manufacturer: $([\varepsilon_{ox}]\lambda_2 A\lambda_1 - [\varepsilon_{ox}]\lambda_1 A\lambda_2)/([\varepsilon_{ox}]\lambda_2 A'\lambda_1 - [\varepsilon_{ox}]\lambda_1 A'\lambda_2) \times 100$, where ε_{ox} is the molar extinction coefficient of the alamarBlue oxidized form, the numerator is the test agent, the denominator is the untreated control, A is the absorbance of the test wells, A′ is the absorbance of the positive-control well (mock infection), λ_1 is 570 nm, and λ_2 is 600 nm.

Received 8 January 2013 **Returned for modification** 4 February 2013
Accepted 8 March 2013
Published ahead of print 18 March 2013
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doi:10.1128/AAC.00056-13

TABLE 1 Comparison of IFA and the alamarBlue assay for enumeration of *C. trachomatis* infection

Dilution of EB	Infection level (%) 24 hpi in IFA	Reducing capability of infected cells relative to the mock-infected cells[a]								
		24 hpi			48 hpi			72 hpi		
		Avg (%)	SD (%)	Z′ factor	Avg (%)	SD (%)	Z′ factor	Avg (%)	SD (%)	Z′ factor
1:1	95.77	26.40	1.36	0.56*	6.90	0.68	0.83*	4.21	0.08	0.98*
1:2	92.94	61.80	3.66	−0.02	18.83	0.89	0.79*	6.13	0.28	0.97*
1:4	90.64	87.84	4.71	−2.47	46.35	0.27	0.72*	13.42	1.59	0.92*
1:8	86.70	103.00	6.16	−14.50	69.27	0.78	0.47	27.96	2.17	0.88*
1:16	63.95	107.29	2.15	−3.74	85.86	2.26	−0.47	49.17	2.91	0.79*
1:32	43.61	109.90	5.82	−3.60	96.11	1.15	−3.50	74.03	2.82	0.60*
1:64	20.80	114.35	4.43	−1.88	106.17	0.42	−1.48	85.67	1.35	0.57*
1:128	14.79	119.60	9.48	−1.88	99.23	5.43	−38.28	88.67	2.00	0.29
1:256	7.20	114.30	0.77	−1.13	102.47	0.49	−5.27	100.06	0.72	−75.83
1:512	3.06	108.15	8.51	−5.58	105.74	0.61	−1.76	100.58	0.47	−4.98
1:1,024	2.16	102.65	4.24	−14.41	103.46	1.90	−4.71	102.67	2.49	−2.57
1:2,048	1.08	120.76	8.34	−1.56	108.26	0.66	−0.94	103.06	0.50	−0.17
1:4,096	0.98	109.81	0.48	−2.01	108.17	2.61	−1.67	105.87	0.66	0.31
R^2		0.52			0.79			0.98		

[a] Z′ factors of <1 and ≥0.5 (marked with asterisks) are indicative of an assay with suitable screening characteristics (14). SD, standard deviation.

Table 1 summarizes the reducing capability of infected samples as well as the infection level determined by IFA. To statistically compare and evaluate the data sets, the Z′ factor was determined. The Z′ factor is a statistical parameter used to evaluate the quality of high-throughput assays, where Z′ factor values greater than 0.5 are indicative of excellent low variation and signal dynamic range (15). At 24 hpi, the reduction capability of the infected sample was mostly comparable to that of mock-infected cells with the exception of the three highest inoculation doses (1:1, 1:2, and 1:4 dilutions). Given that cell lysis upon completion of the developmental cycle takes place 48 to 72 hpi, this decrease at the high inoculation doses is likely to be a result of cytotoxicity due to overinfection rather than completion of the developmental cycle. At 48 and 72 hpi, the expected dose-dependent decrease in reduction capability was observed to range from 108.17 to 6.90% and 105.87 to 4.21%, respectively, over increasing inoculation doses. The Z′ factor for 48-hpi analysis was determined to be above 0.5 for the three highest inoculation doses, which corresponds to 95.77 to 90.64% infection reported by IFA. When infection was allowed to proceed for an additional 24 h, the 7 highest inoculation doses, corresponding to 95.77 to 20.8% infection levels, resulted in a Z′ factor greater than 0.5, indicating that the analysis in this infection range can be performed with a high degree of confidence at 72 hpi. In order to utilize this assay in measuring infection level or assessing anti-*Chlamydia* activity of compounds, a sufficient dynamic range must be provided, and therefore, 72 hpi was chosen as the time point for the subsequent analysis. In addition, when a correlation coefficient (R^2) was used to determine the degree of similarity between the two methods, linear regression analysis between the data sets obtained from the alamarBlue assay (72 hpi) and IFA resulted in a correlation coefficient of 0.98, indicating a strong correlation between the two analyses and further supporting that enumeration of *Chlamydia* infection by the alamarBlue assay is highly comparable to results with the conventional IFA.

To begin validating this method as an anti-*Chlamydia* compound screening tool, inhibition of *C. trachomatis* infection by two well-established inhibitors, tetracycline hydrochloride (USB Corporation, Cleveland, OH) and polymyxin B sulfate (PMB; Enzo Life Science, New York, NY), was assessed (16–18). Tetracycline is membrane permeant and inhibits bacterial translation, whereas PMB primarily affects surface molecules (lipopolysaccharide [LPS]/lipooligosaccharide [LOS]) on bacteria and negatively perturbs membrane integrity. *Chlamydia* infection was performed as described above to achieve approximately 90% infec-

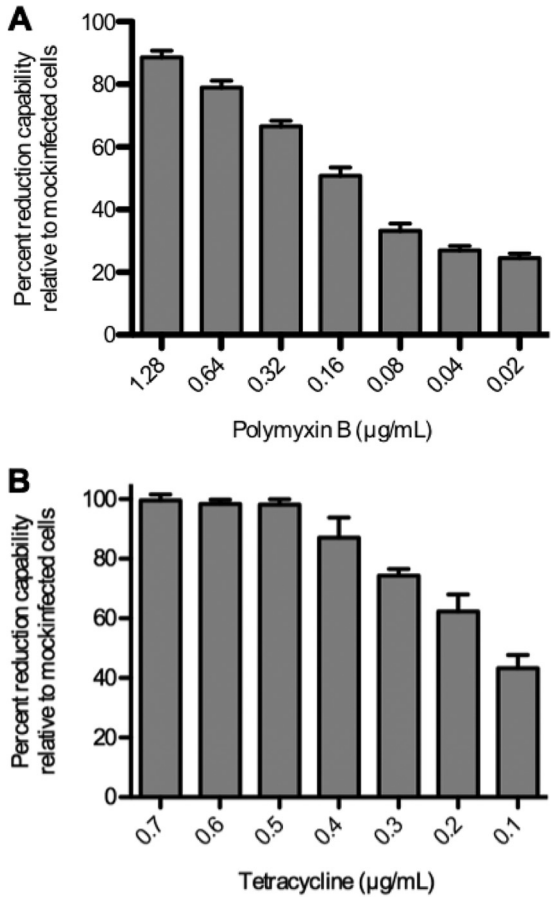

FIG 1 Dose-dependent inhibition of *C. trachomatis* infection (72 hpi) by polymyxin B (A) or tetracycline (B). Error bars indicate standard deviations of the triplicate infections.

tion in the untreated control sample. For PMB treatment, purified *C. trachomatis* cells were diluted in HBSS containing a 2-fold dilution of PMB ranging from 0.02 to 1.28 μg/ml and incubated for an hour prior to proceeding to infection as described previously (17). For tetracycline treatment, tissue culture medium containing various concentrations of the compound ranging from 0.10 to 0.70 μg/ml was added to the cells following EB inoculation. In both PMB- and tetracycline-treated samples, the compound concentration and reduction capability of infected cells resulted in a positive linear correlation, with R^2 values of 0.97 and 0.99, respectively (Fig. 1). For tetracycline, a concentration range above 0.50 μg/ml was not included in determining this linear regression since the inhibition plateaued at 0.50 μg/ml. Importantly, the plateau of the reduction capability of infected cells at this concentration is consistent with a previously reported observation by IFA that 0.51 μg/ml was sufficient to achieve 100% inhibition of *C. trachomatis* serovar L2 (19). Furthermore, no significant effect on the reduction capability was observed when cells were incubated with antibiotics alone (data not shown).

Together, these data demonstrate that measuring host cell viability of infected cells can be used as an indirect enumeration of *Chlamydia* infection, as it reflected the infection level determined by the conventional IFA. Although the method requires a 72-hour incubation time, the high-throughput capacity of a microplate reader considerably reduces the active time required for analysis. While further studies are clearly required, this method may provide a rapid and cost-effective approach to the discovery of novel anti-*Chlamydia* compounds with the added benefit of dual function (i.e., host-cell toxicity analysis and *Chlamydia* inhibitory properties).

ACKNOWLEDGMENT

This study was funded by an NIH Microbicide Innovation Program grant (NIH AI082697).

REFERENCES

1. **Gerbase AC, Rowley JT, Mertens TE.** 1998. Global epidemiology of sexually transmitted diseases. Lancet **351**(Suppl 3):2–4.
2. **Wang S, Indrawati L, Wooters M, Caro-Aguilar I, Field J, Kaufhold R, Payne A, Caulfield MJ, Smith JG, Heinrichs JH.** 2007. A novel automated method for enumeration of Chlamydia trachomatis inclusion forming units. J. Immunol. Methods **324**:84–91.
3. **Beeckman DS, Meesen G, Van Oostveldt P, Vanrompay D.** 2009. Digital titration: automated image acquisition and analysis of load and growth of Chlamydophila psittaci. Microsc. Res. Tech. **72**:398–402.
4. **Osaka I, Hills JM, Kieweg SL, Shinogle HE, Moore DS, Hefty PS.** 2012. An automated image-based method for rapid analysis of *Chlamydia* infection as a tool for screening antichlamydial agents. Antimicrob. Agents Chemother. **56**:4184–4188.
5. **de Fries R, Mitsuhashi M.** 1995. Quantification of mitogen induced human lymphocyte proliferation: comparison of alamarBlue assay to 3H-thymidine incorporation assay. J. Clin. Lab. Anal. **9**:89–95.
6. **Ahmed SA, Gogal RM, Jr, Walsh JE.** 1994. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. J. Immunol. Methods **170**:211–224.
7. **Al-Nasiry S, Geusens N, Hanssens M, Luyten C, Pijnenborg R.** 2007. The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells. Hum. Reprod. **22**:1304–1309.
8. **Voytik-Harbin SL, Brightman AO, Waisner B, Lamar CH, Badylak SF.** 1998. Application and evaluation of the alamarBlue assay for cell growth and survival of fibroblasts. In Vitro Cell. Dev. Biol. Anim. **34**:239–246.
9. **O'Brien J, Wilson I, Orton T, Pognan F.** 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. **267**:5421–5426.
10. **Baker CN, Tenover FC.** 1996. Evaluation of Alamar colorimetric broth microdilution susceptibility testing method for staphylococci and enterococci. J. Clin. Microbiol. **34**:2654–2659.
11. **Tiballi RN, He XG, Zarins LT, Revankar SG, Kauffman CA.** 1995. Use of a colorimetric system for yeast susceptibility testing. J. Clin. Microbiol. **33**:915–917.
12. **Collins L, Franzblau SG.** 1997. Microplate Alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob. Agents Chemother. **41**:1004–1009.
13. **Pettit RK, Weber CA, Pettit GR.** 2009. Application of a high throughput Alamar blue biofilm susceptibility assay to Staphylococcus aureus biofilms. Ann. Clin. Microbiol. Antimicrob. **8**:28.
14. **Hybiske K, Stephens RS.** 2007. Mechanisms of host cell exit by the intracellular bacterium Chlamydia. Proc. Natl. Acad. Sci. U. S. A. **104**:11430–11435.
15. **Zhang JH, Chung TDY, Oldenburg KR.** 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. **4**:67–73.
16. **Chopra I, Roberts M.** 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. **65**:232–260.
17. **Lampe MF, Ballweber LM, Isaacs CE, Patton DL, Stamm WE.** 1998. Killing of *Chlamydia trachomatis* by novel antimicrobial lipids adapted from compounds in human breast milk. Antimicrob. Agents Chemother. **42**:1239–1244.
18. **Matsumoto A, Higashi N, Tamura A.** 1973. Electron microscope observations on the effects of polymixin B sulfate on cell walls of *Chlamydia psittaci*. J. Bacteriol. **113**:357–364.
19. **Walsh M, Kappus EW, Quinn TC.** 1987. In vitro evaluation of CP-62,993, erythromycin, clindamycin, and tetracycline against *Chlamydia trachomatis*. Antimicrob. Agents Chemother. **31**:811–812.