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Optimization of a Rapid Viability Assay for *Mycobacterium avium* subsp. *paratuberculosis* by Using alamarBlue[▽]

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A microtiter alamarBlue assay was adapted and optimized for *Mycobacterium avium* subsp. *paratuberculosis*. Using cell concentrations ranging from 10^4 to 10^8 CFU/ml, a minimum incubation time to indicate viability was obtained after 24 h. Rifampin (rifampicin) was used to demonstrate that this method has applications for high-throughput screening against *M. avium* subsp. *paratuberculosis*.

Mycobacterium avium subsp. *paratuberculosis* is a chronic enteric pathogen which is widely distributed throughout the food chain (4, 16). Its association with Johne's disease in cattle is economically significant, with the United States alone suffering losses of $1.5 billion a year (6). Furthermore, its potential to cause human disease is disconcerting and controversial (7, 10, 11, 13). Therefore, the identification and development of novel anti-*M. avium* subsp. *paratuberculosis* agents are urgently required. In order to facilitate this, it is important to have available rapid anti-*M. avium* subsp. *paratuberculosis* assays permitting high-throughput analysis. A microtiter alamarBlue assay (currently untested for *M. avium* subsp. *paratuberculosis*) is a reliable means of determining cellular viability in bacteria (9). This rapid and inexpensive assay lends itself to a high-throughput screening format and has been shown to be applicable to some species of mycobacteria (3, 15). Furthermore, its correlation with other more expensive methods for determining mycobacterium viability is high, between 93 and 100% (1, 5, 15). These include the Mycobacteria Growth Indicator Tube, the Bactec radiometric method, and luciferase reporter systems. This study set out to establish and optimize a microtiter alamarBlue assay for a broad range of *M. avium* subsp. *paratuberculosis* titers and to evaluate applications for this assay, including high-throughput screening of novel anti-*M. avium* subsp. *paratuberculosis* compounds, and antibiotic resistance profiling of *M. avium* subsp. *paratuberculosis*.

M. avium subsp. *paratuberculosis* (CIT03) was isolated from the feces of an infected cow, as described by Ristow et al. (12), and cultivated on Herrold's egg yolk medium agar supplemented with mycobactin J (2 μl/ml), amphotericin B (50 μg/ml), vancomycin (50 μg/ml), and nalidixic acid (50 μg/ml) for 16 weeks at 37°C. The identity of *M. avium* subsp. *paratuberculosis* was confirmed using acid-fast staining, mycobactin dependency, and PCR analysis as previously described (2, 8, 14). To generate sufficient biomass, *M. avium* subsp. *paratuberculosis* was subsequently grown in Middlebrook 7H9 broth (MB broth), supplemented with oleic acid, albumin, dextrose, catalase (10%; Becton Dickinson), glycerol (0.2%), and mycobactin J (0.2%). This generally took 16 weeks.

Prior to the assay, 10 ml of the 16-week culture was centrifuged at 15,000 rpm for 20 min. The pellet was washed in fresh MB broth and resuspended in 10 ml of fresh supplemented MB broth containing 0.2% mycobactin J. The turbidity was adjusted to match McFarland standard no. 1 (3×10^8 CFU/ml). From this suspension, a series of 1:5 dilutions ranging from 3×10^8 to 9.6×10^4 CFU/ml was set up in MB broth (5-ml volumes), using sterile Falcon tubes. The microtiter plate was organized into rows B, C, D, E, F, and G. Two-hundred-microliter aliquots of 3×10^8 CFU/ml *M. avium* subsp. *paratuberculosis* were added to 10 wells in row B. Two-hundred-microliter aliquots of 6×10^7 CFU/ml were added to row C, 1.2×10^7 CFU/ml to row D, 2.4×10^6 CFU/ml to row E, 4.8×10^5 CFU/ml to row F, and 9.6×10^4 CFU/ml to row G. This assay was allowed to progress over a period of 11 days. Twenty microliters (10% of the volume in the well) of a fresh alamarBlue reagent (AbD Serotec) was added, with mixing, to each column on each sampling day. Plates were covered and resealed with Parafilm and incubated at 37°C after the addition of the dye. Absorbance readings at 570 and 600 nm were then taken at 6 h, 24 h, and 48 h for each column. The assay was performed in triplicate, and percent reduction values of alamarBlue were determined using the appropriate formula (www.biokom.com.pl/files/alamarblue.pdf).

In terms of optimization, this assay examined the influence of cell numbers, the cellular incubation time, and the optimal incubation time with alamarBlue. All three had a significant impact on color development and percent reduction of the dye. For the highest concentration of cells (3×10^8 CFU/ml) (Fig. 1a), a strong reduction of the dye was observed after 1 day of cellular incubation. Further incubation of the cells or incubation with the dye did not result in an appreciable increase in dye reduction values. Indeed, a decrease in the percent reduction was noted, most likely due to buffering agents reaching their maximum buffering efficiencies in the reagent mix (www.abdserotec.com/about/alamarblue). At the mid-range cellular levels (2.4×10^6 CFU/ml) (Fig. 1b), detectable dye reduction occurred after 2 days. The reduction was substantial after day

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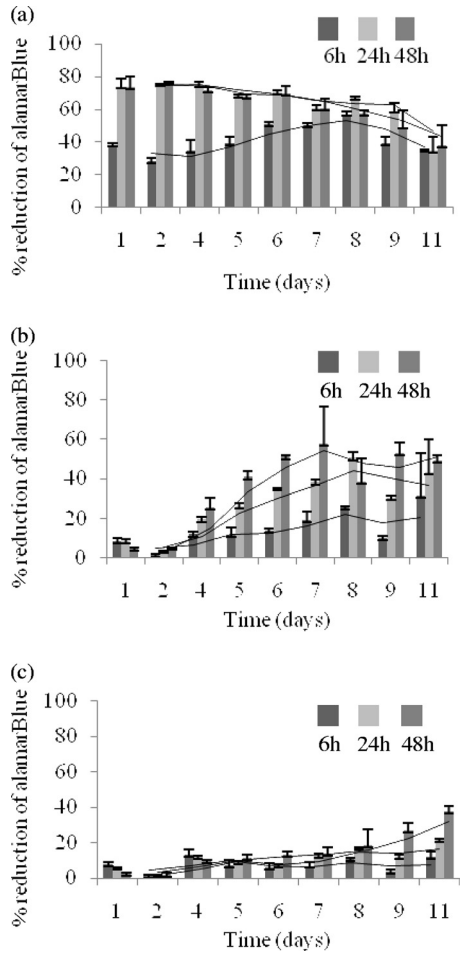

FIG. 1. Optimization of alamarBlue conditions using 3×10^8 CFU/ml (a), 2.4×10^6 CFU/ml (b), and 9.6×10^4 CFU/ml (c) over 11 days. Following the addition of alamarBlue, readings were taken at 6, 24, and 48 h.

4. At the lowest concentration of cells (9.6×10^4 CFU/ml) (Fig. 1c), a noticeable change in dye reduction was observed after 9 days. While longer incubation with alamarBlue led to greater dye reduction with a given cell titer, as shown with each suspension at 6, 24, and 48 h, the 24-h reading was considered sufficient to give a clear indication of viability.

Percent reduction of the dye was standardized to 10, 20, 40, and 60% for each concentration of cells (Table 1). These values serve as definitive indicators of metabolic activity and may be used for multiple applications, such as comparing the relative viabilities of strains, or the influence of media composition or environmental stress on *M. avium* subsp. *paratuberculosis*. In particular, we feel that this assay is suited to comparing the relative efficacies of multiple anti-*M. avium* subsp. *paratuberculosis* compounds and/or antibiotic resistance profiling in a high-throughput screening format. The success of this assay requires strict adherence to specific cell numbers, growth phases, and their equivalent incubation times.

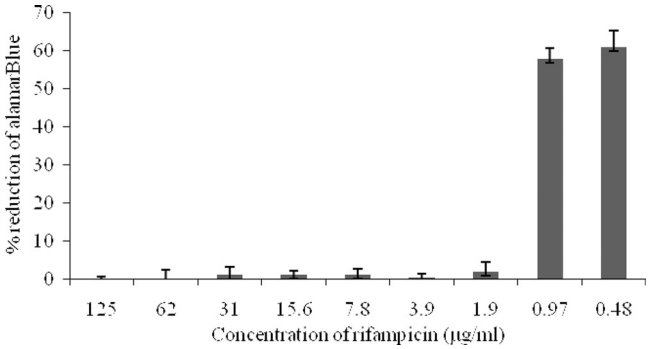

FIG. 2. Determination of the MIC_{90} of rifampin against *M. avium* subsp. *paratuberculosis*

Traditionally, detection of viability with the alamarBlue assay has been achieved on the basis of the pink-blue color change (1, 5, 9, 15). However, this method for *M. avium* subsp. *paratuberculosis* is also quantitative, as it determines the threshold percent reduction values required for visual color change of the medium (<12% blue, 12 to 18% purple, and >20% pink). Visually, all wells with calculated values below 12% after the addition of the dye were consistently blue, most probably due to insufficient metabolism needed to indicate viability. All wells which were purple were recorded as having percent reduction values of 12 to 18%. Wells with values over 20% were consistently pink, which is indicative of cellular metabolism. The time taken to indicate definitive viability varied significantly for each dilution of cells, ranging from 1 day plus 6 h with the dye (3×10^8 CFU/ml) to 9 days plus 48 h with the dye (9.6×10^4 CFU/ml), as seen in Table 1.

To demonstrate the application of the assay, it was used to assess the susceptibility of *M. avium* subsp. *paratuberculosis* to rifampin (rifampicin). Rifampin was prepared in dimethyl sulfoxide at double the maximum concentration required. The

TABLE 1. Required time taken for *M. avium* subsp. *paratuberculosis* to reduce alamarBlue

M. avium subsp. *paratuberculosis* cell concn (CFU/ml)	Time (h) required to reach indicated % reduction[a]							
	10		20		40		60	
	Cellular	alamarBlue	Cellular	alamarBlue	Cellular	alamarBlue	Cellular	alamarBlue
3×10^8	24	6	24	6	24	24	24	24
6×10^7	24	6	24	24	24	48	96	24
1.2×10^7	24	24	96	24	96	24	144	48
2.4×10^6	96	6	96	48	120	48	N/A	N/A
4.8×10^5	96	6	144	48	216	48	264	48
9.6×10^4	96	6	216	48	>264	N/A	N/A	N/A

[a] N/A, not applicable.

assay was carried out using 6×10^7 CFU/ml over a period of 4 days, plus a 24-h incubation with alamarBlue in supplemented MB broth containing 0.2% mycobactin J. The final antibiotic concentration ranged from 125 to 0.48 μg/ml. The MIC_{90} of rifampin for *M. avium* subsp. *paratuberculosis* was determined as 1.92 μg/ml, which correlates well with other studies (17) (Fig. 2). This result highlights the assay's potential as a high-throughput screening platform for antibiotic resistance profiling and the identification of novel anti-*M. avium* subsp. *paratuberculosis* agents.

Given the association of *M. avium* subsp. *paratuberculosis* with both animal and human diseases, such rapid, quantitative, and inexpensive (approximately 10 euro cents per sample) viability assays will be very useful for rapid screening of anti-*M. avium* subsp. *paratuberculosis* compounds and antibiotic profiling.

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