JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2010, p. 3725–3728 Vol. 48, No. 10
0095-1137/10/$12.00 doi:10.1128/JCM.01482-10


# NOTES

## Development of a Rapid ATP Bioluminescence Assay for Biocidal Susceptibility Testing of Rapidly Growing Mycobacteria[▽]

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Received 21 July 2010/Accepted 5 August 2010

**An ATP-based biocide susceptibility assay for mycobacteria was developed by optimizing the cell lysis and assay conditions. Compared to the conventional agar plating method, the assay was rapid (1.5 h) and showed high sensitivity and specificity as determined by receiver operating characteristic (ROC) analysis. The test species, *Mycobacterium immunogenum*, *M. chelonae*, and *M. abscessus*, showed various susceptibilities to the glutaraldehyde- and isothiazolone-based test biocides.**

Nontuberculous mycobacteria (NTM) are ubiquitously distributed. Here we focus on members of the *Mycobacterium chelonae-Mycobacterium abscessus* (MCA) complex that have been frequently associated with hospital infections or pseudo-outbreaks (5, 6, 7, 22, 26, 34) and occupational pulmonary diseases (4, 23, 34). Biocide-based disinfection is practiced for the control of these environmental pathogens. For instance, Cidexplus (3.4% alkaline glutaraldehyde) is used for mycobacterial and other disinfection capabilities for a wide range of contaminated hospital materials and medical instruments (3, 8, 29). Isothiazolone-based formulations, such as Kathon, are among the common biocides used for control of mycobacterial colonization of metalworking fluids (31, 32).

The methods currently used for testing the mycobactericidal activity of a test biocide are time-consuming (2, 33), requiring long incubation periods (3 to 5 days for rapid growers and 7 to 14 days for slow growers), and they provide a semiquantitative estimate of the biocide efficacy (1, 9, 17). Use of a bioluminescence assay based on bacterial ATP has been proposed for assessment of microbial load (10, 18) or antimicrobial efficacy (14, 19). However, when applied to mycobacteria, a major limitation of this method is the inefficient lysis of the cell wall, resulting in inaccurate measurement of cellular ATP (20, 25). In this study, we developed an improved cell lysis protocol for intracellular ATP-based biocide susceptibility testing of mycobacteria. Furthermore, both cell suspension (pregrown) and growth-based formats were optimized for the assay.

Reference strains and isolated genotypes (15) of the member species of MCA complex listed in Tables 1 and 2 were included as the test strains for this study. The mycobacterial cultures were grown in Middlebrook 7H9 (MB7H9) broth, supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (BD Biosciences, Sparks, MD) and 0.05% Tween 80 as described previously (30) or Sauton's broth medium (28).

Extraction of the intracellular ATP from mycobacterial cells was optimized using the following treatments and their combinations. Chemical extraction treatments were as follows: the single-tube cell lysis method (16), boiling Tris-EDTA buffer (27), lysozyme (Sigma, St. Louis, MO) treatment (3 mg/ml; 37°C, 1 h), and Bactozyme treatment (MRC, Inc., Cincinnati, OH) (37°C, 1 h). Physical extraction treatments were as follows: bead beating using 0.1-mm glass beads in a minibeadbeater (BioSpec Products, Bartlesville, OK) for 3 min (alternating 30-s pulses and 10-s cooling intervals) at the maximum speed setting (mix) or sonication for 1 min (with 10-s alternate pulses of sonication and cooling) using a probe sonicator (Ultrasonic processor XL2020; Misonix Inc., Farmingdale, NY) at 20% amplitude. The bead beating and sonication treatments were also tested in combination with lysozyme or Bactozyme. ATP released from the cells was quantified using the BacTiter-Glo assay kit (Promega, Madison, WI) and expressed as relative light units (RLU). The unlysed cells were used as a control. Statistical means were compared using two-sided *t* tests, and *P* values of $<0.05$ were considered statistically significant.

For evaluation of the developed ATP assay, Cidexplus (Johnson and Johnson Co., United Kingdom) was used as the test biocide for cell suspension-based susceptibility testing as described by Hernandez et al. (12), except that we used a relatively lower test inoculum ($\sim 10^6$ to $10^7$ CFU/ml). Kathon 886 MW (Rohm & Haas Co., Philadelphia, PA) was used as the test biocide for optimizing the growth-based susceptibility testing format.

For the growth-based assay, conditions were selected by testing two media, MB7H9 broth and Sauton's medium (28), and two inoculum sizes ($10^4$ and $10^6$ cells/ml). The MICs of Kathon against all test strains were determined, using various concentrations (10 to 40 ppm). Receiver operating characteristic (ROC) curve analysis was performed on the ATP data. The RLU cutoff value that yielded the maximum sensitivity

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▽ Published ahead of print on 18 August 2010.

TABLE 1. Mycobactericidal activity of Cidexplus as measured in a cell suspension assay format using the agar plate count method and the developed ATP bioluminescence assay

| *Mycobacterium* species | Cidexplus concn[a] (% [vol/vol]) | Log initial count (log CFU) | Log CFU reduction factor | | | % reduction in RLU[b] | | |
|---|---|---|---|---|---|---|---|---|
| | | | 10 min | 20 min | 30 min | 10 min | 20 min | 30 min |
| *M. chelonae* ATCC 35752 | 1 | 6.4 | 6.4 | 6.4 | 6.4 | 100 | 100 | 100 |
| | 2 | | 6.4 | 6.4 | 6.4 | 100 | 100 | 100 |
| *M. abscessus* ATCC 19977 | 1 | 7.2 | 1.5 | 1.72 | 2.8 | 88.3 | 99.03 | 100 |
| | 2 | | 7.2 | 7.2 | 7.2 | 100 | 100 | 100 |
| *M. immunogenum* ATCC 700506 | 1 | 6.3 | 0.58 | 1.8 | 2.23 | 97.15 | 99 | 100 |
| | 2 | | 6.3 | 6.3 | 6.3 | 100 | 100 | 100 |

[a] The test concentrations (1% and 2%) were selected based on the fact that 2% is the maximum concentration limit suggested for hospital disinfection applications.
[b] The values represent % reduction in the normalized value (compared to the untreated control); normalization was done by subtracting the residual ATP value.

and specificity compared to the conventional CFU was chosen as the criterion to determine the MIC.

Among the chemical extractants tested, our "single-tube cell lysis reagent" (16) yielded the highest ATP release (Fig. 1). Overall, a combination of lysozyme with bead beating yielded the highest ATP and thus was chosen as the preferred cell lysis method. Using this method, the ATP assay revealed a linear relationship ($r^2 = 0.99$) between intracellular ATP (RLU) and the cell number (CFU/ml) in the CFU range of $\sim 10^3$ to $10^7$ CFU/ml. The observed minimum limit of detection (LOD) for the three species was between $10^3$ and $10^4$ CFU/ml (data not shown), a level consistent with the inherent LOD of the ATP quantification methods (13).

In evaluation studies on the developed ATP assay, Cidexplus showed a rapid bactericidal effect (100% reduction of log CFU or RLU), within 10 min, on all three test species (Table 1). To our knowledge, except in the case of *M. chelonae* (12, 21), there have been no such previous studies on the effect of glutaraldehyde on MCA member species.

In growth-based susceptibility testing, the choice of a growth medium (32) and inoculum size (25) is critical. For instance, the presence of albumin in the complete MB7H9 medium causes a reduced efficacy of the Kathon 886 biocide due to the degradation of its active component, 5-chlor-2-methyl-2*H*-isothiazol-3-one (32). In this study, Sauton's medium proved to be a medium of choice considering that it is albumin free (thus avoiding interaction with isothiozolone) and supported better growth, peaking on day 4, as confirmed by both CFU and RLU analyses (data not shown). Nevertheless, the possibility that the concentration of Tween 80 used in growth media, though low (e.g., 0.05% in MB7H9 and 0.1% in Sauton's medium), may have a secondary effect on biocide susceptibility (11, 24) cannot be excluded. For inoculum size, our results (Fig. 2) on susceptibility analysis showed that MIC determination can be achieved in a much shorter time using the small inoculum (25 ppm at day 2) versus the larger inoculum (100 ppm at day 6). The former inoculum level ($10^4$ CFU/ml) was therefore preferred for the assay.

The optimized ATP assay (growth based) showed high overall sensitivity (90 to 100%), specificity (93 to 100%), and accuracy (96 to 100%) compared to the conventional plate count method (Table 2). *Mycobacterium immunogenum* showed relatively higher Kathon resistance (MIC = 20 to 30 ppm) than *M. chelonae* and *M. abscessus* (MIC = 10 ppm for both species). To our knowledge, relative biocide susceptibility patterns between *M. abscessus* and other MCA species have not been investigated (32).

Taken together, the developed ATP bioluminescence assay showed a good correlation with the conventional plate count method. The results of the ATP assay were available in just 1.5 h, however, in contrast to the 3 to 5 days required for the plate count method. Moreover, the use of a bioluminescence assay replaces the laborious and often lengthy plating procedures for CFU determination.

In conclusion, the study led to the optimization of a cell lysis protocol for maximum release of intracellular ATP from my-

TABLE 2. Sensitivities and specificities of the developed ATP bioluminescence assay for determining the MIC of Kathon

| Test strain | RLU cutoff value[a] | MIC (ppm) of Kathon[b] | Sensitivity[c] (%) | Specificity[d] (%) | Accuracy[e] (%) |
|---|---|---|---|---|---|
| *M. chelonae* ATCC 35752 | ≤200 | 10 | 100 | 100 | 100 |
| *M. abscessus* ATCC 19977 | ≤200 | 10 | 100 | 100 | 100 |
| *M. immunogenum* ATCC 700506 | ≤600 | 20 | 91 | 100 | 96 |
| *M. immunogenum* MJY-4 | ≤300 | 30 | 100 | 93 | 96 |
| *M. immunogenum* MJY-13 | ≤40 | 20 | 90 | 100 | 96 |

[a] RLU cutoff for a test species was based on receiver operating characteristic (ROC) curve analysis.
[b] The MIC was measured after 4 days of incubation in the presence of the biocide and was defined as the lowest concentration of biocide causing complete inhibition of growth, as indicated by an absence of any colonies on MB7H10 agar plates (CFU/ml = 0) and an RLU value equivalent to the cutoff limit.
[c] Defined as $TP/(TP + FN)$, where $TP$ = number of samples showing no growth on MB7H10 agar plates and an RLU ≤ cutoff limit, and $FN$ = number of samples showing no growth on MB7H10 agar plates and an RLU > cutoff limit.
[d] Defined as $TN/(TN + FP)$, where $TN$ = number of samples showing growth on MB7H10 agar plates and an RLU > the cutoff limit, and $FP$ = number of samples showing growth on MB7H10 agar plates and an RLU ≤ the cutoff limit.
[e] Defined as $(TP + TN)/N$, where $N$ = the total number of samples.

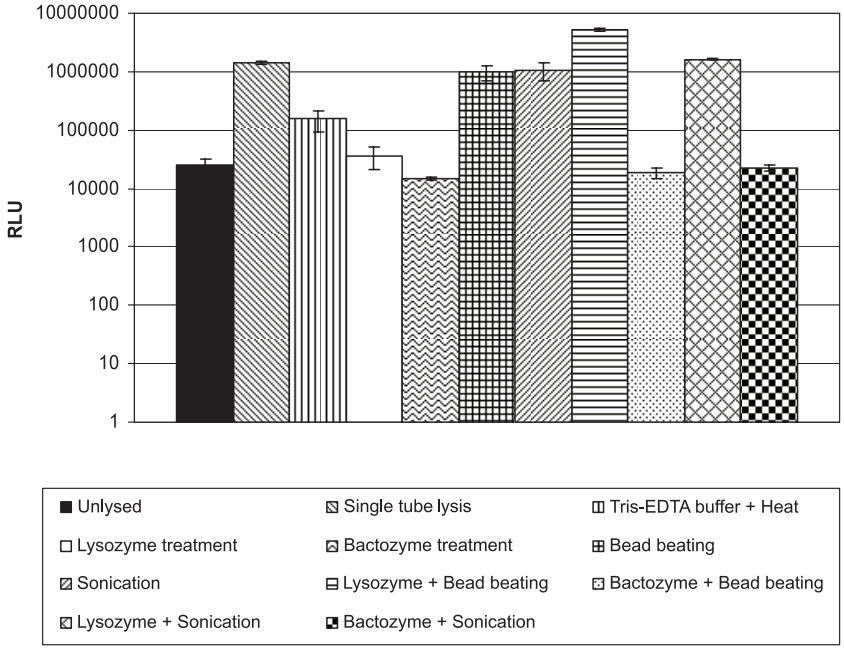


FIG. 1. Comparison of different cell lysis protocols for ATP extraction from *M. immunogenum* ATCC 700506 ($\sim 10^6$ cells). Values presented are means ± standard deviations ($n = 3$). ATP release using lysozyme treatment was significantly improved ($P$ value $< 0.05$) when combined with bead beating.

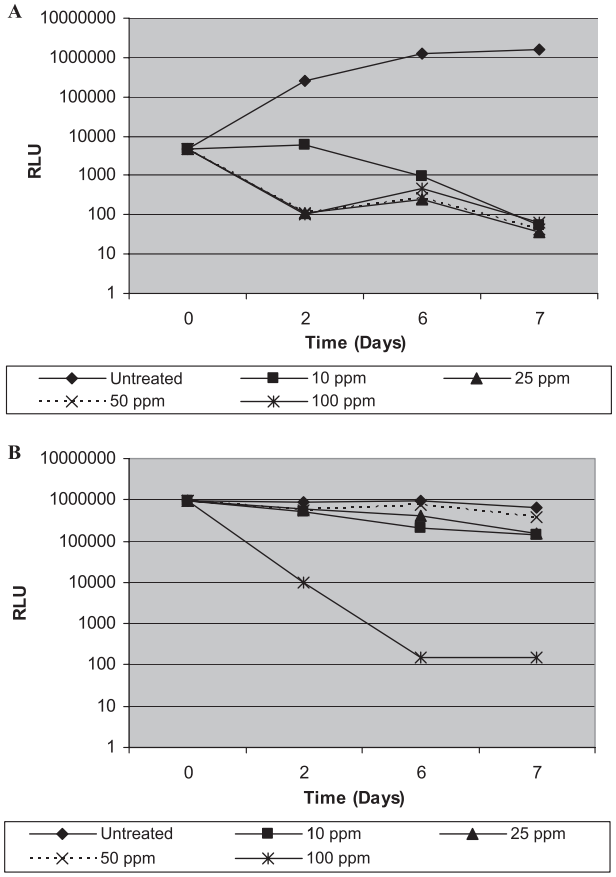


FIG. 2. Optimization of the inoculum size for Kathon susceptibility testing of *M. immunogenum*. (A) Small inoculum ($10^4$ CFU/ml). (B) Large inoculum ($10^6$ CFU/ml). Sauton's medium was used for culturing the test strain (*M. immunogenum* ATCC 700506).

cobacteria. This, along with selection of appropriate growth conditions, yielded an optimized ATP bioluminescence assay for use in biocide susceptibility testing of mycobacteria. The ATP assay is rapid with a high-throughput potential and thus could serve as a preferred method for routine susceptibility testing of mycobacteria as applied to hospital settings and industrial establishments. Future application studies should also evaluate mycobacteria biofilms to assess their effect on biocidal efficacy in the field.

This study was supported by the research grant 2R01OH007364 (to J.S.Y.) from the National Institute of Occupational Safety and Health, Centers for Disease Control and Prevention.

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