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Novel Antibiotic Susceptibility Tests by the ATP-Bioluminescence Method Using Filamentous Cell Treatment

NORIAKI HATTORI,[1]* MOTO-O NAKAJIMA,[1] KOJI O'HARA,[2] AND TETSUO SAWAI[2]

Research and Development Division, Kikkoman Corporation, Chiba 278-0005,[1] and Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Chiba 263-8522,[2] Japan

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Antimicrobial susceptibility testing by the ATP-bioluminescence method has been noted for its speed; it provides susceptibility results within 2 to 5 h. However, several disagreements between the ATP method and standard methodology have been reported. The present paper describes a novel ATP method in a 3.5-h test which overcomes these deficiencies through the elimination of false-resistance discrepancies in tests on gram-negative bacteria with β-lactam agents. In our test model using *Pseudomonas aeruginosa* and piperacillin, it was shown that ATP in filamentous cells accounted for the false resistance. We found that 0.5% 2-amino-2-methyl-1,3-propanediol (AMPD) extracted ATP from the filamentous cells without affecting normal cells and that 0.3 U of adenosine phosphate deaminase (APDase)/ml simultaneously digested the extracted ATP. We used the mixture of these reagents for the pretreatment of cells in a procedure we named filamentous cell treatment, prior to ATP measurements. This novel ATP method with the filamentous cell treatment eliminated false-resistance discrepancies in tests on *P. aeruginosa* with β-lactam agents, including piperacillin, cefoperazone, aztreonam, imipenem-cilastatin, ceftazidime, and cefsulodin. Furthermore, this novel methodology produced results which agreed with those of the standard microdilution method in other tests on gram-negative and gram-positive bacteria, including *P. aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Enterococcus faecalis*, for non-β-lactam agents, such as fosfomycin, ofloxacin, minocycline, and aminoglycosides. MICs obtained by the novel ATP method were also in agreement with those obtained by the agar dilution method of susceptibility testing. From these results, it was shown that the novel ATP method could be used successfully to test the activities of antimicrobial agents with the elimination of the previously reported discrepancies.

Bacteria resistant to multiple antibiotics, such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* spp., have been isolated with increasing frequency, and health care institutions are in need of a rapid antimicrobial susceptibility test for therapeutic, epidemiologic, and economic reasons. Standard susceptibility methods involving liquid media or agar plates, however, require 18 to 24 h of incubation. The ATP-bioluminescence method is an alternative technique that has been adopted in the quest for a methodology which produces rapid results, and it has been widely utilized for sanitation and hygiene monitoring (3, 24, 26). Antimicrobial susceptibility testing by the ATP-bioluminescence method, which requires only 2 to 5 h to perform, was first described in work originating at the U.S. National Aeronautics and Space Administration (6) and in Sweden (8) in 1976. Although this method has been noted for its speed, it is not now widely employed due to a lack of suitable instrumentation, the prohibitive cost of reagents, and disagreement with results obtained with standard methodology. *Pseudomonas aeruginosa* is clinically one of the most important bacteria involved in opportunistic infection and hospital infection, and rapid susceptibility testing allowing the selection of suitable chemotherapy would be a valuable tool. Many other species of bacteria include an increasing number of strains resistant to many kinds of antibiotics. These problems demonstrate the strong need for a rapid and practical means to determine susceptibility to antimicrobial agents. The ATP-bioluminescence method has been applied to the susceptibility testing of gram-negative bacteria, including *P. aeruginosa*; however, several discrepancies were noted when results obtained by the ATP-bioluminescence method were compared to those obtained by standard methodology in tests for some β-lactam agents, including those that are considered primary choices in chemotherapy directed against *P. aeruginosa*. These disagreements, in which strains were found resistant by the ATP susceptibility method but susceptible by the standard method, were labeled false resistance (6, 31). It has been suggested that these disagreements may be the result of the delayed lysis of protoplasts or spheroplasts. The objective of this study was to develop a rapid and simple procedure to eliminate the false-resistance discrepancies noted with gram-negative bacteria, especially *P. aeruginosa*, and β-lactam agents. Moreover, the general applicability of the rapid method was evaluated by comparing it with the standard method in tests on other bacteria and antimicrobial agents.

* Corresponding author. Mailing address: Noriaki Hattori, Research and Development Division, Kikkoman Corporation, 399 Noda, Noda City, Chiba Pref. 278-0005, Japan. Phone: 81-471-23-5522. Fax: 81-471-23-5550. E-mail: 8345@mail.kikkoman.co.jp.

MATERIALS AND METHODS

Bacteria and culture medium. Four reference strains, *P. aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *S. aureus* ATCC 25923, and *Enterococcus faecalis* ATCC 29212, were used. The culture medium was prepared from Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) to which were added 50 mg of Ca^{2+} and 25 mg of Mg^{2+} per liter (20).

Antimicrobial agents. The following β-lactam agents were tested: piperacillin (Sankyo Co., Ltd., Tokyo, Japan), cefoperazone (Pfizer, Inc., New York, N.Y.), aztreonam (Eizai Co., Ltd., Tokyo, Japan), imipenem-cilastatin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), ceftazidime (Tanabe Seiyaku Co., Ltd., Osaka, Japan), cefsulodin (Takeda Chemical Industries, Ltd., Osaka, Japan), cefazolin (Fujisawa Pharmaceutical Co., Ltd., Tokyo, Japan), ampicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), and aspoxicillin (Tanabe Seiyaku Co., Ltd.). The following non-β-lactam antimicrobial agents were tested: fosfomycin (Meiji Seika Kaisha Ltd.), gentamicin (Schering-Plough Corporation, Madison, Wis.), tobramycin (Shionogi & Co., Ltd., Tokyo, Japan), ofloxacin (Daiichi Pharmaceutical

Co., Ltd., Tokyo, Japan), minocycline (Lederle [Japan], Ltd., Tokyo, Japan), erythromycin (Dainabot Co., Ltd., Tokyo, Japan), and chloramphenicol (Sankyo Co., Ltd.).

Growth curve by the ATP-bioluminescence method. Several colonies of bacteria from an overnight blood agar plate (Eiken Chemical Co., Ltd., Tokyo, Japan) culture, incubated at 37°C, were suspended in 1 ml of sterilized saline. The density of the suspension was adjusted to McFarland standard 0.5, corresponding to approximately 10^8 CFU/ml. After a further 10-fold dilution, 50 μl was inoculated into 5 ml of broth with or without 5 μg of piperacillin per ml and was incubated at 37°C. A luciferin–luciferase-based bioluminescence assay of ATP was performed every hour with a Lucifer LU plus kit (Kikkoman Corporation, Chiba, Japan) according to the following protocol. A 100-μl sample from the culture was mixed with an equal volume of the kit's constituent ATP extractant. After 20 s, 100 μl of bioluminescence reagent (luciferin-luciferase) was added to the mixture, and the emitted light was measured with a luminometer, Lumat LB-9501 (EG & G Berthold, Wildbad, Germany). The intensity of the bioluminescenct light was expressed as relative light units (RLU), and it is well established that the light intensity is proportional to the bacterial count (2). Morphological observation was carried out simultaneously by using an aliquot withdrawn at the same time, which was examined under a phase-contrast microscope, model BHS (Olympus Optical Co., Ltd., Tokyo, Japan).

Screening of specific substances for ATP extraction from filamentous cells of *P. aeruginosa*. Thirty-seven substances which have an amphipathic property, including surfactants and emulsifying agents, were screened for their ability to extract ATP from filamentous cells of *P. aeruginosa*. The filamentous cells were obtained by incubating *P. aeruginosa* in broth with 5 μg of piperacillin per ml at 37°C for 3 h. A 50-μl solution containing one of the amphipathic extractants at a concentration of 0.005 to 0.2% (wt/vol) in 25 mM Tricine buffer (pH 7.75) was added to 100 μl of the filamentous cell cultures. The cultures were left to stand for 30 min at room temperature, and 50 μl of bioluminescence reagent was added to the mixture, which was measured for emitted light. In parallel, the same procedure was performed with *P. aeruginosa* cells in broth cultures without piperacillin to obtain reference values. ATP extraction was calculated according to the following equation: percent ATP extraction = (bioluminescence after the addition of the extractant substance/bioluminescence after the addition of the buffer alone) × 100.

Antimicrobial susceptibility test. Testing by the novel ATP-bioluminescence susceptibility method was performed according to the following procedure. A 5-μl inoculum from a culture containing approximately 10^7 CFU of test bacteria/ml was added to each well of a white 96-well microtitration plate (Dynex Technologies, Inc., Chantilly, Va.) containing 100 μl of broth with or without antimicrobial agents. After incubation at 37°C for 3 h, 50 μl of filamentous cell treatment solution, consisting of 0.5% 2-amino-2-methyl-1,3-propanediol (AMPD), 0.3 U of adenosine phosphate deaminase (APDase)/ml, and 5 mM EDTA in 25 mM Tricine buffer (pH 7.75), was added, and the plate was left standing for 30 min at room temperature. Subsequently, 50 μl of ATP extractant, consisting of 0.2% benzalkonium chloride in 25 mM Tricine buffer (pH 7.75), was added. After a further 20 s, 50 μl of bioluminescence reagent reconstituted in 2.5% α-cyclodextrine solution was added, and the emitted light was measured with a 96-well microtitration plate luminometer, ML-3000 (Dynex Technologies, Inc.). APDase included in the filamentous cell treatment was denatured by the benzalkonium chloride added in the subsequent ATP extraction step, thereby protecting the ATP extracted from normal cells. Although benzalkonium chloride would usually denature the luciferase in the bioluminescence reagent added later, α-cyclodextrine neutralizes this effect by forming an inclusion complex (16). The ATP-bioluminescence was expressed as an ATP index; ATP index = (bioluminescence in broth with antimicrobial agent/bioluminescence in broth without antimicrobial agent) × 100. The results were classified as negative (ATP index ≤ 40), or positive (ATP index > 40). The MIC was determined in broth with a twofold-dilution series of antimicrobial agents in the range of 0.1 to 100 μg/ml. The MIC was defined as the lowest concentration of antimicrobial agent which resulted in a negative ATP index in ATP-bioluminescent testing. The values were considered equivalent when they agreed, within 2 twofold-dilution values, with those obtained by the standard methods.

Standard ATP-bioluminescence susceptibility testing was performed in the same way as testing by the novel ATP method described above, except that the filamentous cell treatment was not used.

In the standard microdilution method (20), 5 μl of inoculum from a culture containing approximately 10^7 CFU of the test bacteria/ml was added to each well of a transparent 96-well microtitration plate (Costar, Cambridge, United Kingdom) containing 100 μl of broth with or without antimicrobial agents and was incubated at 37°C for 18 to 20 h. Wells in each antibiotic dilution series, consisting of twofold dilutions of antimicrobial agent in the range of 0.1 to 100 μg/ml, were classified as negative (no growth), or positive (growth). The MIC was recorded as the lowest concentration of antimicrobial agent that inhibited visible growth.

In the standard agar dilution method (10), a bacterial inoculum from a culture containing approximately 10^6 CFU/ml was transferred with a multipoint inoculator to Mueller-Hinton agar plates containing twofold dilutions of antimicrobial agents in the range of 0.1 to 100 μg/ml and was incubated at 37°C for 18 to 20 h. The MIC was recorded as the lowest concentration of antimicrobial agent that inhibited visible growth.

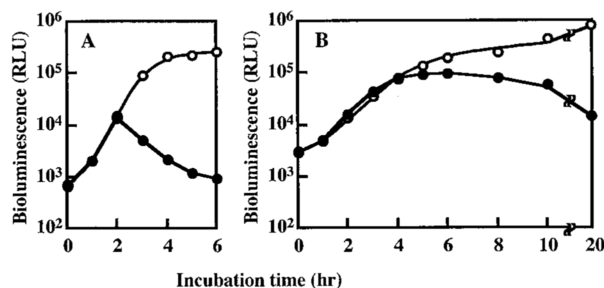

FIG. 1. Growth curves of *E. coli* ATCC 25922 (A) and *P. aeruginosa* ATCC 27853 (B) by the standard ATP-bioluminescence method in broth with (solid circles) or without (open circles) 5 μg of piperacillin per ml, corresponding to 1.5 times the MIC for both organisms.

RESULTS

Investigation of the basis of the false resistance demonstrated by the standard ATP method. Growth curves of *E. coli* and *P. aeruginosa* were determined by measurements made by the ATP-bioluminescence method in broth containing the β-lactam agent piperacillin at 5 μg per ml (Fig. 1). Both microorganisms had the same piperacillin MIC of 3.13 μg/ml, and therefore the concentration of piperacillin used for this study corresponds to about 1.5 times the MIC for both microorganisms. A concentration near the MIC is most suitable for analyzing the mechanism of false-resistance discrepancies in further detail. With *E. coli*, bioluminescence in the medium containing piperacillin paralleled that of the control culture without piperacillin for the first 2 h of incubation; however, it then decreased with further incubation. The bioluminescence of the culture containing piperacillin, following 3 h of incubation, decreased to less than 1/10 of that without piperacillin, with a resulting ATP index of less than 10%. *E. coli* was therefore classified as negative (or susceptible) in a 3-h test. These data agreed with the result expected from the MIC. In contrast, with *P. aeruginosa*, bioluminescence in the medium containing piperacillin remained at the same level as in the drug-free culture for 4 h and subsequently decreased little, even after several hours of further incubation. This result indicated false resistance when compared with that expected from the MIC. Following 20 h of incubation, however, which is the incubation time required for the standard methodology, the bioluminescence of the culture containing piperacillin decreased to about 1/100 of that without piperacillin. The resultant ATP index was about 1%, and accordingly, the susceptibility result by the ATP-bioluminescence method, following the extended incubation time, now correlated with that expected from the known MIC.

When we microscopically observed cultures of *P. aeruginosa* exposed to piperacillin for 3 h, we discovered filamentous cells which were approximately 30 times longer than normal cells. The cells continued to elongate with extended incubation. In further studies it was also confirmed that other β-lactam agents, such as cefoperazone, aztreonam, imipenem-cilastatin, ceftazidime, cefsulodin, cefazolin, carbenicillin, latamoxef, cefotaxime, flomoxef, sulbactam-cefoperazone, and cefuzonam, induced similar filamentation of cells. These results, taken together, suggested that the cells of *P. aeruginosa* remained viable for several hours following their conversion to the filamentous form. We therefore concluded that false-resistance discrepancies in rapid tests on *P. aeruginosa* with β-lactam agents were due to the delayed lysis of the filamentous cells formed in these cultures.

TABLE 1. Specific ATP extraction from filamentous cells of *P. aeruginosa* ATCC 27853

Extractant substance	ATP extraction (%)[a]	
	Without piperacillin	With piperacillin[b]
Triton X-100	122	209
Amphitol	91	223
AMPD	117	457
Buffer[c]	100	100

[a] Calculated as (bioluminescence after the addition of the extractant substance/bioluminescence after the addition of the buffer alone) × 100.
[b] Five micrograms of piperacillin per milliliter, corresponding to 1.5 times the MIC for *P. aeruginosa*, was used to obtain filamentous cells.
[c] A 25 mM Tricine buffer (pH 7.75) was used.

Elimination of ATP in filamentous cells formed by β-lactam agents. We considered that the false-resistance discrepancies between the ATP-bioluminescence method and the standard microdilution method could be resolved by extracting and eliminating ATP from the filamentous cells. We examined this possibility using filamentous cells of *P. aeruginosa* which resulted from exposure to 5 μg of piperacillin/ml for 3 h. We began with an investigation of whether surfactants and emulsifying agents, which destroyed cell membrane integrity, could extract ATP from filamentous cells. From the data obtained, three substances with the desired property were selected; these are shown in Table 1. ATP extraction above 100% means that the substance extracted ATP from cells in an amount exceeding that extracted by buffer alone. In a control culture without piperacillin, ATP extraction by either Triton X-100, Amphitol, or AMPD was almost at the same level as that obtained by the use of buffer alone. In contrast, in a culture containing piperacillin, the levels of ATP extraction by these substances were about 2 to 5 times higher than that by the buffer alone. From these results, it was shown that these substances extracted ATP selectively from filamentous cells and not from morphologically normal cells. It was found that AMPD combined this selectivity with the greatest effectiveness, and it was therefore used in subsequent experiments.

The optimal concentration of AMPD and the time required to extract ATP from filamentous cells were next investigated. In Fig. 2A, the results summarized show that increasing the concentration of AMPD past 0.5% did not enhance the bioluminescence over the peak levels reached at this concentration. The final selection of a concentration of 0.5% was further supported by the increasing inhibition of luciferase activity with an increased concentration of AMPD (data not shown). In Fig. 2B, it can be seen that ATP was efficiently extracted from the filamentous cells following 20 to 30 min of exposure. It was therefore concluded that treatment for 30 min using 0.5% AMPD was well suited for extraction of ATP from the filamentous cells. APDase has previously been reported as a most effective agent for the elimination of ATP (25). We confirmed that 0.3 U of APDase/ml could remove the ATP extracted from filamentous cells by AMPD (data not shown). The combination of reagents and procedures outlined above provided us with a simple and rapid pretreatment, which we named the filamentous cell treatment, that might be used to provide valid results from ATP-bioluminescence susceptibility testing.

A comparison of ATP-bioluminescence results with results of the standard microdilution method. The speed advantage of the novel 3.5-h ATP-bioluminescence susceptibility method was of value only if the accuracy of the modified testing could be validated. In tests on *P. aeruginosa* using seven different β-lactam agents, it was investigated whether the novel ATP-bioluminescence method would eliminate false-resistance discrepancies compared with the standard method. The susceptibility results were compared to those obtained by the standard ATP-bioluminescence method and those obtained by the microdilution method, as presented in Table 2. The standard ATP-bioluminescence susceptibility method gave positive results, indicating resistance, at all the concentrations of the agents tested, whereas the microdilution method indicated complete susceptibility at all concentrations of the same agents except for cefazolin (resistance at all concentrations) and 3 μg of aztreonam/ml. These results showed that the standard ATP-bioluminescence susceptibility method caused many false-resistance discrepancies compared with the microdilution method in almost all tests. It was noted that the bioluminescence from cultures including antimicrobial agents was greater than that of those without, and especially with piperacillin and cefsulodin, the dose-dependent effect was not observed. With the novel ATP-bioluminescence method, in which ATP from filamentous cells was digested, bioluminescence reflected only the ATP from normal cells and the dose-dependent effect was observed. The results obtained by this novel method were in perfect agreement with those obtained by the microdilution method.

The correlation between the three methods was further investigated in tests on *P. aeruginosa* with non-β-lactam agents and on three bacteria other than *P. aeruginosa* with piperacillin, aztreonam, ampicillin, imipenem-cilastatin, minocycline, and ofloxacin (Table 3). In tests on *P. aeruginosa* using fosfomycin, false-resistance results were noted by the standard ATP-bioluminescence susceptibility method, but this false resistance was eliminated in the novel ATP-bioluminescence susceptibility method. This result showed that the novel method also has a beneficial effect in tests on fosfomycin, a non-β-lactam agent which inhibits cell wall synthesis as well as β-lactams do (7, 11, 30). With *E. coli*, the standard ATP-bioluminescence method indicated false resistance with positive ATP indices at 3 and 10 μg of aztreonam/ml; however, the novel ATP-bioluminescence method could resolve these. In contrast, on the gram-positive bacteria, *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212, the results of the standard ATP method were in agreement with those of the standard microdilution method. The results of the novel ATP method also correlated well with those of the standard method.

MICs for *P. aeruginosa* determined by the novel ATP-bioluminescence susceptibility method were compared with those obtained by standard procedures, including the microdilution and agar dilution methods (Table 4). The MICs obtained by the novel ATP-bioluminescence method were in agreement with those obtained by the standard methods within 2 twofold-

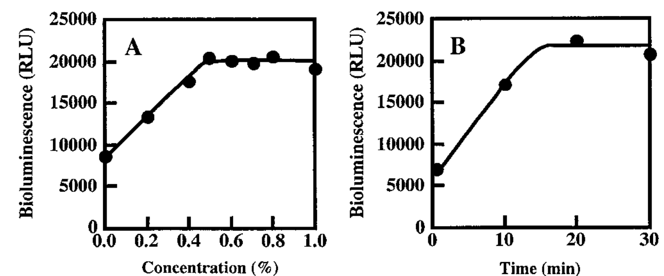

FIG. 2. Determination of optimal conditions for the extraction of ATP from filamentous cells using AMPD. The optimal concentration of AMPD (A) and the time required for extraction of ATP by 0.5% of AMPD (B) are shown.

TABLE 2. Antimicrobial susceptibility tests of *P. aeruginosa* ATCC 27853 and β-lactam agents by the novel ATP-bioluminescence method, the standard ATP-bioluminescence method, and the microdilution method

Antimicrobial agent (MIC [μg/ml][a])	Concn (μg/ml)	Novel ATP method			Standard ATP method			Result with microdilution method[d]
		Bioluminescence (RLU)	ATP index[b]	Result[c]	Bioluminescence (RLU)	ATP index[b]	Result[c]	
Piperacillin (3.13)	5	63	13.3	−	310	76.0	+	−
	10	81	17.2	−	516	126.5	+	−
	30	40	8.5	−	208	51.0	+	−
Cefoperazone (6.25)	2	128	27.1	−	471	115.4	+	−
	10	58	12.3	−	443	108.6	+	−
	25	45	9.5	−	489	119.9	+	−
Aztreonam (6.25)	3	241	51.1	+	495	121.3	+	+
	10	57	12.1	−	517	126.7	+	−
	50	52	11.0	−	560	137.3	+	−
Imipenem-cilastatin (1.56)	3	35	7.4	−	408	100.0	+	−
	10	27	5.7	−	280	68.6	+	−
	50	25	5.3	−	289	70.8	+	−
Ceftazidime (1.56)	3	54	11.4	−	578	141.7	+	−
	10	66	14.0	−	568	139.2	+	−
	100	21	4.4	−	566	138.7	+	−
Cefsulodin (1.56)	5	80	16.9	−	274	67.2	+	−
	15	91	19.3	−	317	77.7	+	−
	40	75	15.9	−	402	98.5	+	−
Cefazolin (≥100)	3	358	75.8	+	226	55.4	+	+
	15	411	87.1	+	220	53.9	+	+
	60	391	82.8	+	241	59.1	+	+
Control[e]	0	472	100.0	+	408	100.0	+	+

[a] Determined by the standard agar dilution method.
[b] Calculated as (bioluminescence in broth with antimicrobial agent/bioluminescence in broth without antimicrobial agent) × 100.
[c] −, ATP index ≤ 40; +, ATP index > 40.
[d] −, no growth; +, growth.
[e] Results for drug-free conditions are shown.

dilutional increments, with the exception of the MIC for minocycline determined by the standard microdilution method. These results validated the accuracy of the novel ATP-bioluminescence susceptibility testing method, which, in a 3.5-h test, could be reliably used to determine MICs in agreement with those obtained by standard microdilution testing requiring overnight incubation.

DISCUSSION

Several approaches have been investigated in the past for the performance of rapid antibiotic susceptibility testing, including ATP-bioluminescence (6, 8, 9, 15, 18, 19, 22, 29, 31, 32), turbidimetry (1), impedance (4), conductance (23), and radiometry (5). The ATP-bioluminescence method was notable, because several difficulties related to reagents and instrumentation had been overcome (12, 13, 27, 28, 32). In this study, we investigated a novel method to eliminate the remaining technical problem of disagreements between the ATP-bioluminescence susceptibility method and standard methodology.

We noted that the disagreements involved false-resistance discrepancies in tests on gram-negative bacteria with β-lactam agents. Several approaches to eliminating false-resistance discrepancies had already been investigated. Wheat et al. used low-osmolality broth to induce the lysis of *Proteus mirabilis* spheroplasts. A much better correlation was achieved with ampicillin and piperacillin (31). Limb et al. extended the time of incubation to 6 h for *Mycoplasma* spp. tested with ciprofloxacin, and good correlation resulted (15). These methods, however, are unsuited to the development of a simple and rapid susceptibility test. In our investigations we began by determining the precise cause of the false-resistance discrepancies. We noted that *P. aeruginosa* cells remained viable following conversion to their filamentous form following 3 h of incubation in the presence of antibiotics, and elongation increased over time. It was clear that ATP in the filamentous cells could account for the false-resistance discrepancies. We subsequently investigated a method to eliminate this ATP from filamentous cells and derived our unique filamentous cell treatment. The treatment reagents consisted of 0.5% AMPD, 0.3 U of APDase/ml, and 5 mM EDTA in 25 mM Tricine buffer (pH 7.75). This combination digested ATP from filamentous cells only without affecting normal cells. In antimicrobial susceptibility testing by the novel ATP-bioluminescence method on *P. aeruginosa* for β-lactam agents, the filamentous cell treatment eliminated false-resistance discrepancies. The results agreed with those obtained by the standard microdilution method, not only in tests on gram-negative and gram-positive bacteria, but also for non-β-lactam agents. These results strongly suggested that the novel ATP-bioluminescence method was a practical test for a variety of bacteria and antimicrobial agents and that it maintained simplicity and speed.

Recently, the ATP-bioluminescence method has been applied to antibiotic susceptibility tests on *Mycobacterium* spp. (2,

TABLE 3. Antimicrobial susceptibility tests by the novel ATP-bioluminescence method, the standard ATP-bioluminescence method, and the microdilution method

Micro-organism	Antimicrobial agent (MIC [μg/ml[a]])	Concn (μg/ml)	Result		
			ATP method[b]		Micro-dilution method[c]
			Novel	Standard	
P. aeruginosa ATCC 27853	Fosfomycin (6.25)	10	−	+	−
		50	−	+	−
		200	−	+	−
	Gentamicin (3.13)	5	−	−	−
		10	−	−	−
		30	−	−	−
	Tobramycin (1.56)	2	−	−	−
		10	−	−	−
		50	−	−	−
	Ofloxacin (6.25)	1	+	+	+
		3	+	+	+
		10	−	−	−
E. coli ATCC 25922	Piperacillin (3.13)	5	−	−	−
		10	−	−	−
		30	−	−	−
	Aztreonam (0.20)	3	−	+	−
		10	−	+	−
		50	−	−	−
	Imipenem-cilastatin (0.78)	3	−	−	−
		10	−	−	−
		50	−	−	−
	Minocycline (0.78)	2	−	−	−
		10	−	−	−
		50	−	−	−
	Ofloxacin (0.05)	1	−	−	−
		3	−	−	−
		10	−	−	−
S. aureus ATCC 25923	Piperacillin (1.56)	5	−	−	−
		10	−	−	−
		30	−	−	−
	Ampicillin (0.39)	2	−	−	−
		5	−	−	−
		20	−	−	−
	Imipenem-cilastatin (0.10)	3	−	−	−
		10	−	−	−
		50	−	−	−
	Minocycline (0.39)	2	−	−	−
		10	−	−	−
		50	−	−	−
	Ofloxacin (0.78)	1	−	−	−
		3	−	−	−
		10	−	−	−
E. faecalis ATCC 29212	Piperacillin (6.25)	5	−	−	−
		10	−	−	−
		30	−	−	−
	Ampicillin (3.13)	2	−	−	−
		5	−	−	−
		20	−	−	−
	Imipenem-cilastatin (1.56)	3	−	−	−
		10	−	−	−
		50	−	−	−
	Minocycline (3.13)	2	−	−	−
		10	−	−	−
		50	−	−	−
	Ofloxacin (6.25)	1	+	+	+
		3	−	−	−
		10	−	−	−

[a] Determined by the standard agar dilution method.
[b] Calculated as (bioluminescence in broth with antimicrobial agent/bioluminescence in broth without antimicrobial agent) $\times$ 100.
[c] −, ATP index $\leq$ 40; +, ATP index $>$ 40.

TABLE 4. MICs for *P. aeruginosa* ATCC 27853 as determined by the novel ATP-bioluminescence method, the microdilution method, and the agar dilution method

Antimicrobial agent	MIC (μg/ml) determined by the following method:		
	Novel ATP	Microdilution	Agar dilution
Aspoxicillin	25	100	50
Piperacillin	1.56	3.13	3.13
Imipenem-cilastatin	0.78	1.56	1.56
Cefazolin	≥100	≥100	≥100
Minocycline	6.25	50	12.5
Erythromycin	100	≥100	≥100
Chloramphenicol	50	≥100	100

21) and on microorganisms in biofilms (14), and for assessing the postantibiotic effect (17). In tests on gram-negative bacteria, poor correlation between the ATP-bioluminescence susceptibility method and standard methods was obtained because of morphological changes such as the production of filamentous cells and spheroplasts. It is expected that the novel ATP-bioluminescence susceptibility method described here would eliminate these disagreements due to the filamentous cell treatment.

The speed of this method further addresses a clinical need for early information regarding susceptibility tests and would allow feedback of the susceptibility data to a physician in one day, resulting in lower health care costs and the selection of better treatment regimens for patients. The novel ATP-bioluminescence method described in this paper is simple to perform because this method, distinct from those previously described, does not require the use of centrifugation and filtration to concentrate bacterial cells. A further advantage of the novel method is its adaptability to fully automated and cost-efficient testing, which results from the use of 96-well microtitration plates in the performance of the test. Such automation has the potential to provide susceptibility results devoid of variation arising from the performance of individual technicians. As described above, this novel ATP-bioluminescence method appears to be a technique ideally suited to the clinical-microbiology laboratory. Our ongoing studies will continue to evaluate the reliability and practicality of the novel method in expanded tests involving more species of organisms and more antimicrobial agents.

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REFERENCES

1. **Bascomb, S., A. A. Glynn, H. Gaya, R. C. Spencer, and P. J. Shine.** 1982. Rapid determination of bacterial susceptibility to antimicrobial agents by a semi-automated continuous flow method. J. Antimicrob. Chemother. **9:**343–355.
2. **Beckers, B., H. R. M. Lang, D. Schimke, and A. Lammers.** 1985. Evaluation of bioluminescence assay for rapid antimicrobial susceptibility testing of *Mycobacteria*. Eur. J. Clin. Microbiol. **4:**556–561.
3. **Bell, C., P. A. Stallard, S. E. Brown, and J. T. E. Standley.** 1994. ATP-bioluminescence techniques for assessing the hygienic condition of milk transport tankers. Int. Dairy J. **4:**629–640.
4. **Colvin, H. J., and J. C. Sherris.** 1977. Electrical impedance measurements in the reading and monitoring of broth dilution susceptibility tests. Antimicrob. Agents Chemother. **12:**61–66.
5. **Deblanc, H. J., P. Charache, and H. N. Wagner.** 1972. Automated radiometric measurement of antibiotic effect on bacterial growth. Antimicrob. Agents Chemother. **2:**360–366.

6. **Gutekunst, R. R.** 1976. Rapid procedure under development and evaluation: bioluminescence and impedance measurement, p. 85–100. *In* A. Bond, J. T. Bartola, and J. E. Prier (ed.), The clinical laboratory as an aid in chemotherapy of infectious disease. Williams and Wilkins, Baltimore, Md.
7. **Hendlin, D., E. O. Stapley, M. Jackson, H. Wallich, A. K. Miller, F. J. Wolf, T. W. Miller, L. Chaiet, F. M. Kahan, E. L. Foltz, H. P. Woodruff, J. M. Mata, S. Hernandez, and S. Mochales.** 1969. Phosphonomycin, a new antibiotic produced by strains of *Streptomyces*. Science **166:**122–123.
8. **Hojer, H., L. Nilsson, S. Ansehn, and A. Thore.** 1976. In-vitro effect of doxycycline on levels of adenosine triphosphate in bacterial cultures. Scand. J. Infect. Dis. **9**(Suppl.)**:**58–61.
9. **Hojer, H., L. Nilsson, S. Ansehn, and A. Thore.** 1979. Possible application of luciferase assay of ATP to antibiotic susceptibility testing, p. 523–530. *In* Proceedings of the International Symposium on Analytical Application of Bioluminescence and Chemiluminescence.
10. **Japan Society of Chemotherapy.** 1981. Method of MIC determination. Chemotherapy (Tokyo) **29:**76–79.
11. **Kahan, F. M., J. S. Kahan, P. J. Cassidy, and H. Kropp.** 1974. The mechanism of action of fosfomycin (phosphonomycin). Ann. N. Y. Acad. Sci. **235:** 364–386.
12. **Kajiyama, N., and E. Nakano.** 1993. Thermostabilization of firefly luciferase by a single amino acid substitution at position 217. Biochemistry **32:**13795–13799.
13. **Kajiyama, N., and E. Nakano.** 1994. Enhancement of thermostability of firefly luciferase from *Luciola lateralis* by a single amino acid substitution. Biosci. Biotechnol. Biochem. **58:**1170–1171. (Note.)
14. **Kumon, H., N. Ono, M. Iida, and J. C. Nickel.** 1995. Combination effect of fosfomycin and ofloxacin against *Pseudomonas aeruginosa* growing in a biofilm. Antimicrob. Agents Chemother. **39:**1038–1044.
15. **Limb, D. I., P. F. Wheat, J. G. M. Hastings, and R. C. Spencer.** 1991. Antimicrobial susceptibility testing of mycoplasmas by ATP bioluminescence. J. Med. Microbiol. **35:**89–92.
16. **Lundin, A., J. Anson, and P. Kau.** 1994. ATP extractants neutralised by cyclodextrins, p. 399–402. *In* Proceedings of the 8th International Symposium on Bioluminescence and Chemiluminescence.
17. **Mackenzie, F. M., I. M. Gould, D. G. Chapman, and D. Jason.** 1994. Postantibiotic effect of meropenem on members of the family *Enterobacteriaceae* determined by five methods. Antimicrob. Agents Chemother. **38:**2583–2589.
18. **McWalter, P. W.** 1984. Determination of susceptibility of *Staphylococcus aureus* to methicillin by luciferin-luciferase assay of bacterial adenosine triphosphate. J. Appl. Bacteriol. **56:**145–150.
19. **McWalter, P. W.** 1984. Rapid susceptibility testing of *Staphylococcus aureus*, p. 17–20. *In* L. J. Kricka, P. E. Stanley, G. H. G. Thorpe, and T. P. Whitehead (ed.), Analytical application of bioluminescence and chemiluminescence. Academic Press, Inc., London, United Kingdom.
20. **National Committee for Clinical Laboratory Standards.** 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 2nd ed. Approved standard M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
21. **Nilsson, L. E., S. E. Hoffner, and S. Ansehn.** 1988. Rapid susceptibility testing of *Mycobacterium tuberculosis* by bioluminescence assay of mycobacterial ATP. Antimicrob. Agents Chemother. **32:**1208–1212.
22. **Park, C. H., D. L. Hixon, C. M. McLaughlin, and J. F. Cook.** 1988. Rapid detection (4 h) of methicillin-resistant *Staphylococcus aureus* by a bioluminescence method. J. Clin. Microbiol. **26:**1223–1224.
23. **Porter, I. A., T. M. S. Reid, W. J. Wood, D. M. Gibson, and G. Hobbs.** 1983. Conductance measurements for determining antibiotic sensitivity, p. 49–60. *In* A. D. Russell and L. B. Quesnel (ed.), Antibiotics: assessment of antimicrobial activity and resistance. Academic Press, London, United Kingdom.
24. **Poulis, J. A., M. de Pijper, D. A. A. Mossel, and P. P. A. Dekkers.** 1993. Assessment of cleaning and disinfection in the food industry with the rapid ATP-bioluminescence technique combined with the tissue fluid contamination test and a conventional microbiological method. Int. J. Food Microbiol. **20:**109–116.
25. **Sakakibara, T., S. Murakami, N. Hattori, M. Nakajima, and K. Imai.** 1997. Enzymatic treatment to eliminate the extracellular ATP for improving the detectability of bacterial intracellular ATP. Anal. Biochem. **250:**157–161.
26. **Siragusa, G. R., C. N. Cutter, W. J. Dorsa, and M. Koohmaraie.** 1995. Use of a rapid microbial ATP bioluminescence assay to detect contamination on beef and pork carcasses. J. Food Prot. **58:**770–775.
27. **Tatsumi, H., N. Kajiyama, and E. Nakano.** 1992. Molecular cloning and expression in *Escherichia coli* of a cDNA clone encoding luciferase of a firefly, *Luciola lateralis*. Biochim. Biophys. Acta **1131:**161–165.
28. **Tatsumi, H., T. Masuda, N. Kajiyama, and E. Nakano.** 1989. Luciferase cDNA from Japanese firefly, *Luciola cruciata*: cloning, structure and expression in *Escherichia coli*. J. Biolumin. Chemilumin. **3:**75–78.
29. **Thore, A., L. Nilsson, H. Hojer, S. Ansehn, and L. Brote.** 1977. Effect of ampicillin on intracellular levels of adenosine triphosphate in bacterial cultures related to antibiotic susceptibility. Acta Pathol. Microbiol. Scand. Sect. B. **85:**161–166.
30. **Waxman, D. J., and J. L. Strominger.** 1982. β-Lactam antibiotics: biochemical modes of action, p. 209. *In* R. B. Morin and M. Gorman (ed.), Chemistry and biology of β-lactam antibiotics. Academic Press, New York, N.Y.
31. **Wheat, P. F., J. G. M. Hastings, and R. C. Spencer.** 1988. Rapid antibiotic susceptibility tests on Enterobacteriaceae by ATP bioluminescence. J. Med. Microbiol. **25:**95–99.
32. **Wheat, P. F., R. C. Spencer, and J. G. M. Hastings.** 1989. A novel luminometer for rapid antimicrobial susceptibility tests on gram-positive cocci by ATP bioluminescence. J. Med. Microbiol. **29:**277–282.