A novel luminometer for rapid antimicrobial susceptibility tests on gram-positive cocci by ATP bioluminescence

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Summary. The susceptibility of 130 clinical isolates of gram-positive cocci to a wide range of antimicrobial agents was assessed by ATP bioluminescence in a 4-h test. ATP assays were performed on a novel luminometer, the Amerlite Analyser, which measures luminescence from microtitration trays. For most organisms tested, there was good correlation (>90%) with conventional MIC values estimated on 18-h cultures. However, a problem was found with detection of penicillin resistance in *Staphylococcus aureus* by the ATP method, 13% of strains showing major disagreement. Methicillin resistance of *S. aureus* was shown reliably for most strains (94%) by ATP assay, provided they were incubated at 30°C. The Amerlite Analyser offers the potential for the development of a semi-automated antimicrobial susceptibility test, with a significant reduction in reagent costs when compared with previously described bioluminescence protocols.

Introduction

The firefly (luciferin-luciferase) assay of adenosine triphosphate (ATP) as a rapid method for determining antimicrobial susceptibility was first described from the USA National Aeronautics and Space Administration (Gutekunst, 1976) and from Sweden (Hojer *et al.*, 1976). Commercial development has been slow, because of lack of suitable instruments and protocols and the prohibitive cost of reagents (Hastings, 1987). We described a protocol for testing Enterobacteriaceae by ATP bioluminescence (Hastings *et al.*, 1987; Wheat *et al.*, 1988). In the present study a novel luminometer (Amerlite Analyser; Amersham International, Little Chalfont, Buckinghamshire HP7 9NA), has been used for clinical isolates of gram-positive cocci.

Materials and methods

Bacteria

We examined 130 clinical isolates of gram-positive cocci, of which 67 were *Staphylococcus aureus*, 20 were coagulase-negative staphylococci (CNS), and 43 were

enterococci. Of the *S. aureus* isolates, 36 (kindly supplied by Dr R. George, Central Public Health Laboratory, Colindale, London) were methicillin resistant (MRSA). The CNS isolates were mainly *S. epidermidis* (8), *S. haemolyticus* (5) or *S. hominis* (5), according to identification by API Staph (API Laboratory Products, Grafton Way, Basingstoke, Hampshire). The enterococci were identified as either *Enterococcus faecalis* (32) or *E. faecium* (11) by API 20 Strep.

Antimicrobial agents

These were obtained as pure powders of stated potency and stored at 4°C: fusidic acid (Leo), gentamicin (Rousell), methicillin (Beecham), penicillin (Glaxo), teicoplanin (Merrell-Dow), vancomycin (Eli-Lilly), ampicillin (Beecham).

Determination of minimum inhibitory concentrations (MICs)

Standard solutions of each antimicrobial agent were prepared in sterile distilled water for use on the same day. The MIC (lowest concentration that inhibited visible growth) was determined on Isosensitest Agar (Oxoid) containing two-fold dilutions of antimicrobial agent. Bacterial inocula of 10^7 – 10^8 cfu/ml were transferred to the agar plates with a multipoint inoculator (Denley Instruments) and incubated for 18 h at 37°C, and also at 30°C for methicillin.

Susceptibility testing by ATP bioluminescence

Inoculum. Three colonies of the test organism from an overnight culture on Columbia blood agar were inoculated into 1 ml of Hartley's Digest Broth (Oxoid) containing glucose 0.1%, and incubated at 37°C in a reciprocating water-bath. After 1 h, the density of cultures was adjusted to McFarland standard 2, and 0.1 ml was inoculated into 2 ml of Isosensitest Broth (Oxoid) with or without antimicrobial agent. This represented an initial inoculum of 10^6 – 10^7 cfu/ml. The cultures were incubated for a further 3 h at 37°C. With methicillin, cultures were incubated also at 30°C. Final concentrations (break-points) were ampicillin 2 mg/L, fusidic acid 0.5 mg/L, gentamicin 2 mg/L, methicillin 5 mg/L, penicillin 0.125 mg/L, teicoplanin 10 mg/L and vancomycin 10 mg/L (Pease *et al.*, 1988).

Assay of bacterial ATP. A 200- μ l sample of the culture was mixed with an equal volume of extraction reagent consisting of trichloroacetic acid 2.5% and 4 mM ethylene diamine tetra-acetic acid (EDTA); after 2 min, 20 μ l of this extract was added to 200 μ l of buffer (Tris-EDTA) and 20 μ l of ATP Monitoring Reagent (luciferase-luciferin; LKB Wallac) in a well of a microtitration tray. Light emission was measured in the Amerlite analyser. ATP-bioluminescence was expressed as a percentage:

$$\frac{\text{luminescence from broth with antimicrobial agent}}{\text{luminescence from control broth}} \times 100.$$

From our previous experience of this assay, we categorised the results empirically as sensitive (<41%), intermediate (41–49%) or resistant (>49%).

Analysis of ATP and MIC susceptibility results

ATP assays were compared with MIC values by error boxes (fig. 1), based on the method of Metzler and DeHaan (1974). Strains in boxes A and B were considered as showing agreement, strains in boxes C and D as showing major disagreement, and strains with 41–49% bioluminescence as showing minor disagreement. Strains whose MIC did not differ by more than one dilution from the chosen breakpoint concentration in the ATP method were classified as showing agreement; however, these were infrequent except with methicillin at 37°C (fig. 2).

Results

Staphylococci

The correlation between MIC and ATP-bioluminescence is summarised in table I. The greatest problem was with penicillin: of 71 strains with MICs >0.5 mg/L, 9 (13%) were sensitive by the ATP assay and, of these, 8 were methicillin-sensitive isolates of *S. aureus*. Repetition of the

Fig. 1. Comparison of ATP-bioluminescence results and MIC values by error-box analyses. Boxes A and B indicate agreement, boxes C and D major disagreement.

Fig. 2. Error-box analyses comparing 18-h MIC values with ATP-bioluminescence results after incubation for 3 h in the presence of methicillin at a 30°C, b 37°C: ● methicillin-resistant *S. aureus*; ○ methicillin-sensitive *S. aureus*; △ coagulase-negative staphylococci.

ATP assay confirmed consistent failure to detect about 10–15% of penicillin-resistant staphylococci.

With gentamicin and fusidic acid, major disagreements were 5% and 10% respectively. However, these figures represent only single strains of CNS which were resistant by MIC; on initial testing by the ATP method they were sensitive, but on repetition they were resistant, and the original

results were presumed to have been caused by manipulative errors.

Staphylococci were tested against methicillin at 30°C and 37°C by both methods (fig. 2). Predictably, MICs at 37°C showed wide variation with the 36 reference strains of MRSA which were said to have MICs >40 mg/L; more than half of these showed MICs ≤ 10 mg/L. In contrast, at 30°C, 34 of the 36

strains showed MICs ≥ 40 mg/L. Bioluminescence results showed a similar pattern; at 37°C, 25% of MRSA strains were sensitive, compared with only three strains at 30°C, and one of these consistently had an MIC of 10 mg/L in our hands. All methicillin-sensitive strains had low MICs and were sensitive by the bioluminescence assay at both temperatures. The CNS strains gave satisfactory results at 37°C, but were not tested at 30°C because of poor growth.

Enterococci

Agreement between MIC and ATP results was good with the enterococci (table II). However, all 43 strains were sensitive to vancomycin and teicoplanin, with MIC₉₀ values of 5 mg/L and 0.6 mg/L respectively.

Discussion

Antibiotic susceptibility results by 4-h ATP-bioluminescence assay correlated well with overnight MIC values for staphylococci tested with fusidic acid, gentamicin, vancomycin and teicoplanin, and for enterococci with ampicillin, teicoplanin and vancomycin.

However, there was a significant number of false-sensitive results by the ATP method for *S. aureus* strains with penicillin, and these false results were reproducible. A similar problem with a rapid test was encountered by Schoenknicht *et al.* (1980) in the Autobac system. They suggested that the discrepancies may result from staphylococci pro-

ducing only low levels of β -lactamase, the organisms being destroyed before sufficient enzyme was available. Rapid estimation of β -lactamase production might avoid this problem (Sykes, 1978).

Tests for susceptibility of staphylococci to methicillin also presented problems. At 37°C, MICs for the MRSA strains varied widely, as did the results with ATP assay; and correlation between the two methods was poor. It is now recognised that the results are influenced by a resistant sub-population which grows poorly at 37°C (McDougall and Thornsberry, 1984). The detection of MRSA is therefore a problem in rapid methods which rely on early detection of bacterial growth; and this was not resolved by the use of sodium chloride 5% or by incubation at 30–32°C (Boyce *et al.*, 1982; Putland and Guinness, 1985). Cleary and Maurer (1978) suggested that isolates which were multi-resistant by the Autobac system should be retested by an accepted procedure. However, with ATP-bioluminescence, we have discriminated between methicillin-sensitive and -resistant strains of *S. aureus* after 3 h at 30°C, and we found only 6% major disagreement with MIC results (table I). CNS strains grew poorly at 30°C but methicillin resistance of these organisms was detected reliably at 37°C (fig. 2b).

Several strains which were sensitive, intermediate or resistant by MIC and ATP methods were retested twice (data not shown); the ATP assays were as reproducible as the MICs.

Rapid tests on *S. aureus* by bioluminescence methods have been described previously. McWalter (1984), with a method similar to ours, tested 100 isolates with methicillin (at 30°C), gentamicin,

Table I. Summary of error-box analyses for the comparison of MIC and ATP-bioluminescence assays of the antibiotic sensitivities of 87 strains of *Staphylococcus*

Antimicrobial agent	ATP breakpoint (mg/L)	Number of strains resistant by MIC assay	Percentage of strains showing					
			agreement		minor disagreement		major disagreement	
			S	R	S	R	S	R
Fusidic Acid	0.5	10	97	90	3	0	0	10
Methicillin 30°C*	5	36	97	94	3	0	0	6
Methicillin 37°C†	5	5	100	100	0	0	0	0
Gentamicin	2	20	97	95	3	0	0	5
Penicillin	0.125	71	100	87	0	0	0	13
Vancomycin	10	0	100	—	0	—	0	—
Teicoplanin	10	0	100	—	0	—	0	—

S = sensitive, R = resistant, by MIC assay.

* 67 strains of *S. aureus* (31 methicillin-sensitive, 36 -resistant, by MIC assay).

† 20 strains of coagulase-negative staphylococci.

Table II. Summary of error-box analyses for the comparison of MIC and ATP-bioluminescence assays of the antibiotic sensitivities of 43 strains of *Enterococcus*

Antimicrobial agent	ATP breakpoint (mg/L)	Number of strains resistant by MIC assay	Percentage of strains showing					
			agreement		minor disagreement		major disagreement	
			S	R	S	R	S	R
Ampicillin	2	11	100	100	0	—	0	—
Vancomycin	10	0	100	—	0	—	0	—
Teicoplanin	10	0	100	—	0	—	0	—

S = sensitive, R = resistant, by MIC assay.

clindamycin, erythromycin and fusidic acid, and found good correlation with standard disk diffusion results and MIC values. Barton (1985) used a novel approach in assaying extracellular release from staphylococci after incubation with methicillin and treatment with lysostaphin; although only 29 strains were examined, the results were similar to those with conventional tests. Unfortunately, this method is applicable only to *S. aureus*, because other staphylococci may not be susceptible to lysostaphin (Barton, 1985). These studies and earlier reports on bioluminescence have relied on single-chamber luminometers (Picciolo and Chappelle, 1977; Thore *et al.*, 1977). Such methods are labour-intensive, and the luciferin-luciferase reagent costs £0.3–0.5 per test. The Amerlite analyser is a sensitive

microtitration-plate reader, designed primarily for luminescence immunoassays. Its application for ATP bioluminescence was first reported by Oxley *et al.* (1987) and Wheat *et al.* (1987). The microtitration technique has advantages: multi-channel dispensers can be used for transfers and additions, and the small volumes reduce the cost per test to less than £0.05.

Although our technique is simpler than previous ATP methods, it still requires improvement. We are now developing a procedure in which all steps are performed in microtitration trays; also, the Amerlite Analyser needs a substantial database and facilities for storage and retrieval of results in epidemiological analysis of antimicrobial patterns.

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