Rapid antibiotic susceptibility tests on Enterobacteriaceae by ATP bioluminescence

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Summary. The susceptibility of 76 clinical isolates of Enterobacteriaceae to ampicillin, piperacillin and gentamicin was assessed by ATP bioluminescence in a 4-h test. For most organisms tested (*Escherichia*, *Klebsiella*, *Enterobacter* and *Serratia*), there was good correlation with traditional MIC values estimated on 18-h cultures. However strains of *Proteus mirabilis* showed false resistance to the β -lactam agents with the ATP method; and concordance was achieved only after manipulation of the growth conditions. Our method is simpler than those described previously, though currently it is still labour-intensive and expensive.

Introduction

Traditional tests for the antibiotic susceptibility of bacteria in liquid media or on agar plates require overnight incubation. The problem of antibiotic-multiresistant hospital bacteria, and the increasing numbers of immunocompromised patients for whom rapid effective therapy is important, have fostered an interest in new techniques to provide results within a few hours of primary isolation (Spencer and Wheat, 1986). Several approaches have been described including turbidimetry (Bascomb *et al.*, 1982), impedance (Colvin and Sherris, 1977), conductance (Porter *et al.*, 1983) and radiometry (Deblanc *et al.*, 1972). The luciferin-luciferase bioluminescence assay for adenosine triphosphate (ATP) is an established measure of bacterial biomass (D'Eustachio and Johnston, 1968). ATP is required in the luciferin-luciferase reaction as a specific energy source, and the amount of light generated is directly proportional to the original concentration of ATP in the reaction mixture. The use of ATP bioluminescence for rapid antibiotic susceptibility testing has been reported by others (Picciolo and Chappelle, 1977; Thore *et al.*, 1977). Although results have been encouraging, the protocols described are too complex for diagnostic laboratories, and only a limited number of antimicrobial agents and bacterial species have been evaluated. In the present study, a simple ATP bioluminescence method was used to provide rapid antibiotic susceptibility results with clinical isolates of Enterobacteriaceae.

Materials and methods

Bacteria

A total of 76 clinical isolates of Enterobacteriaceae were examined: *Escherichia coli* (17), *Klebsiella* spp. (4), *Enterobacter* spp. (7), *Serratia* spp. (10) and *Proteus mirabilis* (38).

Antibiotics

Ampicillin (Beecham Research Laboratories), piperacillin (Lederle Laboratories) and gentamicin (Roussel Laboratories) were provided as pure powders of stated potency and stored at 4°C.

Minimum inhibitory concentrations (MICs)

Standard antibiotic solutions were prepared in sterile distilled water for use the same day. MICs were determined on Isosensitest Agar (Oxoid) with antibiotic concentrations of 0.25–32 mg/L. Bacterial inocula of 10^7 – 10^8 colony forming units (cfu)/ml were transferred to the agar plates with a multipoint inoculator (Denley Instruments), and incubated for 18 h at 37°C. The MIC was recorded as the lowest concentration of antibiotic that inhibited visible growth.

Susceptibility testing by ATP bioluminescence

Inoculum preparation. Three colonies of the test organism from an overnight culture on MacConkey agar were inoculated into 1 ml of Hartley's Digest Broth (Oxoid) containing glucose 0.1%, and incubated at 37°C in a shaking 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 (ISB; Oxoid) with or without antibiotic. This represented an initial inoculum of 10^6 – 10^7 cfu/ml. Final antibiotic concentrations were ampicillin 2 mg/L, piperacillin 8 mg/L and gentamicin 2 mg/L. The cultures were incubated for a further 3 h at 37°C.

In some experiments with *P. mirabilis* several modifications were made: increasing the time of incubation in antibiotic to 6 h, reducing the osmolality of the ISB from 358 mOsm/L to 176 mOsm/L by the addition of distilled water, and substituting the usual 8-mm diameter glass test-tubes with 7-ml screw-capped glass bottles, 18 mm diameter.

Assay of bacterial ATP. A 200- μ l sample of the culture was mixed with an equal volume of extraction reagent—trichloroacetic acid 10% and 4 mM ethylene diamine tetra-acetic acid (EDTA)—and left for 2 min; 20 μ l of this extract was then added to 400 μ l of buffer (Tris acetate, EDTA) and 100 μ l of ATP Monitoring Reagent (luciferin-luciferase; LKB Wallac) in a plastic cuvette. Light emission was measured in a luminometer (1250; LKB Wallac) as a mV reading. Results could be converted to picomoles of ATP by comparison with the mV deflection resulting from the addition of 10 μ l of ATP standard (0.01 mM; LKB Wallac) to each cuvette. However, this conversion was unnecessary; but the addition of ATP standard was continued as a control measure. Results of ATP bioluminescence susceptibility tests were expressed in relation to bacterial growth in control broths:

Percentage bioluminescence =

$$\frac{\text{luminescence from broth with antibiotic (mV)}}{\text{luminescence from control broth (mV)}} \times 100$$

Fig. 1. For 38 non-proteus strains of Enterobacteriaceae, comparison of 18-h MIC results (mg/L) with ATP bioluminescence after incubation for 3 h in (A) gentamicin 2 mg/L, (B) ampicillin 2 mg/L, (C) piperacillin 8 mg/L.

Results

Comparison of ATP bioluminescence susceptibility tests with MIC values

Rapid antibiotic susceptibility tests performed by the ATP assay with gentamicin, ampicillin and piperacillin (figs. 1A, B, C) showed good correlation with standard 18-h MICs for Enterobacteriaceae, except for strains of *P. mirabilis*. A fairly good correlation was observed for *P. mirabilis* strains with gentamicin (fig. 2A), but rapid ATP results compared poorly with MIC values when the same *P. mirabilis* strains were tested with the β -lactam agents ampicillin and piperacillin (figs. 2B, C). Many sensitive strains of *P. mirabilis* (MIC <1 mg/L) grew, according to the ATP assay criteria, in the presence of ampicillin 2 mg/L or piperacillin 8 mg/L.

Influence of incubation time and container size on ATP results

Seven strains of *P. mirabilis*, six sensitive and one resistant to ampicillin, were selected for more detailed studies. Prolonged incubation in antibiotic (6 h instead of 3 h) did not alter the ATP ampicillin-susceptibility results (figs. 3A, B).

Glass test-tubes (diameter 8 mm) had been used in all the previous experiments. Agreement between the rapid ATP method and MIC values was

Fig. 2. For 38 strains of *P. mirabilis*, comparison of 18-h MIC results (mg/L) with ATP bioluminescence after incubation for 3 h in (A) gentamicin 2 mg/L, (B) ampicillin 2 mg/L, (C) piperacillin 8 mg/L.

achieved by using glass containers with a wider diameter (18 mm), but only after incubation for 6 h (figs. 3C, D). Similar results were found when piperacillin was substituted for ampicillin in these experiments (results not shown).

Effect of osmolality on ATP results

Gram-stained smears of *P. mirabilis* grown for 3 h in the presence of ampicillin 2 mg/L or piperacillin 8 mg/L showed abundant spheroplasts. The possibility that lowering the osmolality of the culture medium might improve the results with the rapid ATP assay was investigated by diluting ISB with distilled water. This had only a marginal effect on the growth kinetics of *P. mirabilis*. Its influence on the ATP results for a single strain of *P. mirabilis* with ampicillin is shown in fig. 4. After 3 h in ampicillin-containing broth, the bioluminescence was significantly reduced by the use of low osmolality broth in either 7-ml screw-capped containers or test-tubes. When the 38 isolates of *P. mirabilis* (figs. 2B, C) were re-tested with ampicillin and piperacillin in low osmolality broth and in 7-ml containers, there was a much closer correlation between ATP results and MIC values, even when the ATP test was applied to cultures incubated in antibiotic for only 3 h (figs. 5A, B).

Discussion

The central aim of the study was to evaluate the ATP bioluminescence assay as a method of rapid antibiotic susceptibility testing. A simple method was chosen that would be suitable for the routine laboratory. Therefore the use of centrifugation and filtration to concentrate bacterial cells, as described by other workers (Gutekunst *et al.*, 1977), was not considered. It has been claimed that measurements of intracellular ATP by the prior destruction of extracellular ATP with apyrase allows a better separation between suppressed and non-suppressed growth patterns (Thore *et al.*, 1977). However, extracellular ATP is destroyed rapidly by simultaneously released apyrase (Nilsson, 1982) and we detected only small amounts in the antibiotic-containing broth incubated for 3 h. Thus total ATP values approximated closely to intracellular ATP levels and represented a simplification of the assay procedure.

In previous reports, overnight broth cultures have been used as starting inocula (Hojer *et al.*, 1976; Thore *et al.*, 1977; Beckers *et al.*, 1984; Kouda *et al.*, 1985). This would be impractical for a rapid method. McWalter (1984) prepared inocula of staphylococci directly from colonies on blood-agar plates. In our protocol, colonies were emulsified in broth and then incubated for 1 h before the addition of antibiotics. Although this increases the

Fig. 3. For seven strains of *P. mirabilis*, comparison of 18-h MIC results (mg/L) with ATP bioluminescence after incubation in ampicillin 2 mg/L in 8-mm diameter glass test-tubes for (A) 3 h, (B) 6 h, or in 18-mm diameter glass 7-ml screw-capped containers for (C) 3 h, (D) 6 h.

total assay time, it has the advantage of allowing standard inocula to be prepared from only a few discrete colonies on the primary isolation plate. Furthermore, test organisms are in early logarithmic growth at the time of antibiotic challenge which is of particular relevance in testing susceptibility to β -lactam antibiotics (Hobby *et al.*, 1944; Tuomanen *et al.*, 1986).

Results with the ATP assay compared favourably with MIC data, and most strains could be clearly defined as sensitive or resistant after 4 h. All the major discrepancies occurred with strains of *P. mirabilis* that showed false resistance to the β -lactam agents. Similar difficulties have been observed by other workers who used either turbidimetric or ATP bioluminescence methods (Greenwood and O'Grady, 1973; Gutekunst *et al.*, 1977). Results were improved when tests were done in 7-ml containers; this may have been related to better oxygenation. However, this improvement

Fig. 4. For one strain of *P. mirabilis*, growth suppression by ampicillin 2 mg/L, assayed by ATP bioluminescence in normomolar (358 mOsm/L) — or low osmolality broth (176 mOsm/L) --- in glass test-tubes (●) or glass 7-ml screw-capped containers (▲).

was only apparent after a further 3 h, an incubation period we considered too long for a rapid same-day susceptibility test.

It has been suggested that false resistance of *P. mirabilis* to β -lactam agents may be the result of delayed lysis of spheroplasts (Greenwood and O'Grady, 1973). Spheroplasts formed by *P. mirabilis* show greater tolerance of low osmolality than those formed by *E. coli* (Greenwood and O'Grady, 1972). Recent observations have attributed this to the continued synthesis of functionally defective peptidoglycan by *P. mirabilis* spheroplasts in the presence of β -lactam agents (Martin, 1984). Reducing the osmolality of ISB significantly decreased

Fig. 5. For 38 strains of *P. mirabilis*, comparison of 18-h MIC results (mg/L) with ATP bioluminescence after incubation for 3 h in low osmolality broth (176 mOsm/L) containing (A) ampicillin 2 mg/L, (B) piperacillin 8 mg/L.

the ATP content in cultures of *P. mirabilis* after incubation for 3 h in the presence of ampicillin (fig. 4). Light microscopy and electronmicroscopy confirmed that this was related to increased lysis. Similar observations were made by Greenwood and O'Grady (1972) who monitored the influence of osmolality on the stability of penicillin-induced spheroplasts of *P. mirabilis* and *E. coli* by turbimetry. With low osmolality broth, a much better correlation between rapid ATP susceptibility results and standard MIC values was achieved for *P. mirabilis* isolates tested with ampicillin and piperacillin.

Although the protocol we have developed and tested is simpler than previously described methods, it remains labour intensive. Recently a luminometer which reads light emission from samples in wells of microtitration plates has been developed by Amer-sham International, Little Chalfont, England. The

microtitration-plate format is well-suited to a flexible semi-automated approach and we are currently adapting our protocol to accommodate microtitration volumes. The cost of luciferin-luciferase reagents has been considered a strong argument against the use of ATP-based methods in routine diagnostic laboratories (Martin *et al.*, 1985). However, the development of more sensitive techniques may allow the amount of reagents used for each ATP assay to be reduced considerably (Hastings, in press). Furthermore, the successful cloning of the luciferase gene has recently been reported (DeLuca, 1986) and this should significantly reduce the cost of ATP assays in the future.

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REFERENCES

- Bascomb S, Glynn A A, Gaya H, Spencer R C, Shine P J 1982 Rapid determination of bacterial susceptibility to antimicrobial agents by a semi-automated continuous flow method. *Journal of Antimicrobial Chemotherapy* **9**: 343–55.
- Beckers B, Lang H R M, Beinke A 1984 Determination of intracellular ATP during growth of *Escherichia coli* in the presence of ampicillin. In: Kricka L J (ed) *Analytical applications of bioluminescence and chemiluminescence*. Academic Press, London, pp. 67–70.
- Colvin H J, Sherris J C 1977 Electrical impedance measurements in the reading and monitoring of broth dilution susceptibility tests. *Antimicrobial Agents and Chemotherapy* **12**: 61–6.
- Deblanc H J, Charache P, Wagner H N 1972 Automated radiometric measurement of antibiotic effect on bacterial growth. *Antimicrobial Agents and Chemotherapy* **2**: 360–6.
- D'Eustachio A J, Johnson D R 1968 Adenosine triphosphate content of bacteria. *Federation Proceedings* **27**: 761.
- DeLuca M 1986 Firefly luciferase. *Journal of Bioluminescence and Chemiluminescence* **1**: 94.
- Greenwood D, O'Grady F 1972 The effect of osmolality on the response of *Escherichia coli* and *Proteus mirabilis* to penicillins. *British Journal of Experimental Pathology* **53**: 457–64.
- Greenwood D, O'Grady F 1973 Comparison of the responses of *Escherichia coli* and *Proteus mirabilis* to seven β -lactam antibiotics. *Journal of Infectious Diseases* **128**: 211–22.
- Gutekunst R R *et al.* 1977. Luminescence assays for the determination of microbial levels. In: *Proceedings of the ATP methodology symposium*. SAI Technology Co., San Diego, pp. 491–524.
- Hastings J G M Luminescence in clinical microbiology. In: Schölermerich J (ed) *Bioluminescence and chemiluminescence*. Proceedings of the 4th International Symposium. John Wiley, Chichester, in press.
- Hobby G L, Dawson M H 1944 Effect of rate of growth of bacteria on action of penicillin. *Proceedings of the Society for Experimental Biology and Medicine* **56**: 181–184.
- Hojer H, Nilsson L, Ansehn S, Thore A 1976 *In-vitro* effect of doxycycline on levels of adenosine triphosphate in bacterial cultures: possible clinical applications. *Scandinavian Journal of Infectious Diseases, Suppl.* **9**: 58–61.
- Kouda M, Ouchi Y, Takasaki Y, Maeda T, Matsuzaki H, Nakaya R 1985 Bioluminescent assay as a potential method of rapid susceptibility testing. *Microbiology and Immunology* **29**: 309–315.
- Martin E T, Cote J A, Perry L K, Martin W J 1985 Clinical evaluation of the Lumac bioluminescence method for screening urine specimens. *Journal of Clinical Microbiology* **22**: 19–22.
- Martin H H 1984 *In-vitro* synthesis of peptidoglycan by spheroplasts of *Proteus mirabilis* grown in the presence of penicillin. *Archives of Microbiology* **139**: 371–75.
- McWalter P W 1984 Determination of susceptibility of *Staphylococcus aureus* to methicillin by luciferin-luciferase assay of bacterial adenosine triphosphate. *Journal of Applied Bacteriology* **56**: 145–50.
- Nilsson L 1982 Rapid bioluminescent assay of serum amikacin. *Journal of Antimicrobial Chemotherapy* **10**: 125–30.
- Picciolo G L, Chappelle E W 1977 Application of firefly luciferase assay for adenosine triphosphate (ATP) to antimicrobial drug sensitivity testing. *NASA Technical Note D-8439*.
- Porter I A, Reid T M S, Wood W J, Gibson D M, Hobbs G 1983 Conductance measurements for determining antibiotic sensitivity. In: Russell A D, Quesnel L B (eds) *Antibiotics: assessment of antimicrobial activity and resistance*. Academic Press, London, pp. 49–60.
- Spencer R C, Wheat P F 1986 Novel mechanisms for determining antibiotic susceptibilities. *Journal of Antimicrobial Chemotherapy* **17**: 404–407.
- Thore A, Nilsson L, Hojer H, Ansehn S, Brote L 1977 Effects of ampicillin on intracellular levels of adenosine triphosphate in bacterial cultures related to antibiotic susceptibility. *Acta Pathologica Microbiologica et Immunologica Scandinavica* **85B**: 161–6.
- Tuomanen E, Cozens R, Tosch W, Zak O, Tomasz A 1986 The rate of killing of *Escherichia coli* by β -lactam antibiotics is strictly proportional to the rate of bacterial growth. *Journal of General Microbiology* **132**: 1297–1304.