Antimicrobial susceptibility testing of mycoplasmas by ATP bioluminescence

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Summary. The susceptibility of 72 mycoplasmas to a range of antimicrobial agents was assessed in a 6-h ATP bioluminescence system. ATP was assayed with the Amerlite Analyser. Correlation with conventionally determined MICs was excellent for erythromycin and tetracycline even at 3 h. However, for ciprofloxacin, correlation was poor unless incubation was extended to 6 h.

Introduction

Mycoplasmas have been implicated as the cause of genital and respiratory tract infections and of life-threatening infections in neonates.[1] Standard antibiotic therapy for mycoplasmal infections includes tetracycline and erythromycin.[2] However, *Mycoplasma hominis* is resistant to erythromycin and tetracycline is contra-indicated in certain clinical conditions. Furthermore, there has been an increasing number of reports of tetracycline-resistant strains of both *M. hominis* and *Ureaplasma urealyticum*.[3, 4] These problems have indicated the need for a rapid, practical means for determining antimycoplasmal susceptibility. The use of the luciferin-luciferase bioluminescence adenosine triphosphate (ATP) assay for the estimation of microbial biomass is well established.[5] We have described previously a protocol for the rapid antimicrobial susceptibility testing of Enterobacteriaceae[6] and gram-positive cocci[7] based on ATP bioluminescence. This protocol has now been modified for testing the susceptibility of mycoplasmas.

Materials and methods

Organisms

A total of 72 clinical isolates of *M. hominis* (36 strains) and *U. urealyticum* (36 strains) was tested. They were identified by arginine hydrolysis and production of urease respectively.[1]

Antimicrobial agents

Ciprofloxacin (Bayer), ofloxacin (Hoechst), flerox-acin (Roche), tetracycline (Lederle) and erythromycin (Abbott) were supplied as standard powders and solutions were freshly prepared before each experiment.

Determination of minimum inhibitory concentrations (MICs)

Antimycoplasmal activity in the range 0·06–32 mg/L was determined by the agar-dilution method[8, 9] with Mycoplasma Agar (Oxoid) containing yeast extract 20% w/v, and supplemented with horse serum 10% v/v and 3 mM urea (filter-sterilised 1 M urea 0·3 ml/100 ml of agar medium) for ureaplasmas. Inocula of c. 10^7 cfu/ml (c. 10^4 cfu/spot) were transferred to the antibiotic-containing agar plates by means of a multipoint inoculator (Denley Instruments) and incubated for 72 h at 37°C in an anaerobic environment (CO_2 10%, H_2 10%, N_2 80%). Ureaplasma colonies were visualised by spraying the plates with calcium chloride-urea stain.[10] The plates were then examined by light microscopy with a low power objective (× 10). The MIC was defined as the lowest concentration of antimicrobial agent that inhibited visible growth.

Microbial growth curve studies

A 24-h broth culture of *M. hominis* was resuspended in equal volumes of Mycoplasma Broth (Oxoid) supplemented with horse serum 10% v/v and yeast extract 20% w/v with or without antimicrobial agent. The concentrations of antimicrobial agents were ciprofloxacin 4 and 8 mg/L, erythromycin 4 mg/L and tetracycline 4 mg/L. ATP measurements were assessed at the start of incubation and after incubation for 3, 6 and 9 h at 37°C.

Susceptibility testing by ATP bioluminescence

Preliminary studies showed that 18–24-h broth cultures of *M. hominis* and *U. urealyticum* were suitable as starting inocula (c. 10^6–10^7 cfu/ml). ATP measurements were assessed in the control and in broths containing 4 mg/L of ciprofloxacin, erythromycin or tetracycline at the start of incubation and after incubation for 3 and 6 h at 37°C.

Assay of ATP. A 200-μl 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. After 2 min, 20 μl of this extract was added to 100 μl of buffer (Tris-EDTA) and 50 μl of ATP Monitoring Reagent (luciferin-luciferase; LKB Wallac) in one well of a microtitration tray. Light emission was measured with an Amerlite analyser (Amersham International, Buckinghamshire). ATP bioluminescence was expressed as a percentage:

$$\frac{\text{luminescence in broth with antimicrobial agent}}{\text{luminescence in control broth}} \times 100\%$$

From our previous experience of this assay, we categorised the results empirically as sensitive ($\leqslant$ 40%), intermediate (41–49%) or resistant ($\geqslant$50%).

Analysis of ATP and MIC susceptibility results

ATP assays were compared with MIC values by error boxes (fig. 1), based on a method we have previously described.[7] For strains in boxes A and B, the tests were considered to show agreement, for strains in box C a major disagreement and for strains in box D a very major disagreement. Strains for which the MIC did not differ by more than one dilution from the chosen concentration of antimicrobial agent in the ATP method were classified as showing agreement.

Results

MIC values determined conventionally are summarised in the table. All strains of *U. urealyticum* were

Table. MIC values of quinolones, erythromycin and tetracycline for mycoplasmas

Species (n)	Antimicrobial agent	MIC (mg/L)		
		50%	90%	Range
M. hominis (36)	Ciprofloxacin	1	1	0·5–2
	Ofloxacin	2	2	1–2
	Fleroxacin	2	2	1–2
	Erythromycin	>32	>32	>32
	Tetracycline	0·5	32	0·25–32
U. urealyticum (36)	Ciprofloxacin	16	32	8–32
	Ofloxacin	2	4	1–8
	Fleroxacin	4	4	2–4
	Erythromycin	2	2	2–4
	Tetracycline	1	2	1–2

susceptible to erythromycin and tetracycline. In contrast, all strains of *M. hominis* were resistant to erythromycin. The majority of *M. hominis* strains were susceptible to tetracycline; five strains were resistant. Of the three fluoroquinolone agents, ciprofloxacin proved to be the most active against *M. hominis* but the least active against *U. urealyticum*.

Mycoplasmal growth curve studies

Growth of *M. hominis* could be detected by ATP bioluminescence after incubation for only 3 h in the antibiotic-free broth (fig. 2). At this time resistance to erythromycin and sensitivity to tetracycline could be demonstrated. In contrast, when ATP levels were measured in the broth containing ciprofloxacin 4 mg/L after incubation for 3 h, levels parallelled those of the control broth and only decreased after incubation for a further 3 h (fig. 2). The broth containing ciprofloxacin 8 mg/L showed no initial increase in ATP readings compared to the control.
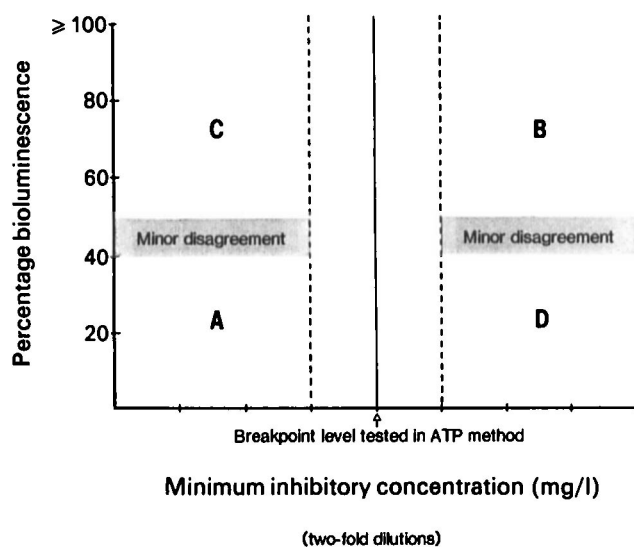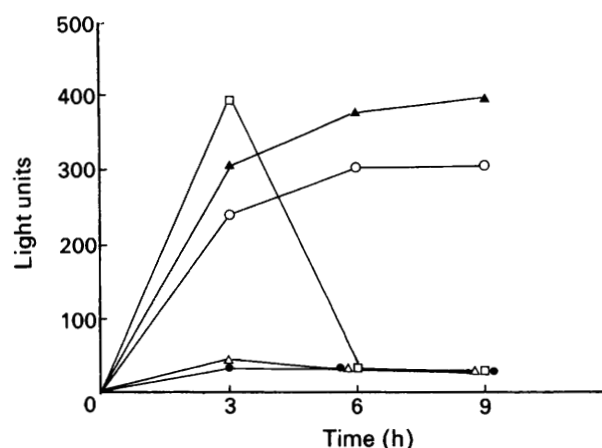

Fig. 1. Comparison of ATP bioluminescence results and MIC values by error box analyses; box C represents major disagreement and box D very major disagreement.

Fig. 2. Growth of *M. hominis* assessed by ATP bioluminescence: (▲) control; (○) with erythromycin 4 mg/L; (●) with tetracycline 4 mg/L; (□) with ciprofloxacin 4 mg/L (△) with ciprofloxacin 8 mg/L.

Comparison of MIC values and ATP results

Overall correlation between MIC values and ATP bioluminescence results was excellent (fig. 3a–c). There were no instances of major or very major disagreement. Predictably, mycoplasma strains tested against ciprofloxacin 4 mg/L required incubation for 6 h before satisfactory results were obtained (fig. 3c).

Discussion

Present antimicrobial susceptibility testing of mycoplasmas is cumbersome and time-consuming, and the end-point determination may be very subjective. The small size of these organisms (c. 0·3 μm) and low maximum harvests of cells (c. 10^7–10^8 organisms/ml) make microscopic and turbidimetric measurements unreliable.[11]

ATP is ubiquitous in all living cells and its assessment is an established method for measuring microbial biomass.[5] This is particularly useful for enumeration of fastidious micro-organisms. In the present study, a rapid susceptibility method for mycoplasmas and ureaplasmas showed excellent correlation with conventional techniques, which are seldom performed on a routine basis in the laboratory. However, before correlation was acceptable for ciprofloxacin, incubation had to be extended to 6 h. This may be similar to a phenomenon seen when *Escherichia coli* is exposed to ciprofloxacin—the organism shows initial filamentation followed by eventual lysis.[12] Studies in our laboratory have shown that *E. coli* exposed to ciprofloxacin for only a few minutes yielded markedly increased levels of ATP.[13]

Stemler *et al.*[11] showed that ATP estimation could be used as a means of measuring the growth of *U. urealyticum*. They described a single-chamber luminometer which utilised larger volumes of reagents than were used in our study. We used a novel luminometer which worked to a microtitration format[13]—this allows a reduction in reagent volume and facilitates the use of multi-channel dispensers for transfers and additions.[7] The protocol described in this study is inexpensive and suitable for use in the diagnostic microbiology laboratory. However, further improvements are necessary if the technique is to be used on a routine basis. We are developing an extraction method in which all steps are performed to a microtitration format, and the addition of a microcomputer database would assist the storage and retrieval of susceptibility data.
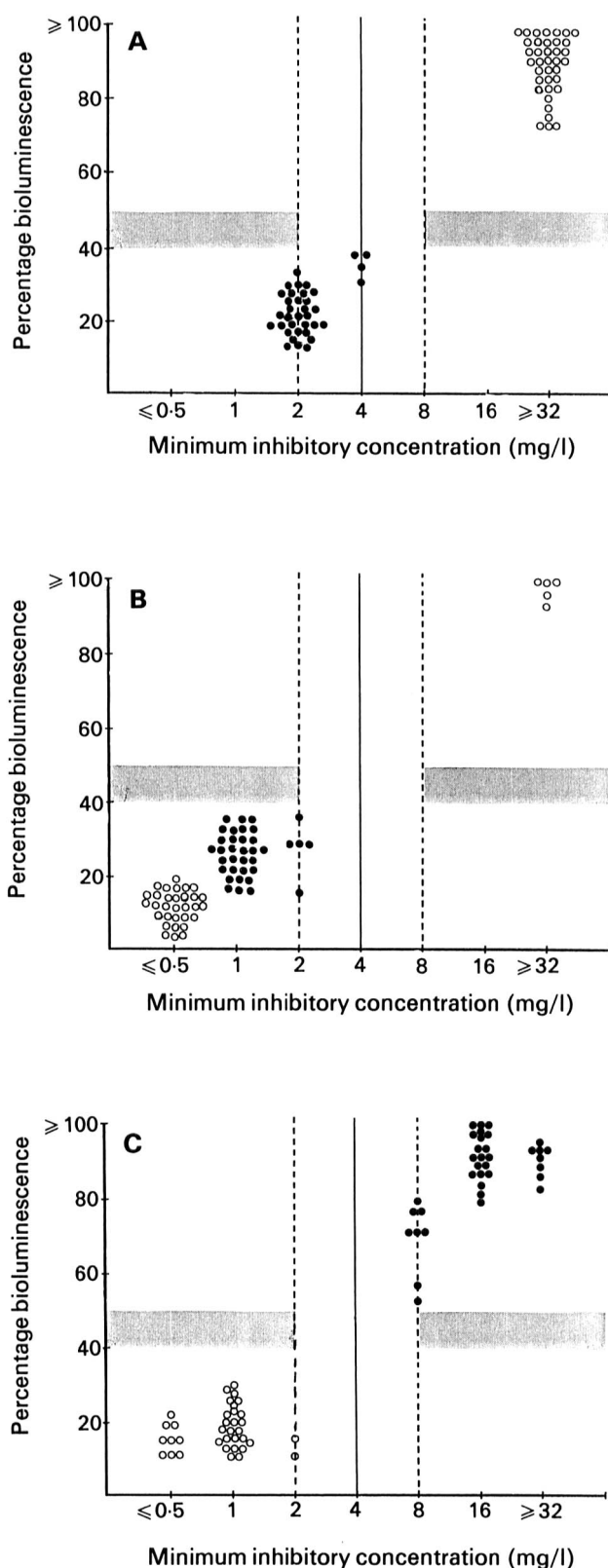
Fig. 3. Comparison of MIC results (mg/L) with 6-h ATP bioluminescence values for 36 strains each of *M. hominis* (○) and *U. urealyticum* (●) with (a) erythromycin 4 mg/L, (b) tetracycline 4 mg/L, and (c) ciprofloxacin 4 mg/L.

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