

Antimicrobial susceptibility testing of intracellular and cell-associated pathogens

Sub-committee on Susceptibility Testing of Intracellular and Cell-associated Pathogens of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID)

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INTRODUCTION

Susceptibility testing for intracellular infections depends not only on the drug used but also on the micro-organism under consideration. The great complexity of the scientific background accounts for this.

The micro-organism

Each intracellular micro-organism develops a specific strategy for surviving within cells. Examples of these strategies, which can combine their effects, include inhibition of the phagosome-lysosome fusion, resistance to lysosomal products once the fusion has occurred, and escape into the cytoplasm and interference with host cell machinery (Table 1).

The drugs

Each antibiotic exhibits specific intracellular kinetics. β -Lactams penetrate poorly into cells, do not accumulate, are associated with organelles, and are found mostly in the cytoplasm. Ampicillin and derivatives, and third generation cephalosporins, are active against salmonellae, and ampicillin and derivatives can form part of the therapy of listeriosis.

Aminoglycosides penetrate very slowly into cells, and significant intracellular concentrations can be observed only after prolonged exposure. Under these conditions, they accumulate in the lysosomes, where very high concentrations can be achieved after several weeks of therapy. These high concentrations are thought to be associated with ototoxicity and nephrotoxicity.

Streptomycin and kanamycin have been used in tuberculosis, and streptomycin in the management of brucellosis.

Quinolones penetrate rapidly into the host cell cytoplasm, where the concentration is typically 5–15 times the extracellular concentration. Release of quinolones from the cell after exposure is rapid. Quinolones do not seem to be associated with any organelles. They are excellent drugs for treating legionellosis and salmonellosis. Fluoroquinolones such as ciprofloxacin and ofloxacin have suboptimal activities against chlamydiae; newer compounds are much more active. Despite good activity in vitro, quinolones are insufficiently active for treating brucellosis. Ofloxacin exhibits some clinical activity in a compassionate context in the management of multiresistant tuberculosis and leprosy.

Macrolides penetrate rapidly into cells, accumulate in the cytoplasm and even more in the lysosome, and are slowly released by the cells. This process is drug dependent: erythromycin < roxithromycin < clarithromycin < azithromycin. These drugs, especially the most recent ones, are active in treating legionellosis, chlamydiosis and rickettsiosis.

LEGIONELLA PNEUMOPHILA

Legionella pneumophila has fastidious growth requirements, with culture medium formulations for primary isolation usually containing activated charcoal, which can bind a range of antimicrobials.

Routine susceptibility testing media, e.g. Mueller-Hinton and Iso-Sensitest, are unsuitable for culturing *L. pneumophila*, and disk diffusion methods are inappropriate because of the

Table 1 Intracellular location and strategies for intracellular survival of some facultative and obligate intracellular bacteria

Intracellular organism	Intracellular location	Main survival strategies
<i>L. pneumophila</i>	Phagosome	I. Inhibit phagolysosome fusion II. Use C3b receptor pathway, which decreases exposure to toxic oxygen derivatives
<i>Brucella</i> spp.	Phagosome	I. Release of adenine and 5'-Guanosine monophosphate (which inhibit myeloperoxidase-peroxide-halide system) II. Produce Cu-Zn superoxide dismutase
<i>Rickettsia</i> spp.	Cytosol	Move out the phagosome after production of phospholipase
<i>C. burnetii</i>	Phagosome	Produce acid phosphatase protecting from enzyme attack
<i>E. chaffensis</i>	Phagosome	I. Inhibit phagolysosome fusion II. Inhibit signal transduction pathway of interferon- γ
<i>M. pneumoniae</i>	Attached to host cell surface	I. Has an adherence organ II. Remains extracellular when attached to ciliated epithelial cell
<i>C. trachomatis</i>	Phagosome	I. Inhibit phagolysosome fusion II. Complex intracellular cycle
<i>L. monocytogenes</i>	Cytosol	Escape into cytosol after production of listeriolysin O

slow growth rate and the need for medium supplementation. Therefore, the most useful susceptibility testing method is determination of the minimum inhibitory concentration (MIC) of an antimicrobial by agar dilution.

L. pneumophila is an intracellular pathogen, so susceptibility testing by determination of MIC should be restricted to those compounds which are known to penetrate macrophages and have been associated, by the use of animal models or clinical studies in humans, with therapeutic success. Such antimicrobials include rifampicin, erythromycin (and other macrolides), the fluoroquinolones and, to a lesser extent, the tetracyclines. Other compounds, such as the β -lactams, may demonstrate in vitro activity in a cell-free system but do not penetrate macrophages efficiently, and are therefore inappropriate for treating infections with *L. pneumophila*.

Cultured macrophages may be used to determine the activity of antimicrobials against intracellular *L. pneumophila*. However, such techniques are neither available nor necessary in routine clinical microbiology laboratories.

Agar dilution MIC determination [1]

Medium: For preparation of 1 L of agar, add 13 g of bacteriological agar no. 1 (Oxoid, Basingstoke, UK) and 10 g of yeast

extract (Oxoid) to 850 mL of distilled water and autoclave at 121 °C for 15 min. Cool to 45–50 °C and add 2 × 50 mL vials of *Legionella* BCYE growth supplement (Oxoid) and 50 mL of water-lysed horse blood (prepared by adding an equal volume of water followed by 3.5 freeze-thaw cycles).

Inoculum: 10⁴ CFU/1- μ L spot.

Incubation: 48–72 h at 35 °C.

Control strain: *L. pneumophila* NCTC 11192, or equivalent.

BRUCELLA SPP.

Brucellosis is a zoonosis caused by several species of the genus *Brucella*. The infection affects domestic animals (sheep, goat, cattle and swine) and is transmitted from infected animal to humans. The main route of transmission is through ingestion of unpasteurized milk and milk products, but contact with infected placentas, aborted ruminants and infected aerosol may also transmit the infection. When infected food is ingested, the organism penetrates through the intestinal mucosa, and is transported to the reticuloendothelial system (liver, bone marrow, lymph nodes and spleen), where it may survive intracellularly for prolonged periods of time.

The most common presentation of brucellosis is a prolonged febrile disease accompanied by night sweats, arthralgias,

myalgias, bone pain and backache, or as localized disease manifested as arthritis, osteomyelitis and epididymo-orchitis.

Brucellae are small (0.5 µm) coccobacillary Gram-negative bacteria. They grow naturally as facultative intracellular organisms, especially in macrophages and monocytes. The genus is taxonomically divided into six species, which are further divided into biovars. The species are *B. melitensis*, *B. abortus*, *B. suis*, *B. neotomae*, *B. canis* and *B. ovis*. The first four have a smooth structure of their lipopolysaccharide (LPS), whereas the last two have the rough-stable form.

Brucellae grow fastidiously on artificial media, and each species has different requirements for growth. CO₂ requirements also vary. Some strains, e.g. *B. melitensis*, require supplementation of basic tryptic soy broth with horse serum to avoid the tendency to dissociate and turn into a rough form. Freshly isolated strains are more antibiotic susceptible than the comparable laboratory-maintained strains.

Two techniques have been used to study antibiotic susceptibility: the agar dilution method [2,3] and the broth dilution technique [4,5]. Neither technique measures the susceptibility of the intracellular organism. However, antibiotics that do not demonstrate activity in host cell-free systems are not even considered candidates for clinical trials.

Agar dilution MIC determination

The preferred agar dilution technique is as follows [6]:

Medium: Mueller–Hinton agar (Oxoid) supplemented with 1% haemoglobin (bioMérieux, Marcy-l'Étoile, France) and 1% PoliViteX (bioMérieux). Alternatively, the medium may be supplemented with 5% sheep blood, or 5% lysed horse blood when testing sulfonamides–trimethoprim.

Inoculum: 10⁴ per spot.

Incubation: 18–48 h at 37 °C in air with 5% CO₂.

Broth macrodilution MIC determination

The preferred broth dilution method is as follows:

Medium: *Brucella* broth supplemented with 1% haemoglobin (bioMérieux) and 1% PoliViteX (bioMérieux).

Inoculum: 1:1000 dilution of a 24-h broth culture (McFarland 0.5).

Incubation: 48 h at 37 °C in air with 5% CO₂ (for *B. ovis*, incubation is for 7 days under the same conditions).

For the determination of MBC, subculture the first two clear tubes on *Brucella* agar incubated for 48 h in 5% CO₂ at 37 °C.

RICKETTSIA SPP.

Rickettsia spp. are strict intracellular pathogens, so antibiotic susceptibilities can only be determined in vitro in cell systems.

The plaque assay

This is currently considered the reference technique [7]. This model is based upon the ability of rickettsiae to induce plaque formation in infected cells in vitro. Vero cells have been most often used. Confluent Vero cells, grown in Petri dishes, are infected with 1 mL of a solution of Eagle minimal essential medium (MEM) supplemented with 4% foetal calf serum and 2 mM L-glutamine, containing approximately 10⁵ plaque-forming units/mL (PFU/mL) of rickettsiae. Titration of the rickettsial inoculum is obtained by inoculation of 10-fold serial dilutions of the primary inoculum to uninfected Vero cells, and counting of plaques after 7–10 days of incubation of cultures, whereas the rickettsial inoculum is frozen at –80 °C during the titration procedure. Because of the long time required for titration of rickettsiae and the necessity to use a frozen inoculum which may influence the antimicrobial susceptibility, we also use a primary inoculum. This is obtained directly after harvesting confluent Vero cell monolayers 100% infected with rickettsiae, and corresponds approximately to 10⁵–10⁶ rickettsiae/mL of medium. After rocking the Petri dishes for 1 h at 22 °C, to allow penetration of rickettsiae within Vero cells, cell monolayers are overlaid with 4 mL of Eagle MEM containing 2% foetal calf serum and 0.5% agar. Antimicrobials are added at a volume of 200 µL in two-fold serial final concentrations. Petri dishes are incubated for 7–10 days at 37 °C in 5% CO₂. The cells are then fixed with 4% formaldehyde, and stained with 1% crystal violet in 20% ethanol. The MIC is the lowest concentration of the agent tested causing complete inhibition of plaque formation, compared with drug-free controls.

The major problem with this methodology is that some rickettsial strains may not induce the formation of plaques in cell cultures. In this case, several passages in various cell lines may allow selection of variants able to produce plaques [8]. Another limitation of the technique is that it is very fastidious and time-consuming.

The microplaque colorimetric assay

This technique (also known as the dye uptake assay) has been proposed as a less fastidious technique than the plaque assay for determination of the antibiotic susceptibility of rickettsial species [7]. Vero cells, grown in MEM supplemented with 4% foetal calf serum and 2 mM L-glutamine in microdilution plates, are inoculated with rickettsiae so that 2000 PFU of rickettsiae are added to each well of the second row, 200 PFU for the third row, and 20 PFU for the fourth row. The first row, containing only Vero cells and MEM, is used as an uninfected control, whereas 2000 PFU of rickettsiae are added to each well of the next eight rows for the antibiotic assay. Two-fold serial dilutions of antibiotics are tested. Plates are incubated for 5–7 days at

37 °C in 5% CO₂. The cell-culture supernatant of each well is then replaced by 50 µL of neutral red dye (0.15% in saline, pH 5.5) and the plates are incubated for 1 h at 37 °C in 5% CO₂. Dye not taken up by the cells is removed by three washes with phosphate-buffered saline (PBS), pH 6.5, and dye absorbed by the cells is extracted by the addition of 100 µL per well of phosphate ethanol buffer (10% ethanol in PBS, adjusted to pH 4.2). The optical density (OD) at 492 nm of the cell supernatant is determined in each well with a spectrophotometer. The MIC is the first dilution for which the mean OD of the corresponding row is between the OD of the cell control row and the OD of the row containing 20 PFU per well.

Results obtained with the dye uptake assay are highly concordant with those of the plaque assay system. The majority of published information on antimicrobial susceptibilities of rickettsiae has been obtained using both models [8], including the activity of the newer macrolides and fluoroquinolone compounds [9,10].

Immunofluorescence assay

An immunofluorescence assay has been reported recently by Ives et al. [11]. In this model, infected Vero cells are grown in slide chambers, in the presence of various concentrations of antimicrobial agents. Growth of rickettsiae is detected after 14 days of incubation of cultures in the presence of the agent tested as compared with a drug-free control, by using an immunofluorescence assay with anti-*Rickettsia* spp. monoclonal antibodies. MICs correspond to the minimum antimicrobial concentration allowing complete inhibition of rickettsial growth.

Results obtained with this technique are comparable to those obtained with the plaque assay system. This technique may be of interest for rickettsial strains that do not produce plaques in vitro. However, in contrast to previous techniques, Ives' technique is less well standardized, and does not allow evaluation of the viability of rickettsiae.

COXIELLA BURNETII

Coxiella burnetii, the agent of Q fever, may induce acute diseases (most often hepatitis and pneumonia) or chronic diseases (mainly endocarditis). In vitro cell systems have been elaborated to test either the bacteriostatic activity or the bactericidal activity of agents against this strict intracellular bacterium. Several cell lines have been used in these models, including murine macrophage-like cell lines (P388D1 and J774) and a murine fibroblast cell line (L929). *C. burnetii*-infected cell cultures may be maintained in vitro for several months. *C. burnetii* was reported to be more resistant to antimicrobials, especially doxycycline, when chronically infected cells (i.e.

infected for more than 400 days) were used in antimicrobial challenges, as compared with acutely infected cells (i.e. infected for less than 30 days) [12]. Consequently, acutely infected cell cultures are used in antimicrobial challenges as models for acute Q fever infection, whereas persistently infected cell cultures are used as models for chronic Q fever infection. Results obtained in the latter models are considered to be more predictive of the antimicrobial activity in chronically infected patients. Because the goal of the antimicrobial treatment of patients with Q fever endocarditis is to eradicate *C. burnetii* from the infected valve, these models were designed to test the bactericidal activity of agents against *C. burnetii*. We currently use one model, the shell vial model [13], to assess the bacteriostatic activity of agents against *C. burnetii*, and two different models, the cycloheximide-blocked L929 cell system [14] and a quantitative model [15], to test their bactericidal activity.

Shell vial model

In this model [13], HEL fibroblast cells are grown in shell vials at 37 °C in a 5% CO₂ atmosphere. Cell monolayers are infected with a *C. burnetii* inoculum previously determined to induce 30–50% infection of HEL cells after 6 days of incubation in the absence of antimicrobial agents, as revealed by an immunofluorescence technique with anti-*C. burnetii* polyclonal antibodies. The percentage of infected cells in antimicrobial-containing cultures is determined after the same incubation time, using the same immunofluorescence procedure. MICs correspond to the minimum antimicrobial concentration allowing complete inhibition of growth, i.e. 0% infected cells after the 6-day incubation period. This method has been used to test the susceptibilities of several *C. burnetii* strains to many agents [13], including the more recently available macrolides [10,16] and fluoroquinolone compounds [10]. This is a very convenient model, allowing concomitant evaluation of the antimicrobial susceptibilities of several strains of *C. burnetii* to newer agents.

Cycloheximide-blocked L929 cell system

In this model [14], L929 cells are persistently infected with *C. burnetii*, and cycloheximide (1 mg/L) is added to the incubation medium to block cell division during the antibiotic challenge. The activity of agents against *C. burnetii* is assessed by daily determination (for a 10-day period) of the percentage of infected cells in antimicrobial-containing cultures as compared with drug-free controls, using an immunofluorescence assay as previously described. Agents are added to the incubation medium when cell monolayers are approximately 100% infected with *C. burnetii*. A significant reduction in the percentage of infected cells over time is interpreted as a bactericidal activity of the antibiotic tested.

The main limitation of the model is the use of cycloheximide, which might theoretically influence bacterial growth, and thus the antimicrobial susceptibility of the tested strain.

Quantitative model

The bactericidal activity of antibiotics against *C. burnetii* is directly evaluated by titration of residual viable bacteria in persistently infected P388D1 cell cultures [17]. A bactericidal activity corresponds to significant reduction in bacterial titers, as compared with the primary inoculum, after 24 h of antimicrobial exposure. This model has the advantage of allowing quantitative analysis of the bactericidal activity of agents against *C. burnetii* without the use of cycloheximide to block cell division. Using this model, Maurin et al. [17] have shown that currently available agents have no bactericidal activity against intracellular *C. burnetii*. They have also confirmed the bactericidal activity of the combination of doxycycline with the lysosomotropic agent chloroquine. The quantitative model is presently regarded as the optimal model for assessing the bactericidal activity of newer compounds against *C. burnetii*.

EHRlichia spp.

Ehrlichiae are strict intracellular pathogens, with a complex intracellular cycle resembling that of chlamydiae. As for chlamydiae, only some intracellular forms of the bacteria can induce new cycles of infection in vitro in cell cultures. Thus, evaluation of the activity of agents against ehrlichiae by titration of residual viable bacteria is not feasible.

Currently available methods correspond to the determination of the percentage of infected cells after several days of culture in the presence of the agents tested as compared to drug-free controls [18,19]. Such techniques allow determination of the bacteriostatic activity of agents. MICs correspond to the minimum concentration of the agent tested giving complete inhibition of growth of ehrlichiae, usually after 3 days of antimicrobial exposure, i.e. the antimicrobial concentration allowing stabilization of the percentage of infected cells over time compared with a drug-free control. The bactericidal activity of antimicrobials (i.e. the eradication of viable ehrlichiae in cultures) may also be evaluated in the same cultures after removal of the agent from cell-culture supernatants. Inhibition of intracellular growth of ehrlichiae despite removal of the agents is interpreted as a bactericidal effect, whereas an increase in the percentage of infected cells within 7 days following antimicrobial removal means that the ehrlichiae were not eradicated from infected cultures.

BARTONELLA spp.

Bartonella species are fastidious, Gram-negative, facultative intracellular bacteria. Such bacteria grow best in blood-enriched

media, at 30 °C (*B. bacilliformis*) or 37 °C (other species), and with or without a CO₂-enriched atmosphere, depending on the species considered.

The MICs of antimicrobials for several strains have been determined in axenic medium, using Columbia base agar supplemented with 5 or 10% horse blood as the antimicrobial assay medium [16,17]. This is not currently considered a reference method, because the presence of blood may affect the activity of some antimicrobials. However, general NCCLS/EUCAST methods for the determination of MICs are not suitable for such fastidious bacteria.

The antimicrobial susceptibility of *Bartonella* spp. has also been evaluated by growth in the endothelial cell line ECV 304 [20]. Although many antimicrobials display low MICs in axenic medium, only the aminoglycosides are bactericidal against intracellular *Bartonella* species. Results obtained in the endothelial cell model are more compatible with the difficulty in eradicating bartonellae in chronically infected patients. Streptomycin is considered the reference antimicrobial therapy for Carrion's disease (caused by *B. bacilliformis*). Thus, results obtained in the endothelial cell culture model may be more relevant to the clinical situation.

MYCOPLASMAS

Mycoplasmas are involved in human respiratory and genital infections, as well as in other infections observed in immunocompromised hosts. *Mycoplasma pneumoniae* is an agent of respiratory infections, whereas *M. hominis*, *M. genitalium* and *Ureaplasma urealyticum* are responsible for a number of genital infections and probably, in the case of *U. urealyticum* and *M. hominis*, for reproductive disorders and neonatal infections. The involvement of other species, such as *M. fermentans* or *M. penetrans*, in human infections is still speculative.

Innate resistance to antibiotics

Mycoplasmas, which belong to the class Mollicutes, have some specific characteristics which have important consequences for the activity of antimicrobial agents and for the methods used for susceptibility testing. Because of the absence of cell walls, they are completely non-susceptible to agents inhibiting peptidoglycan synthesis, such as β -lactam antibiotics and glycopeptides. In addition, mycoplasmas are resistant to polymyxin, rifampicin, sulfonamides, trimethoprim and nalidixic acid. The antimicrobials exhibiting the highest inhibitory effect against mycoplasmas are the tetracyclines, macrolides and related antibiotics, including ketolides, and the newer fluoroquinolones. Aminoglycosides possess less inhibitory activity and are not used in vivo against these organisms, which are often located inside the cells. Chloramphenicol may be useful in some specific situations, such as neonatal meningitis.

Some variation in inhibitory response can be observed within mycoplasmas with certain antimicrobials. For instance, innate resistance to erythromycin and to macrolides with a 14-atom ring, and to azalides, is observed in *M. hominis* and, to a lesser extent, in *M. fermentans*, while josamycin, a 16-member macrolide, is highly active. In contrast, *U. urealyticum* is naturally susceptible to macrolides but not to lincomycin.

Acquired resistance

Acquired resistance to antimicrobial agents has been described in the different *Mycoplasma* species found in humans [21]. No case of tetracycline resistance has been reported in *M. pneumoniae*. Exceptional cases of acquired resistance to macrolides have been described in this species in treated patients. In vitro studies demonstrated that the resistance affected macrolides, lincosamides and streptogramin B agents, and was shown to occur as the result of point mutations in the fifth domain of the 23S rRNA gene. No case of acquired resistance to fluoroquinolones has been reported in *M. pneumoniae*.

With genital mycoplasmas, acquired resistance is only known for *M. hominis* and *U. urealyticum*. Acquired resistance to tetracyclines is not uncommon and is mediated by the *tetM* determinant [22]. The extent to which tetracycline resistance occurs varies geographically (about 5% in France, and higher in some parts of the USA). Acquired resistance to macrolides and to fluoroquinolones has been reported in clinical isolates of *M. hominis* and *U. urealyticum*. Mutations in *gyrA*, *parC* and *parE* were found in *M. hominis* strains isolated from fluoroquinolone-treated patients [23]. The possibility of antimicrobial resistance in *M. genitalium* is not known because of the very low number of strains studied.

Culture requirements for antimicrobial susceptibility testing

Mycoplasmas require specific growth media; some components of these and pH may interfere with the activity of antimicrobial agents. It is not possible to standardize on a simple medium or pH, since growth requirements and metabolic properties differ among species, but the presence of serum (20% horse or foetal calf serum) is a common feature of all proposed media.

Hayflick modified medium can be used for *M. pneumoniae* or *M. hominis*. It contains heart broth infusion (1.7%), fresh yeast extract (2.5%), horse serum (20%) and phenol red indicator (0.001%). For *M. pneumoniae*, broth contains glucose (0.5%), and the final pH is 7.4–7.5. For argininolytic species such as *M. hominis*, arginine (0.5%) is added and the final pH is 7.2–7.3. Agar media usually contain 1% purified agar. SP-4 medium (broth and agar), a more complex medium, may be used for fastidious species such as *M. genitalium* [24], with addition of glucose to the broth medium.

U. urealyticum does not grow on Hayflick or SP-4 media. Specific media used for this organism include Shepard's 10 B broth and A8 agar, both at pH 6.0 [25,26]. Both media contain yeast extract, 20% serum, and urea that will be metabolized by *U. urealyticum*. The pH indicator used is usually phenol red or bromothymol blue. *M. hominis* can grow on the media proposed for *U. urealyticum*.

Culture conditions proposed for mycoplasmas are far from the standardized conditions used for conventional bacteria and may affect antimicrobial activity. A typical example is given by the reduction of antimicrobial activity by the low pH of *U. urealyticum* media, observed for some macrolides. This phenomenon has to be taken into account. When testing a new agent, the effect of mycoplasma culture conditions on it must be controlled by comparing MICs under these conditions for a reference bacterial strain with MICs under standard conditions used for the reference strain.

Growth detection in antimicrobial susceptibility testing

In broth media, mycoplasma growth is detected by colour change of the pH indicator, with an acidic change due to the fermentation of glucose for *M. pneumoniae* or *M. genitalium*, and an alkaline change due to the degradation of arginine for *M. hominis*, or of urea for *U. urealyticum*. Cell numbers present in the medium are expressed as colour-changing units (CCUs).

On agar, growth is detected by the appearance of colonies that are usually very small, requiring a microscope with a low-power objective. Colonies have a typical fried-egg appearance for *M. hominis*, and are more irregular for *M. pneumoniae*. For *U. urealyticum*, colonies are very small and irregular and must be distinguished from artefacts and various debris. Cell numbers are expressed as colony-forming units (CFUs).

Broth microdilution method

The broth dilution method used for mycoplasmas is an application of the metabolism inhibition technique. It detects the inhibition of mycoplasma metabolism in the presence of the antimicrobial. As for classical bacteria, the test is performed in microdilution plates (96 well) in a volume of 200 µL for each well. Final inoculum concentrations are in the range of 10^4 – 10^5 CCU/mL. Plates are sealed with an adhesive cover, and the wells should be vented by puncturing the cover prior to incubation. Incubation of microdilution plates is at 35–37 °C and does not require CO₂. The MIC is read as the highest concentration of antimicrobial that prevents a colour change at the time the growth control first shows a colour change. The MIC will be generally available for *U. urealyticum* at 24 h, for *M. hominis* at 48 h, and for *M. pneumoniae* after 5 or more days.

This technique is the most practical and widely used method. It may be adapted to all *Mycoplasma* species and allows testing of several antimicrobials in the same plate with determination of mycoplasmicidal activity. However, the number of organisms in the inoculum and the time of reading may influence the results and reproducibility. Continued incubation of the plate may result in an increased MIC value. In consequence, some authors describe an initial MIC (read at the time of the colour change in the control) and a final MIC (read at the time the colour change is stable). Some attempt at standardization is necessary to avoid differences in results from one laboratory to another. This phenomenon is particularly important for *U. urealyticum*.

Broth microdilution with commercial kits

Kits designed for susceptibility testing of *U. urealyticum* and *M. hominis* are available in Europe [27]. They consist of microwells containing dried antimicrobials, in one or two concentrations corresponding to the breakpoint concentrations proposed for conventional bacteria to classify a strain as susceptible, intermediate or resistant to an agent. Satisfactory results were observed when comparing the results obtained with one of these kits inoculated with a defined inoculum and established MIC determination [28]. However, some kits combine detection and identification of genital mycoplasmas with antimicrobial susceptibility testing. Absence of control of the inoculum size can be a source of error. When used with a controlled inoculum obtained after a primary culture, the products give useful results.

Agar dilution method

Standard agar dilution methods have been adapted for mycoplasmas. Agar plates containing several antimicrobial concentrations are inoculated with a multipoint replicator, a micropipette or a calibrated loop to deliver 10 µL. Each spot should give 30–300 colonies per spot on the control plate without antibiotics. Too heavy an inoculum can mask the presence of growth. Usually, the equivalent of about 10 µL of a 1 : 10 dilution of *M. pneumoniae* culture or a 1 : 100 dilution of *M. hominis* will yield the desired result. Because of the very small size of colonies of *U. urealyticum*, the agar dilution method is not very well adapted to this species.

Agar plates are incubated in an atmosphere of air with 5% CO₂ at 35–37 °C. Prevention of desiccation is necessary if prolonged incubation is required. Plates are examined microscopically. The MIC is the lowest concentration of antimicrobial that prevents colony formation at the time when the antimicrobial-free control plate shows growth. The growth usually occurs in 2–4 days for *U. urealyticum*, 2–4 days for *M. hominis*, and 5 or more days for *M. pneumoniae*. Incubation time is usually slightly longer than in broth medium, and MIC results may be slightly higher (one dilution difference). Compared

with the broth dilution method, agar dilution has the advantage of a more stable endpoint, and it is convenient for testing a large number of organisms. However, it is time-consuming for a small number of strains and is more affected than broth dilution by the quality of the test medium.

Etest

Agar disk diffusion is not recommended for antimicrobial susceptibility testing of mycoplasmas, because of the slow growth of some species on agar and because of the very small size of colonies given by species such as *U. urealyticum*. However, the agar gradient technique, the Etest, has been adapted for *M. hominis* susceptibility testing for tetracyclines and fluoroquinolones [24]. A 0.5-mL suspension of at least 10⁵ CCU/mL is used as inoculum. The MIC is read under the microscope. Staining plates with Dienes stain helps visualization of the ellipse. The Etest is well-adapted for testing single isolates. It has also been proposed recently for testing in vitro susceptibilities of *Ureaplasma* spp.

Interpretation of results

Breakpoints proposed for other bacteria are usually used to interpret the MIC results for mycoplasmas. However, this practice should be used with care, because no specific breakpoint has been definitely recognized for mycoplasmas.

The distinction between susceptible and resistant strains is clear in some cases, and less clear in others. For instance, *tetM*-related tetracycline resistance is usually easy to confirm in *U. urealyticum* and *M. hominis*, and there is a clear difference between susceptible (MIC usually 0.1–1 mg/L) and resistant (MIC usually ≥8 mg/mL) strains.

U. urealyticum is often considered as being naturally intermediate in susceptibility to fluoroquinolones such as ciprofloxacin and ofloxacin, without evidence of therapeutic failure. The number of acquired resistance reports is still exceptional for both *U. urealyticum* and *M. hominis*. For macrolides, the MICs for *U. urealyticum* are around 0.5–2 mg/L (except for clarithromycin), which often results in intermediate classification when considering the breakpoints used for other bacteria. However, in view of the low pH of the medium, which affects the activity of macrolides, these MICs are probably indicative of clinical susceptibilities [26].

Test of mycoplasmacidal activity

Mycoplasmacidal activity may be studied using the classical procedures used for bacteria. No standardized method is available. Experiments are performed with test tubes containing 2 mL of Hayflick modified broth medium or Shepard's broth medium. The MIC is recorded, and then 100-µL aliquots are

transferred from tubes without colour change to 5 mL of fresh antimicrobial-free broth. The MBC is the lowest antimicrobial concentration inhibiting a colour change in this culture within 4–10 days, according to the species.

With the exception of the newer fluoroquinolones and ketolides, the antimicrobials usually used for mycoplasmas are bacteriostatic.

Indications for antimicrobial susceptibility testing

Indications differ according to the species and the clinical situation. Susceptibility testing of *M. pneumoniae* is not currently indicated, because of its predictable character. The evaluation of in vitro susceptibility is necessary only when new drugs are proposed. The situation is totally different for *U. urealyticum* and *M. hominis*. When these species are estimated to be responsible for infections, it is necessary to evaluate their susceptibility to tetracyclines, macrolides, lincosamides, streptogramins and fluoroquinolones. This evaluation is particularly important when the strains are isolated from immunocompromised patients.

CHLAMYDIA TRACHOMATIS AND CHLAMYDIA (CHLAMYDOPHILA) PNEUMONIAE

The advent of cell-culture techniques for the propagation of *Chlamydia trachomatis* also considerably simplified determination of the effects of antimicrobial agents on the organism. Previously, it had been necessary to use egg titration, which used \log_{10} concentrations of antimicrobials. The method was cumbersome, and the endpoint based on plots of increased survival time against \log_{10} dose [29]. Cell culture allows the use of doubling dilutions, bringing assays more in line with conventional bacterial MIC/MBC methods. However, there are several variables that have resulted in various methods being described. These variables were reviewed by Ehret and Judson [30].

Standardization of testing

Variables in chlamydial susceptibility testing include:

1. Types of culture cell used, and treatment of cells to improve susceptibility to chlamydial infection.
2. Type of culture container, e.g. shell vial, WHO trays, microdilution trays.
3. Centrifugation speed and duration.
4. Timing of exposure of cultures to antimicrobials, and duration of exposure.
5. Staining of inclusions.
6. Endpoint determination.
7. Passage number for MBC determination.

Despite the variety of methods used, the agreement between results reported in the literature by established researchers in this

field is surprisingly good. However, Peeling et al. [31] injected a note of caution when they reported wide variability between results from six laboratories using the same strains against a set panel of antimicrobial agents. For the future, the use of new technologies will need to be considered, e.g. reverse transcriptase PCR [32] and other molecular-based tests. These are likely to be less subjective, but more expensive. The following methods are based on those that have been used successfully at University College London Hospital (UCLH) for over 25 years, and form the basis for discussion on standardization.

Determination of MICs for *C. trachomatis*

Two methods are used for the determination of the MIC. A flat-bottomed microdilution plate technique is used with immunofluorescence staining, and a tube dilution method with coverslip monolayers is used for iodine staining. Two staining methods are used to ensure that the antimicrobials do not affect the staining reaction in one method compared with the other. The methods have been modified from Ridgway et al. [33]. Clinical isolates plus the SA₂f (control strain) of *C. trachomatis* are tested. The SA₂f strain is an LGVII serotype with serologic similarities to the genital serotypes.

Microdilution plate method

Doubling dilutions of the antimicrobial agent are prepared in antimicrobial-free MGM growth medium (Eagle minimal essential medium) supplemented with 1% glutamine 30 g/L, 1% vitamins, 14 mL of 7.5% sodium bicarbonate/L and 10% foetal calf serum supplemented with 1% 3 M glucose. One hundred microlitres of each antimicrobial dilution (double strength) is added to an appropriate well of a 96-well flat-bottomed microdilution plate containing a monolayer of 6-iodo-2-deoxyuridine (IUDR)-treated McCoy cells. One hundred microlitres of the chlamydial suspension made up in antimicrobial-free MGM growth medium, containing approximately 300 inclusion-forming units per mL (IFU/mL), is added to the relevant well. After centrifugation at 1800 rev/min for 1 h, the microtitre plate is incubated in a 10% CO₂ atmosphere for 38–40 h at 35 °C. The fluid is aspirated from the microtitre plates, and the cell monolayer fixed with methanol and stained with an immunofluorescent (IF) culture confirmation test, following the manufacturer's instructions (MicroTrak Syva Corp., PA, CA, USA), directed against *C. trachomatis* major outer membrane protein (MOMP). The MIC is taken as the lowest concentration completely inhibiting the formation of normal inclusions. Tests are performed in duplicate.

Tube dilution method

Tubes containing coverslip monolayers of IUDR-treated McCoy cells are prepared in advance. Prior to inoculation, the medium is aspirated and the monolayer inoculated with

250 µL of chlamydial suspension and 250 µL of the relevant antimicrobial dilution (double strength), centrifuged at 3600 rev/min for 1 h, and incubated as above. After incubation, the coverslips are fixed with methanol and stained with iodine. The MIC is taken as the lowest concentration completely inhibiting the formation of inclusions. Tests are performed in duplicate.

Determination of MICs for *C. pneumoniae*

The method is essentially as above for *C. trachomatis*, using cycloheximide-treated McCoy cells [34]. Coverslips are stained with an LPS-specific fluorescent monoclonal antibody (DAKO Laboratories, Ely, Cambridgeshire UK). Clinical isolates and the SA₂f (control strain) of *C. trachomatis* are tested.

Determination of MBCs for *C. trachomatis*

Clinical isolates and the SA₂f (control strain) of *C. trachomatis* are tested.

Four tubes containing coverslip monolayers of IUDR-treated McCoy cells are prepared for each dilution of antimicrobial, and processed as above. After incubation, the coverslips from each set of tubes (two tubes) are fixed and stained with IF and iodine, respectively. The medium is removed from the remaining two tubes and replaced with 1 mL of medium free from antimicrobial agent. The cells are scraped off the coverslip with a sterile pastette and resuspended in the medium, and each is used to inoculate two tubes containing fresh coverslip monolayers of IUDR-treated McCoy cells. After centrifugation, tubes are incubated and the process is repeated. This passage procedure is repeated as required, and the MCC taken as the lowest concentration of antimicrobial agent preventing demonstrable inclusions after several passages.

Strict precautions are taken to prevent cross-contamination with *C. trachomatis*. No other work with this organism is undertaken while passage experiments are being performed, safety cabinets are always used, and work proceeds from higher to lower concentrations of antimicrobial agent [35].

Determination of MBCs for *C. pneumoniae*

The method used is as described above for *C. trachomatis*, with modifications as described above for MICs for *C. pneumoniae*.

CONCLUSIONS

Testing the susceptibility of cell-associated pathogens requires very specific conditions, different in each case. Most of these bacteria are fastidious, requiring special conditions for in vitro cultivation. The different intracellular locations (in a phagosome, in the cytosol or, in some rare cases, in the nucleus of the

host cell) add another degree of complexity. The strategies developed by these micro-organisms for surviving in the hostile intracellular environment may also alter dramatically the physicochemical conditions found in the immediate vicinity of intracellular bacteria. For example, the phagosome-lysosome fusion is followed by a fall in pH, which compromises the optimal efficacy of agents such as macrolides and quinolones. In addition, the intracellular pharmacokinetics of the different antimicrobials used for treating infections with intracellular bacteria depend on the particular compound. Taken together, these distinctive in vivo conditions cannot be faithfully reproduced in the laboratory, and the interpretation of in vitro susceptibility tests must also take clinical experience into consideration. Many examples are known where clinical efficacy does not correlate with apparently susceptible results in vitro, such with as *Brucella* spp. and quinolones, or *Chlamydia* spp. and ciprofloxacin.

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