Bacterial Persistence

Methods and Protocols

Second Edition

Edited by

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Chapter 16

In Vitro Models for the Study of the Intracellular Activity of Antibiotics

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Abstract

Intracellular bacteria are poorly responsive to antibiotic treatment. Pharmacological studies are thus needed to determine the antibiotics which are the most potent or effective against intracellular bacteria as well as to explore the reasons for poor bacterial responsiveness. An in vitro pharmacodynamic model is described, consisting of (1) phagocytosis of preopsonized bacteria by eukaryotic cells, (2) elimination of noninternalized bacteria with gentamicin, (3) incubation of infected cells with antibiotics, and (4) determination of surviving bacteria by viable cell counting and normalization of the counts based on sample protein content. The use of strains expressing fluorescent proteins under the control of an inducible promoter allows to follow intracellular bacterial division at the individual level and therefore to monitor bacterial persisters that do not multiply anymore.

Key words Intracellular infection, Gentamicin, Antibiotic, Phagocytosis, Opsonization, Pharmacodynamics, Efficacy, Relative potency

1 Introduction

Intracellular survival of bacteria is now recognized as a major factor associated with dissemination, persistence, or recurrence of infections [1–7]. When residing inside eukaryotic cells, bacteria are indeed protected from the host humoral immune defenses and often adopt a dormant lifestyle less responsive to antibiotic action. Studies conducted over the last 10 years suggest that these dormant bacteria may correspond to bacterial persisters [8, 9]. Moreover, in order to exert their activity against intracellular bacteria, antibiotics have to gain access to the infected compartment within the cells and to express their activity in this specific environment [10, 11]. For these reasons, intracellular activity of antibiotics is unpredictable based on the simple evaluation of their activity against extracellular bacteria in broth and of their accumulation within eukaryotic cells.

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Appropriate models need to be developed for the correct assessment of the capacity of antibiotics to act upon intracellular bacteria.

We present here an in vitro model which allows studying the pharmacodynamics of antibiotics against intracellular bacteria. This model is highly flexible, being adaptable to several bacterial species or strains [12–15] as well as to many cell types [14, 16–18]. It has been used to compare the activity of commercially available antibiotics [12, 19] and of molecules in preclinical or clinical development (most of which are now registered or in the late phases in clinical trials [14, 16, 19–26]), with the aim of predicting their potential interest for the treatment of persistent infections. In the case of *Staphylococcus aureus* infections, it has been validated vs. animal models of intracellular infection [27, 28].

2 Materials

2.1 Equipment

1. Laminar flow hood: Work is performed in a laminar flow hood in a room with biosafety level adapted to the pathogenicity of the microorganism under investigation [29].
2. CO_2 incubator.
3. Bacteriology incubator.
4. Hemocytometer.
5. Spectrophotometer.

2.2 Reagents

1. Culture medium adapted for eukaryotic cell line use: Usually RPMI-1640 or DMEM, supplemented with 10% fetal calf serum.
2. Cation-adjusted Mueller-Hinton Broth (CA-MHB) and tryptic soy agar plates (TSA) (or any other specific medium more adapted to the bacterial species investigated).
3. Sterile distilled water.
4. Sterile phosphate buffer saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 1 L distilled water. Adjust to pH 7.4.
5. Human serum from healthy volunteers (for bacterial opsonization).
6. 40 mg/mL gentamicin stock solution.
7. Stock solution of the antibiotic under study.
8. Reagents (*see* **Note 1**) or kit (several kits are commercially available) for protein assay according to the Folin–Ciocalteu method, also referred to as Lowry's method [30].
9. Reagents (*see* **Note 2**) or kit for cell viability assay (e.g., trypan blue exclusion assay [31], or release of the cytosolic enzyme lactate dehydrogenase (LDH) [32]).

3 Methods

The method described is illustrated in Fig. 1.

3.1 Preparation of Bacterial Suspension and of Media

1. The day before the experiment, prepare an overnight bacterial culture in 15 mL of MHB (37 °C; agitation) to obtain a stationary-phase culture.
2. Unfreeze human serum.
3. Prewarm culture medium, sterile water, and PBS at 37 °C.

3.2 Opsonization of Bacteria

Opsonization is a process by which bacteria are marked by opsonins, which are serum proteins (like antibodies or complement proteins) bridging bacteria to the cell surface in order to favor phagocytosis (*see* **Note 3**).

1. Centrifuge the overnight culture to pellet bacteria (7 min at 3200 × *g*).
2. Resuspend in 1 mL of human serum; dilute with 9 mL of eukaryotic cell culture medium (not supplemented with fetal calf serum in this case, since human serum at a final concentration of 10% is present). Do not vortex.
3. Incubate for 30–60 min at 37 °C under gentle agitation (130 rpm) [12, 33].

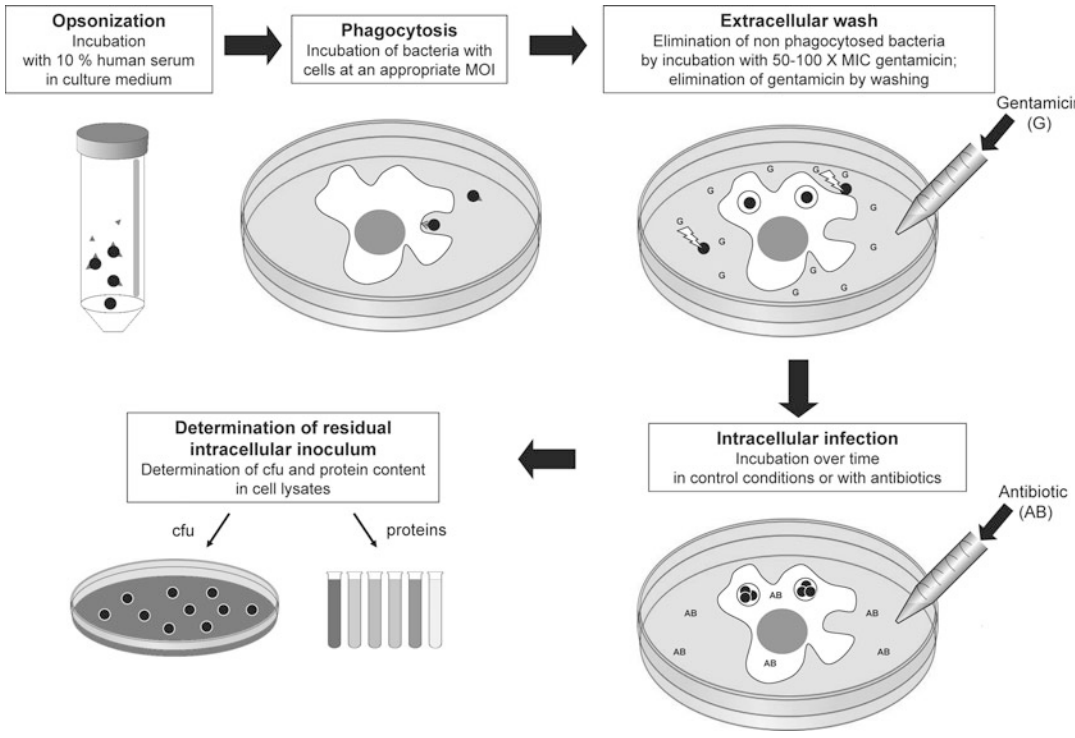

Fig. 1 In vitro model for the assessment of intracellular activity of antibiotics

3.3 Preparation of Eukaryotic Cells and Bacteria for Infection

1. If using eukaryotic cells in suspension, count them (for example using a hemocytometer) in order to obtain a density of 500,000–750,000 cells/mL (*see* **Note 4**).
2. If using adherent cells, plate them in multiwell plates. They should have reached 80% confluence at the time of the experiment. Prepare extra wells to be used for cell counting at the time of the infection.
3. Centrifuge opsonized bacteria for 7 min at 3200 × *g* and remove supernatant. Resuspend the pellet in 2 mL of PBS or culture medium and calculate the bacterial concentration, based on a calibration curve establishing the correlation between colony forming unit (CFU) counts and OD_{620nm} or on the turbidity of the bacterial suspension (McFarland).

3.4 Phagocytosis

This step is critical, in the sense that it is specific for each bacterial strain or species [12, 13, 19, 33, 34] and for the cell type to use for infection [14, 16, 17, 21] and should be adapted by the experimenter (Fig. 2). The objective is to obtain after phagocytosis an intracellular inoculum that is high enough to allow further growth of the bacteria but low enough to avoid killing the host cells (typically 10^6 CFU/mg cell protein). The general principle of this part of the protocol is explained hereafter.

1. Phagocytosis: Add bacterial suspension to cell suspension or to adherent cells in order to obtain the desired multiplicity of infection (MOI; number of bacteria/cell); when setting up the model, use in parallel different MOI (typically 1:1; 5:1; 10:1; 20:1; 50:1). Incubate at 37 °C in a CO_2 incubator for appropriate times; when setting up the model, compare different incubation times (typically 0.5 h, 1 h, 2 h).
2. Eliminate nonphagocytized bacteria either by centrifugation (cells in suspension; 7 min at 340 × *g*) or by elimination of the medium (adherent cells).
3. Reincubate infected cells during 45–60 min (37 °C; CO_2 incubator) in cell culture medium (without serum) containing gentamicin at high concentration (typically 50–100 times the minimal inhibitory concentration (MIC) for the bacterial strain used [12, 19]) in order to eliminate nonphagocytized bacteria that may adhere to the cell surface (*see* **Note 5**).
4. Wash three times with PBS at room temperature to eliminate bacterial debris and gentamicin.
5. Collect infected cells in 1 mL of sterile water in order to lyse them and allow for release of phagocytized bacteria.

6. Prepare logarithmic dilutions of the cell lysates in PBS and plate 50 µL on TSA or any other appropriate agar plate; proceed to colony counting after 24 h incubation.
7. In parallel, determine protein content of the cell lysates by the Folin–Ciocalteu method [30], using a commercial kit or the method described in **Note 1**.
8. Express the data as CFU/mg of cell protein and select for further experiments the conditions for which you obtain approximately 10^6 CFU/mg cell protein (*see* **Note 6**).

3.5 Intracellular Growth

1. Reincubate the infected cells in cell culture medium supplemented with 10% fetal calf serum. For control conditions, add gentamicin at a concentration close to the MIC (as measured in the culture medium used for the experiment) to avoid extracellular growth (Fig. 2) and, in case of cell killing, release of a small

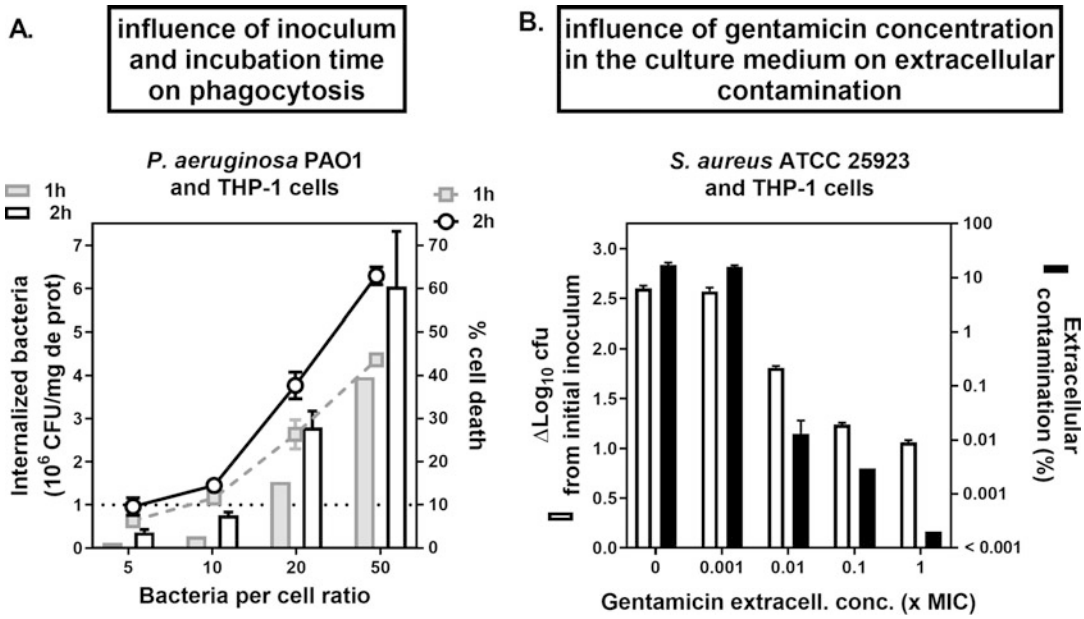

Fig. 2 Setting up a model of intracellular infection. (**a**) Determination of the optimal bacterial inoculum and phagocytosis time, as exemplified for *P. aeruginosa* PAO1 (adapted from [12]). Cells were incubated for 1 or 2 h with PAO1 at increasing bacteria-to-cell ratios (left axis). The percentage of mortality of THP-1 cells was assessed at the end of the phagocytosis period (right axis). Data for 1 h: gray symbols and bars; data for 2 h: open symbols and bars; the back bar and black dot correspond to the conditions considered as optimal for this model (dotted line: 10^6 CFU/mg protein with $<10\%$ cell toxicity). (**b**) Determination of the optimal concentration of gentamicin to add to culture medium of controls during incubation to avoid extracellular contamination, as exemplified for *S. aureus* ATCC25923 (adapted from [19]). Left axis: change in intracellular inoculum (log scale) after 24 h of incubation of infected cells in the presence of increasing concentrations of gentamicin (expressed in multiples of the MIC. Right axis: percentage of contamination of the extracellular medium in these conditions as assessed by the counting of colonies after plating of pooled culture fluids and washing media (limit of detection: 0.001%)

number of bacteria into the medium [19]. For experimental conditions, add the antibiotic you wish to test at the appropriate concentration in the culture medium (*see* **Notes 7** and **8**).

2. At the end of the incubation period, wash the cells three times in PBS and collect them in sterile distilled water as explained above (Subheading 3.4). Proceed to plating, CFU counting and protein assay.

3.6 Assessment of Antibiotic Intracellular Activity

The model described here allows to monitor antibiotic activity against intracellular bacteria over time or as a function of the extracellular concentration of the antibiotic (Fig. 3) [12, 19].

1. Considering time effects, bacterial growth is often delayed inside the cells (lag phase of a few hours), so that bacterial killing by antibiotics occurs slower than in broth. Moreover, the rate of bacterial killing by antibiotics is often biphasic, with

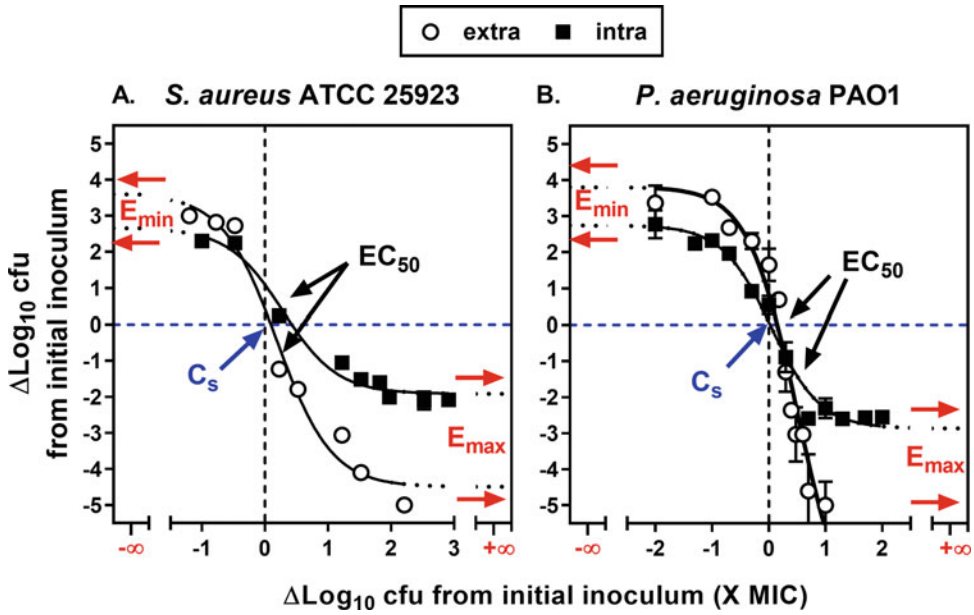

Fig. 3 Concentration–effect relationship for the extracellular and intracellular activity of antibiotics, exemplified for moxifloxacin against *S. aureus* (**a**) and *P. aeruginosa* (**b**). Comparison of the activity of moxifloxacin after 24 h incubation with moxifloxacin in broth (extracellular activity; open symbols) or in infected THP-1 cells (closed symbols). The ordinate shows the change in the number of CFU per mL (extracellular) or per mg cell protein (intracellular) compared to the postphagocytosis inoculum (blue horizontal line at 0). The abscissa shows the antibiotic concentration expressed as the $\log_{10}$ of its MIC in broth. The dotted line shows the MIC value. Data are used to fit Hill equations (slope factor = 1) and derive the pertinent key pharmacodynamic parameters, namely (1) E_{min} (change in CFU for an infinitely low antibiotic concentration; in red); (2) E_{max} (relative efficacy; maximal reduction in inoculum as extrapolated for an infinitely large concentration, in $\log_{10}$ CFU units compared to the original inoculum; in red); (3) EC_{50} (relative potency; concentration causing a reduction of the inoculum halfway between E_{min} and E_{max}, in black); (4) C_s (static concentration; concentration resulting in no apparent bacterial growth; in blue). Constructed based on data presented in [12, 15]

a first rapid drop in the number of CFUs, followed by a slower killing, which can even correspond to a plateau (no further decrease in CFUs). This is one of the hallmarks of persisters (Fig. 4).

2. Considering concentration effects, performing experiments with broad ranges of extracellular concentrations (from sub-MIC values to many times the MIC) allows obtaining full concentration–response curves for fitting with sigmoid regressions (Fig. 3).
3. Using the corresponding Hill's equation, key pharmacological descriptors of activity can be calculated.
   (a) The relative minimal efficacy (E_{min}; in $\log_{10}$ CFU units), that is, the increase in the number of CFU for an infinitely low antibiotic concentration compared to the original postphagocytosis inoculum.
   (b) The relative maximal efficacy (E_{max}; in $\log_{10}$ CFU units), that is, the decrease in the number of CFU for an infinitely large concentration of antibiotic.
   (c) The relative potency (EC_{50}; in mg/L or in multiples of MIC), that is, the concentration of antibiotic yielding a response half-way between E_{min} and E_{max}.
   (d) The static concentration (C_s; in mg/L or in multiple of MIC), that is, the concentration of antibiotic resulting in no apparent bacterial growth compared to the original inoculum [15].
4. Three major observations have been made with this type of model (Figs. 3 and 4).
   (a) First, the relative minimal efficacy is in general similar in the extracellular and intracellular models for facultative intracellular bacteria. Intracellular E_{min} should be considered as an "apparent" intracellular value, because in this case, the presence of extracellular bacteria that are not killed in the medium by subinhibitory concentrations of antibiotic cannot be excluded.
   (b) Second, the static concentration against intracellular bacteria (i.e., the antibiotic concentration preventing bacterial growth) is in most cases close to the MIC, suggesting that the potency of the drug is not directly correlated with its accumulation inside the cells, possibly because of poor intracellular bioavailability. The molecular reasons for this loss of potency inside the cells still remain to be established.
   (c) Third, the antibiotic maximal efficacy is in most cases much lower against intracellular bacteria than against extracellular bacteria, suggesting poor bacterial

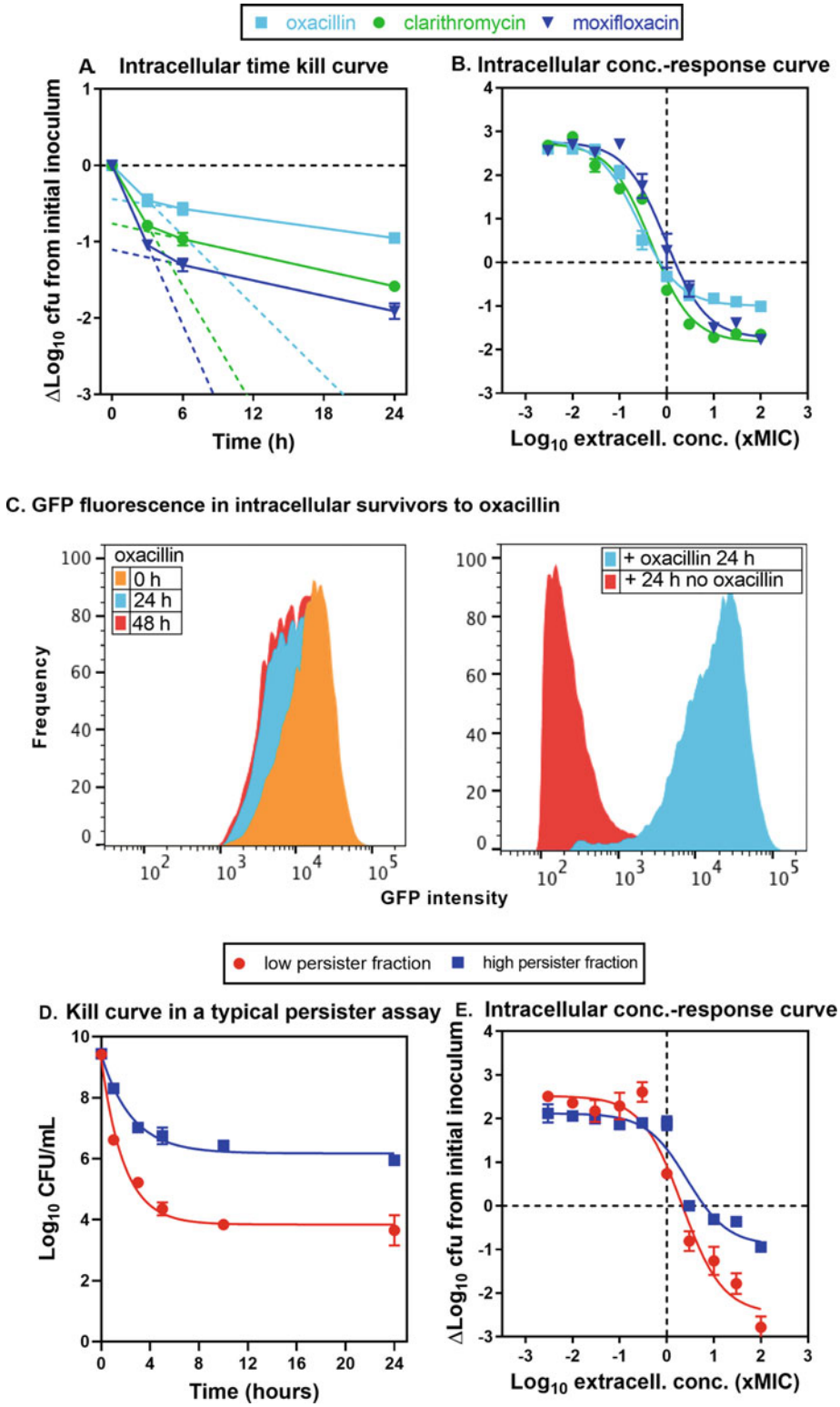

Fig. 4 Demonstration of intracellular persisters in *S. aureus* upon exposure to antibiotics at high concentrations. (**a**) Time-kill curves of *S. aureus* SH1000 in J774 mouse macrophages incubated with oxacillin,

responsiveness to antibiotic action in the intracellular environment. By means of a fluorescence dilution technique (described in Chapter 18), this has been recently ascribed for *S. aureus* to the fact that intracellular survivors have adopted a persister phenotype, characterized by a nondividing state, and reversible in permissive cells as soon as the antibiotic pressure is relieved [18]. The E_{max} value differs from one antibiotic to the other against a same strain, but may differ from one strain to the other with a same antibiotic. These discrepancies could find their explanation in the capacity of different antibiotics or strains to generate persisters [35]. Again the reasons for these differences need to be established.

4 Notes

1. Protein assay can be performed without any commercial kit, using the protocol described by Lowry [30]. Reagents required are Biuret reagent (extemporaneous mixture of 100 mL 2% Na_2CO_3, 1 mL 2% potassium sodium tartrate, 1 mL 1% $CuSO_4.5H_2O$), 2 N Folin–Ciocalteu reagent (diluted to 1 N), 1 N NaOH, and a standard (100 μg/mL bovine albumin). In brief, incubate 0.5 mL of cell lysate (or dilution thereof), blank (medium in which cells were collected), water (solvent of albumin standard) or albumin standard during 30–120 min with 0.5 mL 1 N NaOH. Subsequently, add

Fig. 4 (continued) clarithromycin, or moxifloxacin at 50× their respective MIC. The graph shows the biphasic killing rate, with a fast killing during the first 3 h and a slower killing thereafter (highlighted by dotted lines). The equation of these linear relationships allows to calculate a minimum duration of killing (MDK) for 90% of the population comprised between 2.9 and 6.6 h for the first phase but longer than 24 h for the second phase (adapted from [18]). (**b**) Concentration–response curves for the same antibiotics after 24 h of incubation of infected cells. A plateau is reached corresponding to a maximal reduction of 1–1.8 $\log_{10}$ CFU from the postphagocytosis, depending on the drug (adapted from [18]). (**c**) Flow cytometric profiles of the frequency of events as a function of GFP intensity over time for samples collected from an experiment similar to that described in panel **b** and incubated 0 h (postphagocytosis), 24 h, or 48 h with 50× the MIC of oxacillin (left) or 24 h with oxacillin then reincubated for 24 h in the absence of antibiotic (right). Cells were infected by SH1000 transformed by a plasmid expressing GFP under the control of an inducible promoter. The inducer is added during the prephagocytosis cultures only. Once the bacteria have been internalized, any dilution of the fluorescence signal can be interpreted as denoting bacterial division (adapted from [18]). (**d**) Time-kill curve of extracellular bacteria in stationary cultures exposed to moxifloxacin at 100× its MIC to calculate their persister fraction. The graph compares two clinical isolates harboring low (red) and high (blue) persister fractions (adapted from [35]). (**e**) Concentration–response curves for the same isolates in an intracellular model of infected THP-1 human monocytes incubated during 24 h with moxifloxacin. The graph shows that the E_{max} of moxifloxacin is higher (more negative) for the isolate harboring the lower persister fraction in stationary-phase culture (adapted from [35])

5 mL of Biuret reagent and incubate for 10–20 min. Next, add 0.5 ml of 1 N Folin reagent to each tube and read absorbance at 660 nm after 30 min of incubation (the last step needs to be done tube by tube and with a timer; incubation time should be strictly the same for each tube). The concentration of proteins in the sample is then calculated as ($[OD_{sample} - OD_{blank}]/[OD_{standard} - DO_{water}]$) × 100 µg/mL [standard concentration] × dilution factor) [3].

2. Viability can be easily assessed using a trypan blue exclusion test (vital colorant excluded from viable cells). To this effect, add 100 µL of cell suspension to 900 µL of trypan blue reagent, incubate for 10 min at 37 °C and determine the proportion of dead cells (colored in blue) by cell counting using a haemocytometer. An alternative method consists of measuring the release of LDH, a cytosolic enzyme, in the supernatant of a cell culture, which occurs upon permeabilization of the cell membrane. LDH viability kits are commercially available. The assay can also be performed using the method of Vassault [32], which measures the consumption of NADH upon reduction of pyruvate in lactate by LDH (Fig. 5).

   In brief, mix 50 µL of culture medium or 10 µL of cell lysate with 2.5 mL of 0.244 mM NADH solution in Tris buffer (81.3 mM Tris, 203.3 mM NaCl). Add 500 µL of 9.76 mM natrium pyruvate (prepared in the same buffer) and follow NADH consumption by measuring optical density at 339 nm immediately and then every min during 5 min. Cell mortality is evaluated by the ratio between LDH activity in the supernatant (estimated by $[OD_{0\ min} - OD_{5\ min}]$/µL of medium × total volume of the culture medium) and the total activity in the culture (sum of total activity in supernatant and total activity in cell lysate estimated as ($[OD_{0\ min} - OD_{5\ min}]$/µL of medium × total volume of cell lysate)).

3. When using obligatory or facultative intracellular organisms which are specifically equipped to use the serum complement to increase phagocytosis, opsonization causes massive infection of the cells [36]. Preopsonization is therefore not systematically required [37] and, alternatively, culture medium could be supplemented with decomplemented serum or calf serum (heated for 30 min at 56 °C [38]) to reduce phagocytosis in order to reach postphagocytosis inocula compatible with maintenance of cell viability for 24 h.

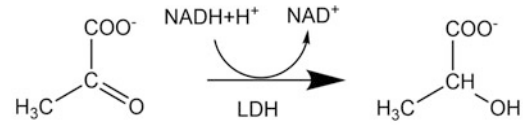

Fig. 5 Conversion of pyruvate to lactate by lactate dehydrogenase (LDH)

4. The number of eukaryotic cells to use depends on the virulence of the bacterial strain. For cytotoxic bacterial strains or species, use a higher eukaryotic cell number in order to keep enough cells after phagocytosis, as some killing may occur during this step [12].
5. A limitation of this assay is that the strain has to be susceptible to gentamicin. This antibiotic is selected for the elimination of nonphagocytized bacteria because it is rapidly bactericidal while at the same time entering only very slowly inside eukaryotic cells. It is therefore important to test for the susceptibility of the bacterial strain to gentamicin (MIC determination) before starting the experiment. Use of lysostaphin as a lytic agent for some extracellular bacteria (*S. aureus*) is also proposed in the literature but we showed that it enters inside the cells and may thus affect intracellular viability [33].
6. Depending on the virulence of the strain and its capacity to multiply intracellularly, it is important to check in parallel for the viability of the cells at the end of the phagocytosis period as well as at the end of the experiment. To this effect, a viability assay (trypan blue exclusion assay or LDH release assay, *see* **Note 2**) should be run in parallel and the postphagocytosis inoculum should be selected so as to guarantee cell viability.
7. Antibiotics or antibacterial agents (or even their solvent if not soluble in water) may also be toxic to eukaryotic cells. Again, it is important to check for cell viability in the presence of the tested agent for correct interpretation of the data. Massive cell death induced by the antibacterial agent can trigger bacterial release into the culture medium and therefore lead to the evaluation of the activity of the tested agent against extracellular bacteria rather than against intracellular bacteria [39].
8. For highly bactericidal antibiotics, check that the amount of carried-over antibiotic does not impair bacterial growth on the plates [33]. This can be done by comparing the number of CFU on plates from lysates preexposed or not to 12.5 mg/L charcoal (adsorbing residual antibiotic) during 10 min [20] or by plating bacteria on agar supplemented with 0.4% charcoal [13].

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