European Journal of Pharmaceutical Sciences 95 (2016) 2–16

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Single-cell level methods for studying the effect of antibiotics on bacteria during infection

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ARTICLE INFO

Article history:
Received 3 March 2016
Received in revised form 17 August 2016
Accepted 23 August 2016
Available online 28 August 2016

Keywords:
Antibiotics
Phenotypic heterogeneity
Antibacterial susceptibility
In vivo animal infection models
Single-cell level methods
Raman spectroscopy
Fluorescence

ABSTRACT

Considerable evidence about phenotypic heterogeneity among bacteria during infection has accumulated during recent years. This heterogeneity has to be considered if the mechanisms of infection and antibiotic action are to be understood, so we need to implement existing and find novel methods to monitor the effects of antibiotics on bacteria at the single-cell level. This review provides an overview of methods by which this aim can be achieved. Fluorescence label-based methods and Raman scattering as a label-free approach are discussed in particular detail. Other label-free methods that can provide single-cell level information, such as impedance spectroscopy and surface plasmon resonance, are briefly summarized. The advantages and disadvantages of these different methods are discussed in light of a challenging *in vivo* environment.

1. Introduction

Antibiotics are among the most influential tools of medicine. As the level of antibiotic resistance increases, measures have to be developed to counteract it. These measures are of two principal types: first, current antibiotics must be used more wisely; and second, new antibiotics must be developed. To achieve these aims, several measures have been suggested involving improved prescription practices and business incentives for developing new drugs. The outcome of antibiotic treatment can be influenced also by antibiotic tolerance that enables bacterial cells to survive a transient exposure to antibiotics at otherwise lethal concentrations (Brauner et al., 2016). In this context, it is essential that we improve our understanding of antibiotic action as well as mechanisms of antibiotic resistance and tolerance. The simple model system for studying the action of an antibiotic reveals its effect on bacterial cultures (Levison and Levison, 2009). The "final truth" about the usefulness of a potential antibiotic or improved dosing regimen comes from clinical trials, which consume considerable resources if time and money are considered (Hesterkamp, 2015). Nowadays, several model systems are deployed between these steps: mammalian cell culture for testing toxicity, co-cultivation of different cell types mimicking human tissues, animal models, *etc.* (Biganzoli et al., 1999; Velkov et al., 2013; Viluksela et al., 1996). In the current review, we focus on methods that could be developed further and used in an animal model, although the tools discussed are often applicable to model systems reconstructed from individual cells. There are several questions we want to answer with help of these systems.

First, the antibiotic has to reach the bacterium. Bulk physicochemical methods are commonly used to study the distribution of the antibiotic in the body, the pharmacokinetics of the drug (Schwameis and Zeitlinger, 2013). However, several infections are intracellular, and some bacteria are encapsulated in extracellular matrices, biofilms, *etc.* Therefore, the concentration of drug in the organ or tissue might not reflect the concentration encountered by the bacterium (Levison and Levison, 2009). Several novel spectroscopic methods allow drug concentration and uptake to be estimated with high spatial resolution. In addition, bacterial reporter systems could be very useful for estimating the actual drug concentration in the microenvironment of the bacterium. These reporters can be either based on the specific binding of the antibiotic to the reporter or measure the general physiological response of the bacterium to the antibiotic action.

Second, from the pharmacodynamics viewpoint, it is important to consider that during infection the bacterial population differentiates into subpopulations with different physiological properties. For example, dormant or actively effluxing bacteria can survive antibiotic treatment (Adams et al., 2011; Helaine et al., 2014). Sometimes, bacteria with a specific range of growth rates contribute most to the persistence of infection (Claudi et al., 2014). For efficient treatment, the different

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http://dx.doi.org/10.1016/j.ejps.2016.08.042
0928-0987/

subpopulations have to be combated; otherwise, even small surviving subpopulation can lead to a relapse or give rise to resistant bacteria (Cohen et al., 2013). It is therefore important to assess the physiological state of bacteria during infection and antibiotic treatment. As there is considerable variation among bacteria, measurements have to be performed at the single-cell level; the population average often describes the real situation very poorly. Several fluorescent protein-based reporters have been developed for bioreporting, and label-free methods for a wide range of applications are developing rapidly.

2. Infection and antibiotic treatment monitoring in animal models

Antibiotic pharmacodynamics is often studied in sole bacterial cultures. However, to understand the effect of the antibacterial drug and also the important interactions between the bacterial and eukaryotic cells infection models have to be employed. For *in vitro* studies, cell lines and primary cells can also be used, but animal models help to reveal the very complex temporal relationships that occur in infectious disease involving the host, its neuroendocrine and immune systems, the pathogen, and antibiotics. The development of a suitable *in vivo* animal model will allow new therapeutic agents or better treatment protocols to be developed.

Animal models have been widely used in studies of infection by such bacteria as enterohepatic *Escherichia coli* (Savkovic et al., 2005), *Helicobacter pylori* (Marchetti et al., 1995), *Mycobacterium tuberculosis* (Lenaerts et al., 2005), and *Salmonella* (Hapfelmeier and Hardt, 2005; Mastroeni and Sheppard, 2004; Santos et al., 2001). Different laboratory animal models are used for such studies, but mouse, rat, and pig models have been most favored. In addition, the vertebrate zebrafish (*Danio rerio*), and non-vertebrate insects and nematodes (*e.g.*, *Caenorhabditis elegans*), are suitable testing some aspects of antibacterial susceptibility during infection (López Hernández et al., 2015). Monitoring of bacteria *in vivo* (progression of infection) is challenging and usually requires invasive methods.

The most commonly used method for determining the drug efficacy is the measured bacterial count after 24 h of treatment, often expressed as the change in log colony forming units (CFU) in the tissue compared with untreated animals (Nielsen and Friberg, 2013). Considerably more information can be collected by methods that also allow antibacterial drug susceptibility to be monitored over time. Several examples in the literature illustrate how antibacterial drug susceptibility can be revealed using *in vivo* whole-body bioluminescence and fluorescence imaging (Andreu et al., 2013; Zelmer et al., 2012). Tissue imaging with two-photon microscopy has higher spatial resolution and has been successfully used in infection studies (Campbell-Valois and Sansonetti, 2014; Richter-Dahlfors et al., 2012; Torstensson et al., 2005). Fluorescence imaging techniques differ from traditional fluorescence microscopy techniques in that living tissues are investigated instead of a fixed sample (Yuste, 2005). *In vivo* optical imaging usually involves the engineering of bacteria with genetic expression constructs for luminescence (*e.g.*, the lux operon) or fluorescent proteins (green fluorescent protein, GFP, mCherry, *etc.*), which are easily exploited for whole-body optical imaging and tracking of the bacteria. Stanton et al. have recently described a novel strategy using endogenous bacterial enzymatic activity to activate an exogenously administered fluorescent imaging probe specifically (Stanton et al., 2015). The authors showed that the dose-responsive bacteria-specific signals obtained *in vitro* with *E. coli*, *Salmonella*, *Listeria*, *Bifidobacterium*, and *Clostridium difficile* were applicable to whole-body imaging in various infection models. Furthermore, using metabolic oligosaccharide engineering and biorthogonal click chemistry, labeled *Bacteroides fragilis* was tracked in mice. It was shown that the bacteria could be located within the intestinal tract and their growth monitored over time, revealing the number of bacteria (Geva-Zatorsky et al., 2015).

If only whole-body imaging methods are used, only the total bacterial load is measured, so information about bacterial population heterogeneity that might lead to heterogeneity in drug action cannot be elicited. Studies at the single-cell level are needed to elucidate bacterial infection and antibiotic action further.

2.1. Tools for separating and analyzing bacterial cells at the single-cell level

There is growing interest in using single-cell tools to monitor the heterogeneity of live cells (Klepárník and Foret, 2013; Wang and Bodovitz, 2010; Yin and Marshall, 2012). Single-cell level methods comprise both separation and analytical techniques (Klepárník and Foret, 2013). Many recent improvements in microscopy have introduced new detection principles and high-throughput formats that allow signals to be collected from large microscopic fields. This enables the detection of bacteria in whole animals, organs, and tissue samples to be improved. During many infections and especially after antibiotic treatment, bacterial counts are often very low but still significant for the treatment outcome. In these cases, the bacteria should be isolated so they can be analyzed. In several cases, the separation technique platforms can be integrated with various analytical tools (optical and spectroscopic). Some examples of conventional separation techniques include centrifugation, capillary electrophoresis, liquid chromatography, microfluidics, and dielectrophoresis (Chrimes et al., 2013; Gagnon, 2011; Kim et al., 2015; Klepárník and Foret, 2013; Li et al., 2012; Liao et al., 2015; Lin et al., 2011; Yin and Marshall, 2012). Techniques that allow single cells to be separated include optical tweezers (Xie et al., 2005a) and flow cytometry (Wang et al., 2013; Watson et al., 2008). In several cases, these could also be used in combination (Huang et al., 2015, 2009; Zhang et al., 2015a,b). Such methods are crucial when real *in vivo* samples are investigated and single-cell level information about the physiological state of the bacteria is needed.

2.2. Physiological heterogeneity of bacteria during infection

Several *in vivo* infection studies have revealed how the phenotypic heterogeneity of a pathogen contributes to the survival of cells during antibiotic treatment. These pioneering studies, briefly described below, demonstrate the importance of implementing bacterial single-cell analysis into studies of antibiotic pharmacodynamics.

It is well established in *in vitro* cultures that bacterial growth rate influences antibiotic sensitivity: non-growing persister cells are more likely to survive antibiotic treatment than growing cells (Lewis, 2010). The earliest *in vivo* studies have suggested that the same is also true during mouse infection. Fluorescent single-cell analysis has enabled antibiotic-tolerant *Salmonella* cells to be identified during infection. It was shown that the vacuolar environment in mouse macrophages induces phenotypic heterogeneity, leading to either bacterial replication or the formation of non-replicating antibiotic-tolerant persisters (Helaine et al., 2014). Another study has confirmed that non-dividing *Salmonella* survived the antibiotic treatment best, but the overall clearance of infection was delayed mainly by the abundant subpopulation of moderately growing *Salmonella* with partial tolerance (Claudi et al., 2014).

In addition, studies of bacterial virulence factors such as the type three secretion system (T3S) have linked T3S expression levels to the rate of bacterial cell growth. It has been shown that virulence gene expression causes a fitness cost to individual bacteria but benefiting the total population. The slow growth of bacteria expressing virulence genes can be compensated by a faster-growing subset of bacteria with low virulence gene expression (Ackermann et al., 2008; Diard et al., 2013; Sturm et al., 2011). Results by Arnoldini and coworkers suggest a general principle of antibiotic evasion by pathogens: the expression of virulence factors often causes metabolic costs and the resulting slow growth could generally increase antibiotic tolerance (Arnoldini et al., 2014).

In experimental infection models, and in patients, the outcome of antibiotic treatment is influenced by the immune system. One general mechanism employed by the immune system to combat invading