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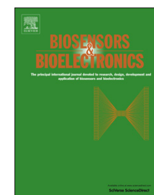

Probing minority population of antibiotic-resistant bacteria

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The evolution and spread of antibiotic-resistant pathogens has become a major threat to public health. Advanced tools are urgently needed to quickly diagnose antibiotic-resistant infections to initiate appropriate treatment. Here we report the development of a highly sensitive flow cytometric method to probe minority population of antibiotic-resistant bacteria via single cell detection. Monoclonal antibody against TEM-1 β-lactamase and Alexa Fluor 488-conjugated secondary antibody were used to selectively label resistant bacteria green, and nucleic acid dye SYTO 62 was used to stain all the bacteria red. A laboratory-built high sensitivity flow cytometer (HSFCM) was applied to simultaneously detect the side scatter and dual-color fluorescence signals of single bacteria. By using *E. coli* JM109/pUC19 and *E. coli* JM109 as the model systems for antibiotic-resistant and antibiotic-susceptible bacteria, respectively, as low as 0.1% of antibiotic-resistant bacteria were accurately quantified. By monitoring the dynamic population change of a bacterial culture with the administration of antibiotics, we confirmed that under the antimicrobial pressure, the original low population of antibiotic-resistant bacteria outcompeted susceptible strains and became the dominant population after 5 hours of growth. Detection of antibiotic-resistant infection in clinical urine samples was achieved without cultivation, and the bacterial load of susceptible and resistant strains can be faithfully quantified. Overall, the HSFCM-based quantitative method provides a powerful tool for the fundamental studies of antibiotic resistance and holds the potential to provide rapid and precise guidance in clinical therapies.

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1. Introduction

Antimicrobial resistance has emerged as one of the greatest threats to human health, and rapid detection of antibiotic-resistant pathogens is urgently needed to guide appropriate clinical treatment (Furuya and Lowy, 2006; Woolhouse and Farrar, 2014). Among many molecular mechanisms that confer antibiotic resistance, production of β-lactamases that catalyze the hydrolysis of β-lactam antibiotics is a major and threatening mechanism (Blair et al., 2015; Lin et al., 2015; Walsh, 2000). On the other hand, it has been reported that individuals could be simultaneously infected with multiple strains of different susceptibility levels (Bailey et al., 2010; Braden et al., 2001; Cohen et al., 2012), and the population of resistant bacteria could be as low as 0.7% (Bailey et al., 2010). However, if the minority population of resistant bacteria cannot be detected in time, an inappropriate prescription of antibiotics is usually a result.

Methods commonly used for antibiotic resistance detection are based upon antibiotic susceptibility testing, such as broth dilution, disk diffusion, and E-test (Schrag et al., 2000; Wiegand et al., 2008). These culture-based bulk assays measure the growth capability of the bacteria under the pressure of antibiotics, and are time-consuming (>48 h) and labor intensive. It is worthy to note that the commercially available automated systems for rapid bacterial identification (ID) and antimicrobial susceptibility testing (AST), e.g. the BD Phoenix system and the bioMérieux VITEK 2 system, are not for use directly with clinical specimens. Isolated colonies of each type of organism should be selected from a primary 18- to 24-hour agar plate, inoculated into a suitable broth medium, and cultivated until it exceeds the turbidity of the 0.5 McFarland standard (usually 2–6 hours) prior to analysis on the system. Particularly the presence of minority population of antibiotic-resistant bacteria can be easily overlooked. During the past few decades, many methods have been developed for the rapid assessment of antibiotic susceptibility, such as mass

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spectrometry to measure the specific peptides or the activity of β-lactamases (Fleurbaaij et al., 2014; Wang et al., 2013), surface plasmon resonance-based biosensors (Chiang et al., 2009; Tawil et al., 2013), and multiplexed microfluid platform (Mohan et al., 2013). Although the antimicrobial resistance can be determined as rapidly as hours, population heterogeneity can hardly be assessed by these ensemble-averaged analyses. Recently, by using GFP as a β-lactamase indicator and acquiring time-lapse images at the single-cell level, a microfluidic device was designed to study antibiotic resistance of β-lactamase bacteria under different nutrition limitations (Wang et al., 2015). Nevertheless, the large heterogeneity of complex and clonal bacterial populations calls for high-throughput measurements of individual cells.

Flow cytometry is a powerful technique for the rapid, quantitative, and multiparameter measurement of single cells or cell-sized particles. However, flow cytometric analysis of individual bacterial cells has been difficult due to their small particle sizes and low quantity of target molecules. Bright fluorescent staining is generally required to discriminate bacterial cells against background signals generated from the instrument noise and the impurity particles in the sheath/sample fluid (Wu et al., 2016). Although flow cytometric analysis of bacteria resistance has been conducted *via* analyzing the efflux pump activity (Farooq et al., 2014) or measuring the bacterial susceptibility upon antibiotic exposure (Soejima et al., 2012), β-lactamase detection in single bacteria has not been reported before. Adopting strategies for single-molecule fluorescence detection in a sheathed flow (Keller et al. 1996), we have recently developed high sensitivity flow cytometry (HSFCM) that is orders of magnitude more sensitive than the conventional flow cytometry (Yang et al., 2009; Zhu et al., 2014; Zhu et al., 2010). Besides the sensitivity gain arising from a reduced detection volume for background reduction and an extending particle transit time through the laser beam for increased photon generation, the significantly reduced sheath flow rate (tens of microliters per minute versus ~15 milliliter per minute) leads to a considerable reduction in the interference of impurity particles. Applications in the detection of specific pathogenic strain (Yang et al., 2010), quantification of bacterial autofluorescence (Yang et al., 2012), analysis of low copy number β-galactosidase (Yang et al., 2013), and bacterial enumeration in drinking water and tea beverages (Yu et al., 2015) have been demonstrated. Particular, employing β-lactamase-induced covalent labeling of fluorescence, we achieved quantitative detection of the resistant bacteria population down to 5% (Shao et al., 2013). Here we report the development of a dual fluorescent staining assay (antibody and DNA) for the quantitative measurement of minority population of antibiotic-resistant bacteria down to 0.1%. The utility of the method in the rapid diagnosis of antibiotic-resistant infection was successfully demonstrated in clinical urine samples without bacterial cultivation.

2. Experimental section

2.1. Reagents and chemicals

Monoclonal antibody (mAb) against β−lactamase was purchased from Abcam (Cambridge, MA, USA). Alexa Fluor 488 (AF488)-conjugated donkey anti-mouse (DAM) IgG (H+L) and SYTO 62 nucleic acid stain were purchased from Molecular Probes (Eugene, OR, USA). Nitrocefin was obtained from EMD Chemicals (Gibbstown, NJ, USA). Fetal bovine serum (FBS) obtained from Hyclone (Logan, Utah, USA) was freshly reconstituted at 1% in PBS. Egg-white lysozyme was purchased from Sigma-Aldrich (St. Louis, MO, USA) and 50 μg/mL solution was freshly prepared in H_2O. Centrifugation (12000 g, 10 min, 4 °C) was carried out prior to the use of FBS or lysozyme solutions. Paraformaldehyde (PFA) was obtained from Alfa Aesar (Ward Hill, MA, USA). PopCulture Reagent was purchased from Novagen (Madison, WI, USA). Müller-Hinton agar was purchased from Hangzhou Binhe Microorganism Reagent (Hangzhou, Zhejiang, China). Bacterial strain *E. coli* ATCC 25922 and antibiotic disks were purchased from Hangzhou Microbial Reagent (Hangzhou, Zhejiang, China). Tryptone, yeast extract, and agar were obtained from Sangon Biotech (Shanghai, China). *E. coli* ATCC 35218 was obtained from the American Type Culture Collection (ATCC). Carbenicillin was purchased from Tiangen Biotech (Beijing, China). Other reagents were purchased from Sinopharm Chemical Reagent (Shanghai, China). Distilled, deionized water supplied by a Milli-Q RG unit was filtered through a 0.22 μm filter and used for buffer preparation.

2.2. Bacterial strains and culture

Bacterial strain *E. coli* JM109 and plasmid pUC19 were obtained from Sangon Biotech (Shanghai, China). Plasmid vector pUC19 has one amp^R gene (ampicillin resistance gene) which encodes TEM-1 β-lactamase. A single colony of *E. coli* JM109 or ampicillin-resistant JM109/pUC19 on a solid Luria-Bertani (LB) (10 g tryptone, 5 g yeast extract, and 15 g agar per liter) agar plate was transferred to a 2 mL liquid LB culture medium without or with 100 μg/mL carbenicillin, respectively. The bacterial samples were cultured overnight in an incubator shaker at 37 °C and 220 rpm. Then 150 μL *E. coli* JM109 or *E. coli* JM19/pUC109 were inoculated in 15 mL fresh LB medium without or with 100 μg/mL carbenicillin, respectively. The samples were grown at 37 °C and 220 rpm until the extinction at 600 nm (OD600) reached 0.8–1.0 (~3.5 h) to the mid log phase. The *E. coli* samples were harvested by centrifugation at 10,000 rpm for 5 min, resuspended in PBS with OD600 adjusted to ~1.2 (concentration ~1×10^9 cells/mL). The harvested bacterial samples were stored at 4 °C and analyzed on the HSFCM within 24 h.

2.3. Dual fluorescent staining of bacterial cells

The harvested bacterial cells were treated as follows: (1) 100 μL of the harvested bacteria were centrifuged and resuspended in 100 μL of 4% PFA and incubated at 37 °C for 10 min. (2) After centrifugation at 5000 g for 5 min, the precipitate was resuspended in 100 μL of 50 μg/mL lysozyme and incubated at 37 °C for 20 min. (3) After centrifugation at 5000 g for 5 min, 100 μL 1% FBS was added to the precipitate to block the bacterial cells at room temperature for 30 min. (4) The bacterial sample was centrifuged at 5000 g for 5 min, and then 100 μL of 5 μg/mL anti-β lactamase mAb was added. The suspension was incubated for 1 h at 37 °C. (5) After washing twice with PBS, 100 μL of 5 μg/mL AF488-DAM IgG was added and incubated at 37 °C for 1 h. (6) The sample was centrifuged, resuspended in 100 μL PBS containing 1 μM SYTO 62, and then analyzed on the HSFCM.

2.4. Disc diffusion test (K-B method) and β-lactamase activity assay

Disc diffusion test (K-B Method) was used for susceptibility test by following the Clinical and Laboratory Standards Institute (CLSI) standards guidelines (detailed procedures are given in the Supplementary Information) and *E. coli* ATCC 25922 was used as the positive control. For the β-lactamase activity assay, bacterial mixtures with different percentages of antibiotic-resistant cells were diluted to 100-fold. Then 10 μL PopCulture Reagent and 1 μL of 1 mg/mL lysozyme were added into the 90 μL 100-fold diluted bacterial cells. After 15 min incubation at the room temperature, 5 μL of 10 mM nitrocefin was added and then incubated at 37 °C for 3 min. After centrifugation at 10000 rpm for 5 min, 80 μL of the supernatant was transferred to a 96-well plate and analyzed by a

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