



An integrated microfluidic chip-mass spectrometry system for rapid antimicrobial resistance analysis of bacteria producing β -lactamases

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ABSTRACT

Bacteria producing β -lactamases have become a major issue in the global public health field. To restrain the development of drug resistance and reduce the abuse of antibiotics, it is very important to rapidly identify bacteria producing β -lactamases and put forward a reasonable treatment plan. Here, an integrated microfluidic chip-mass spectrometry system was proposed for rapid screening of β -lactamase-producing bacteria and optimization of β -lactamase inhibitor dosing concentration. The concentration gradient generator followed by an array of bacterial culture chambers, as well as micro-solid-phase extraction columns was designed for sample pretreatment before mass analysis. By using the combination system, the process of the hydrolysis of antibiotics by β -lactamase-producing bacteria could be analyzed. To validate the feasibility, four antibiotics and two antibiotic inhibitors were investigated using three strains including negative control, SHV-1 and TEM-1 strains. SHV-1 and TEM-1 strains were successfully distinguished as the β -lactamase producing strains. And the acquired optimal concentrations of β -lactamase inhibitors were in accordance with the results by that obtained from the traditional microdilution broth method. The total analysis time only needed around 2 h, which was faster than conventional methods that require a few days. The technique presented herein provides an easy and rapid protocol for β -lactamase resistance related studies, which is important for the inhibition of antimicrobial resistance development and the reduction of antibiotics abuse.

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Antimicrobial resistance (AMR) is considered an increasingly serious threat to global public health by the World Health Organization [1]. Nowadays, about 700,000 deaths all over the world every year are due to resistant pathogens [2]. Unfortunately, the development of AMR is being accelerated because of the abuse of antibiotics including misuse and overuse by people and animals [3]. Because of the high activities and low side effects of β -lactam antibiotics, they account for about 60% of antibiotic usage [4], which makes the treatment of bacteria resistant to β -lactam antibiotics the most significant among resistant pathogens. What's more, in Gram-negative bacteria, the synthesis of β -lactamases is the major mechanism of resistance to β -lactam antibiotics [5]. To inhibit the AMR development of bacteria producing β -lactamases, rapid and

precise tools for their AMR analysis containing identification and antibiotic susceptibility test are in urgent need.

The most common methods used for AMR analysis include plate culture methods and genotypic tests. As the gold standard, culture-based methods, like broth microdilution and disk diffusion, determined the susceptibility of strains according to their growth under antibiotics. However, these methods take 24–48 h [6], which is time-consuming, and not suitable for clinical testing. As for genotypic methods such as PCR, although resistance genes could be detected using very few bacteria, new resistance genes couldn't be detected and the phenotypic expression of genes may be modified by other factors [7,8]. Considering the limitations of the existing methods, it is still necessary to explore novel and rapid methods for AMR analysis.

Microfluidic chip used for bacteria analysis has shown great potential to overcome the above problems due to many attractive features [9], such as high throughput, real-time, fast analysis, and low consumption of reagents. AMR analysis has already been carried out on microfluidic-based platforms by characterization of

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the growth [10–13] or morphological changes [14,15] of bacteria. However, these methods based on morphological changes or the analysis of bacteria still need more exploration to verify their practical clinical applications. For bacteria producing β -lactamases, β -lactamases could hydrolyze the amide bond of the β -lactam ring, thereby inactivating β -lactam antibiotics [16]. The hydrolysate of β -lactam antibiotics could be used as an important marker for identifying β -lactamase-producing microbial strains and screening for β -lactamase inhibitors. Based on the above drug resistance mechanism, mass spectrometry (MS) is an ideal selection for AMR analysis because of its high sensitivity and high resolution, and can provide structural information for metabolites analysis. Besides, MS has already been successfully used for AMR analysis [17–19]. Although the microfluidic chip-mass spectrometry (chip-MS) system has been widely used for cellular drug metabolism studies [20–22], the application of AMR analysis based on metabolites of antibiotics is rarely reported. Only Zhang *et al.* developed a system based on the detection of β -lactam antibiotic hydrolysates for the screening of bacteria producing extended spectrum β -lactamases (ESBLs) [23], which proved effective for AMR analysis of bacteria producing ESBLs. However, they lacked the sample pretreatment, which is generally a necessary procedure to remove undesired compounds such as salts, and buffers that interfere with MS to obtain highly sensitive signals. Micro-solid-phase extraction (μ SPE) columns are an important and widely used method in the sample preparation, pre-concentration, and cleanup interferences from analytical samples. Up to now, the μ SPE columns have been successfully integrated into microfluidic devices for various applications [24,25].

In this work, we presented an integrated microfluidic system with three functional parts including a Christmas-tree concentration gradient generator, a bacterial culture chamber, and the μ SPE columns, which were directly coupled to electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS) (Fig. 1) for AMR analysis. The channel of the Christmas-tree concentration gradient generator was 100 μ m wide and 100 μ m long with two inlets and seven outlets for generating seven concentration gradients. Meanwhile, the chip for bacteria culture and drug treatment consisted of seven parallel channels (2 mm wide, 10 mm long, and 100 μ m high). The μ SPE columns were 2.5 mm wide, 18 mm long, and 100 μ m high with 30 μ m intervals at the end of micro-column array for the immobilization of SPE particles. The design layout is shown in Fig. S1 (supporting information). Also, the fabrication of chip-MS system is shown in the Supporting information. Moreover, the three parts could be flexibly connected by polytetrafluoroethylene tubes. For the identification of bacteria producing β -lactamases, the bacteria were cultured in the culture chamber and incubated with antibiotics for a certain time. For the investigation of the optimal β -lactamase inhibitor dosing concentration, the concentration gradient generator for the generation of β -lactamase inhibitor concentration gra-

dient was connected with the bacteria culture chamber to incubate with the same concentration of bacteria. When detecting the hydrolysis product of antibiotics by bacteria, the bacteria culture chambers were connected with μ SPE columns. After elution, the μ SPE columns were directly coupled to ESI-Q-TOF-MS for online detection. Our established system constructed an integrated tool for rapid screening of β -lactamase-producing bacteria and optimization of β -lactamase inhibitors dosing concentration, providing a new approach to β -lactamase-related research.

Three strains were utilized including the negative control (NC) strain, SHV-1 strain, and TEM-1 strain. All strains were constructed with *Escherichia coli* (*E. coli*) DH5 α as the host strain and the pET28a(+) plasmid as the vector. NC strain did not produce β -lactamases, while SHV-1 strain and TEM-1 strain could produce β -lactamase because they express the SHV-1 gene and the TEM-1 gene separately [26]. The construction of bacterial strains and gene sequences of SHV-1 and TEM-1 are shown in Supporting information. The relationship between bacterial concentrations of the obtained SHV-1 strain and the optical density at 600 nm (OD₆₀₀) was then determined by the dilution plate counting method, and the fitting formula was $y = 9.77x$, $R^2 = 0.986$ (Fig. S2 in Supporting information).

To verify the function of the concentration gradient generator, ultrapure water and 75 μ mol/L sodium fluorescein solution both containing NC strain with OD₆₀₀ of 0.5 were pumped into the two inlets of the chip respectively. Then, the fluorescence intensities at the outlets of the dilution unit were detected by a fluorescent inverted microscope. As shown in Fig. S3 (Supporting information), the fitting formula was $y = 15.3x - 20.5$, $R^2 = 0.980$. Results indicated the concentration gradient generator could generate a precise concentration gradient. Next, different SPE materials filled in the μ SPE columns, compositions of the eluent solvent, formic acid content, and flow rates of the eluent solvent that had great effects on the response intensity of the ESI-Q-TOF-MS were optimized. According to the results shown in Fig. S4 (Supporting information), HLB SPE particles were the ideal SPE material, 80% methanol containing 0.5% formic acid was the optimal elution solvent, and the best flow rate of the eluent was 15 μ L/min. In addition, standard solutions with 10 μ g/mL of ampicillin (AMP), cloxacillin (CLO), cephalixin (LEX) and cefotaxime (CTX) were analyzed to optimize the ESI-Q-TOF-MS parameters used in the multi reaction monitoring method, and the parameters were outlined in Table S1 (Supporting information).

AMP was chosen as a model drug to identify bacteria producing β -lactamase because it can be hydrolyzed by almost all β -lactamases. The reaction of AMP hydrolysis by β -lactamases was shown in Fig. 2a. After incubating 10 μ g/mL AMP with SHV-1 bacteria in a PE tube for 30 min, the medium was pumped into the

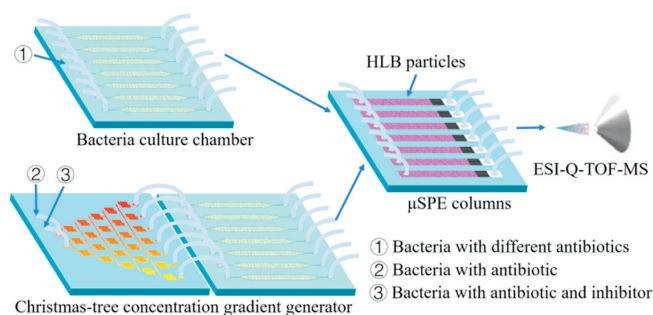


Fig. 1. Schematic diagram of the microfluidic chip-mass spectrometry system including a Christmas-tree concentration gradient generator, a bacteria culture chamber, and micro-solid-phase extraction (μ SPE) columns, coupled with ESI-Q-TOF-MS.

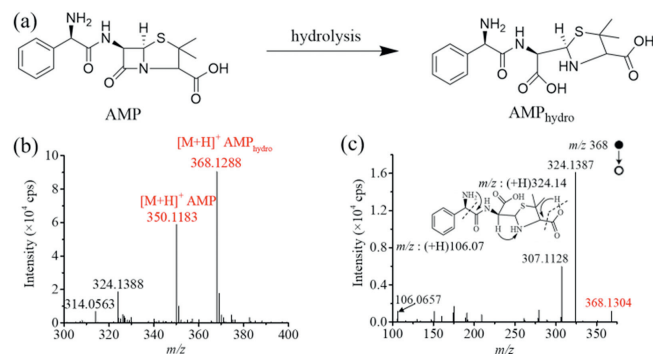


Fig. 2. (a) The hydrolysis reaction of ampicillin (AMP). (b) The mass spectrum of the hydrolysis reaction of AMP, and (c) the MS/MS spectrum of the hydrolysate of AMP.

μ SPE columns, and finally analyzed by ESI-Q-TOF-MS. As shown in Fig. 2b, the protonated AMP ion $[\text{AMP}+\text{H}]^+$ was observed at the peak of m/z 350.12, and the peak of m/z 368.13 corresponded to protonated hydrolysate of AMP $[\text{AMP}_{\text{hydro}}+\text{H}]^+$. Moreover, as shown in Fig. 2c, the ion of $[\text{AMP}_{\text{hydro}}+\text{H}]^+$ was further analyzed in the tandem mass spectrometry (MS/MS) mode, and the monitoring ions of m/z 368.13 and its fragments of m/z 324.13 and 106.07 confirmed the presence of $[\text{AMP}_{\text{hydro}}+\text{H}]^+$. These results were consistent with the previously reported data [27], indicating that the peak of m/z 368.13 was indeed the hydrolysate of AMP ($\text{AMP}_{\text{hydro}}$).

The concentration of bacteria on the microfluidic chip is an important parameter for AMR analysis, because too few bacteria would make slower hydrolysis of the antibiotic, resulting longer analysis time. However, too many bacteria would fail to achieve detection by ESI-Q-TOF-MS due to a large number of antibiotics absorbed into bacteria with very little or no antibiotics left in the culture medium. To optimize this parameter, different concentrations of bacterial solution of the SHV-1 strain were loaded onto the chip and incubated for 30 min, and then detected online by ESI-Q-TOF-MS. As shown in Fig. S5 (Supporting information), as the bacterial concentration increased, the number of β -lactamases increased, and thus more AMP was hydrolyzed resulting in the decrease of AMP intensity and increase of $\text{AMP}_{\text{hydro}}$ intensity. 7.38×10^8 CFU/mL was selected as the optimal bacterial concentration due to the highest $\text{AMP}_{\text{hydro}}$ intensity. 30 min was adequate for analysis due to none of the AMP remaining, which was used in subsequent experiments.

Firstly, four different experimental groups including BC group with no strains, NC group, SHV-1 strain group, and TEM-1 strain group were set up to explore whether the established chip-MS system could rapidly identify bacteria capable of producing β -lactamases. They were incubated with AMP in the bacteria culture chamber separately. Then, the chip was incubated at 37 °C for 30 min to make bacteria fully interact with antibiotics. Next, μ SPE columns were used not only for the removal of bacteria, salts, and other possible interferents but also for the enrichment of antibiotics and their hydrolysates. Finally, antibiotics and their hydrolysates were online analyzed by ESI-Q-TOF-MS.

As shown in Fig. 3a, compared with the BC group, the NC group showed a lower intensity of AMP and almost the same intensity of $\text{AMP}_{\text{hydro}}$. The decrease in AMP intensity indicated a portion of AMP was absorbed into bacteria. However, AMP was almost not

hydrolyzed in the NC group because $\text{AMP}_{\text{hydro}}$ did not have significant change, which indicated that the NC strain was unable to produce β -lactamase. Meanwhile, the SHV-1 and the TEM-1 groups had an extremely low intensity of AMP and a much higher intensity of $\text{AMP}_{\text{hydro}}$, suggesting that AMP was completely hydrolyzed. The results demonstrated that the SHV-1 and TEM-1 strains were identified as strains producing β -lactamases, and the whole analysis time was less than 1.5 h including 30 min incubation time. Moreover, as shown in Fig. S6 (Supporting information), results on the chip-MS system were consistent with traditional experiments which required 24–48 h.

To broaden the feasibility of the established chip-MS system for rapid screening of β -lactamase-producing bacteria, the AMP was replaced by three other β -lactam antibiotics, including CLO (a penicillin antibiotic stable to penicillinase), LEX (a first-generation cephalosporin), CTX (a third-generation cephalosporin), and incubated with the four experimental groups for antibiotics and their metabolites studies. As shown in Figs. 3b–d, the results of the BC and NC groups are similar to the results when using AMP, further indicating the NC strain couldn't produce any β -lactamase. As for the SHV-1 group, compared with the NC group, the intensity of CLO slightly decreased and that of the hydrolysate of CLO ($\text{CLO}_{\text{hydro}}$) slightly increased, suggesting that a small portion of CLO was hydrolyzed. However, the hydrolysates of LEX ($\text{LEX}_{\text{hydro}}$) and CTX ($\text{CTX}_{\text{hydro}}$) could not be detected, which indicated that neither LEX nor CTX were hydrolyzed. Similarly, for the TEM-1 group, a large portion of CLO and a small portion of LEX were hydrolyzed, while CTX was not hydrolyzed. The above significant results obtained from the SHV-1 and TEM-1 strains showed that the chip-MS system could further distinguish the strains containing different types of β -lactamase, which was useful for future clinical relevant medication guidance.

Next, the established chip-MS system was evaluated to whether it could be used for rapid screening of suitable concentrations of inhibitors. To investigate the influence of incubation time, the SHV-1 strain was incubated with 10 $\mu\text{g}/\text{mL}$ AMP and 3 $\mu\text{g}/\text{mL}$ tazobactam over different times on the microfluidic device, and analyzed by the chip-MS system. As shown in Fig. S7a (Supporting information), the AMP and $\text{AMP}_{\text{hydro}}$ intensities were similar during the incubation time from 30 min to 150 min, indicating the incubation time of 30 min was sufficient to inhibit bacteria for evaluating the β -lactam inhibitors.

The Christmas-tree concentration gradient generator was used to generate concentration gradients of subactam and tazobactam against SHV-1 strain. Both water and 15 $\mu\text{g}/\text{mL}$ subactam (or 6 $\mu\text{g}/\text{mL}$ tazobactam) solutions containing SHV-1 strain with OD_{600} of 0.125 and 10 $\mu\text{g}/\text{mL}$ AMP were pumped into the two inlets on the chip respectively, and incubated for 30 min. After incubation, AMP and $\text{AMP}_{\text{hydro}}$ were detected as the methods mentioned above. As shown in Fig. 4a, when the concentration of subactam increased from 0 $\mu\text{g}/\text{mL}$ to 10 $\mu\text{g}/\text{mL}$, the intensity of AMP gradually increased and the intensity of $\text{AMP}_{\text{hydro}}$ gradually decreased. As the

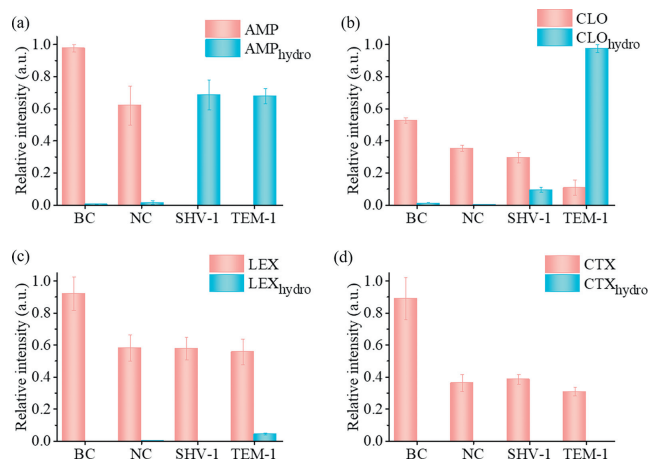


Fig. 3. The relative intensities of β -lactam antibiotics and their hydrolysates after 10 $\mu\text{g}/\text{mL}$ β -lactam antibiotics were incubated without bacteria (BC) and with 7.38×10^8 CFU/mL bacterial solution of the NC, SHV-1, and TEM-1 strains. (a) AMP and its hydrolysate, (b) cloxacillin and its hydrolysate, (c) cephalixin and its hydrolysate, (d) cefotaxime and its hydrolysate.

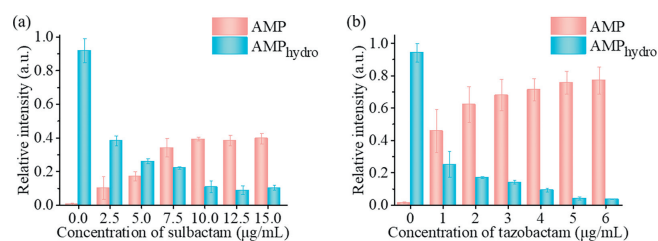


Fig. 4. The relative intensities of AMP and its hydrolysate after 10 $\mu\text{g}/\text{mL}$ AMP and different concentration of β -lactamase inhibitors was incubated with 7.38×10^8 CFU/mL bacterial solutions of the SHV-1 strain: (a) Sulbactam and (b) tazobactam.

concentration of sulbactam increased from 10 $\mu\text{g}/\text{mL}$ to 15 $\mu\text{g}/\text{mL}$, the intensities of AMP and AMP_{hydro} remained about the same. The results indicated that the inhibitory effect of sulbactam on the SHV-1 β -lactamase gradually strengthened as the concentration of sulbactam increased from 0 $\mu\text{g}/\text{mL}$ to 10 $\mu\text{g}/\text{mL}$. When the concentration exceeded 10 $\mu\text{g}/\text{mL}$, the strongest inhibitory effect of sulbactam was reached and stayed almost the same. Therefore, sulbactam at 10 $\mu\text{g}/\text{mL}$ was determined as the optimal dosing concentration for the SHV-1 strain. Similarly, as shown in Fig. 4b, tazobactam at 5 $\mu\text{g}/\text{mL}$ was determined as the optimal dosing concentration for the SHV-1 strain. From the above results, we proposed that the established chip-MS system had the potential to determine the optimal dosing concentration of β -lactamase inhibitor, and the whole analysis time was less than 2.5 h including 30 min of incubation time.

To verify the accuracy of the optimal concentration of β -lactamase inhibitor measured on the chip-MS system, the traditional broth microdilution method where the growth of bacteria under different concentrations of antibiotics in 96-well plates was used for comparison. As shown in Fig. S7b (Supporting information), the minimum inhibitory concentrations (MICs) of the SHV-1 strain to AMP were both 4 $\mu\text{g}/\text{mL}$ under 10 $\mu\text{g}/\text{mL}$ sulbactam and 5 $\mu\text{g}/\text{mL}$ tazobactam respectively. According to the Clinical and Laboratory Standards Institute, the results suggested that the SHV-1 strain was sensitive (MIC \leq 8 $\mu\text{g}/\text{mL}$) to AMP under 10 $\mu\text{g}/\text{mL}$ sulbactam and 5 $\mu\text{g}/\text{mL}$ tazobactam respectively. Therefore, the combinations of 10 $\mu\text{g}/\text{mL}$ AMP and 10 $\mu\text{g}/\text{mL}$ sulbactam, as well as 10 $\mu\text{g}/\text{mL}$ AMP and 5 $\mu\text{g}/\text{mL}$ tazobactam, were both effective for the SHV-1 strain. As shown in Figs. S7c and d (Supporting information), the results demonstrated that MICs of the SHV-1 strain to sulbactam and tazobactam were respectively 7.5 $\mu\text{g}/\text{mL}$ and 2 $\mu\text{g}/\text{mL}$ respectively, and the inhibitory effect of tazobactam was stronger than sulbactam, which was consistent with the results obtained on the chip-MS system. However, both MICs were lower than optimal concentrations obtained from the chip-MS system. The optimal concentrations measured by the chip-MS system are more stringent than the MICs, because bacterial hydrolysis of antibiotics is minimized at the optimal inhibitor concentration measured by the chip-MS system. However, the MICs measured under the conventional method only represent the inhibition of bacterial growth and the degree of antibiotic hydrolysis is not minimized.

In summary, the established chip-MS system exhibited a powerful ability to rapidly screen β -lactamase-producing bacteria and assess the optimal dose of β -lactamase inhibitors for antibiotic treatment, which provides a new approach to β -lactamase-related research. The system distinguished two strains capable of producing β -lactamase successfully and the total analysis time was less than 1.5 h. Besides, the optimal concentrations of two β -lactamase inhibitors of sulbactam and tazobactam against SHV-1 strains were also evaluated with an analysis time of less than 2.5 h. And the results of the chip-MS system were roughly consistent with those of the conventional method. In conclusion, the chip-MS system

can provide a powerful tool for rapid screening of bacteria producing β -lactamases and its optimal inhibitors dosing, which is of great significance for the treatment and prevention of infectious diseases, that may realize technological innovation in assays.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ccllet.2022.107790.

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