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SHORT REPORT

Rapid Determination of Benzylpenicillin Resistance in *Staphylococcus aureus* Bacteraemia Model

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Abstract: Rapid determination of antimicrobial susceptibility/resistance is an important factor in selecting an appropriate antimicrobial treatment and eradicating infections promptly. Conventional antimicrobial susceptibility tests (ASTs) are very time consuming. Thus, we developed a liquid chromatography-mass spectrometry (LC-MS/MS) method for rapidly determining the resistance of *Staphylococcus aureus* to penicillin-G in an animal-infection model. This technique will be able to detect those resistant strains whose resistance mechanism specifically controlled by penicillinase. The resistance status of *S. aureus* against penicillin-G was determined by conventional AST. Cultured *S. aureus* cells were inoculated to chicken for developing bacteraemia. The solution of penicillin-G was intravenously administered (10 mg/kg b.w.) to chickens just after infection detection. Blood samples were collected at different intervals after drug administration. The concentration of active penicillin-G and its metabolites were determined from the bacteria-free blood supernatant by utilizing the LC-MS/MS method. Evidence of infection in chicken was observed within 5 h of bacterial inoculation. The penicillinase enzyme generated by *S. aureus* transforms the active penicillin-G to an inactive metabolite by hydrolysis, which is evident by the mass shift from 335.10600 to 353.11579 Da as quantified using liquid chromatography quadrupole time-of-flight mass spectrometry (LC/Q-TOF/MS). The signal intensity of inactive/hydrolysed penicillin-G is several-fold greater than that of the active penicillin-G in the blood sample of chicken infected with resistant strain and treated with penicillin-G. The antimicrobial resistance index (ARI) value of resistant *S. aureus* strain was more than 1, demonstrating the penicillin-G-resistance pattern of that strain. This method is able to determine the extent of β-lactam antimicrobial resistance within 1.5 h from the patient's blood and is complementary with those existing AST methods which are usually practicing in the evaluation of β-lactam antibiotic resistance.

Keywords: β-lactamase, spectrometry, antibacterial susceptibility test, chicken infection model, antibacterial resistance

Introduction

The crisis of antibiotic resistance by *Staphylococcus aureus* in poultry is worsening due to the abuse and overuse of antibiotics.[1] The successful treatment of bacterial infections is largely reliant on the promptness of diagnosis to identify the most efficacious antimicrobials for the successful eradication of the infecting pathogens. The existing methods of antibacterial susceptibility testing (AST) have numerous shortcomings; including long turnaround times, excessive consumption of samples and reagents, and laborious.[2] These aspects impede the timely application of correct

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antibiotics, which complicates the management of infection and worsens the development of antibacterial resistance.[3] Therefore, a consistent diagnostic method is necessary for the rapid, sensitive and specific detection of the antibacterial resistance of pathogenic bacteria.

S. aureus is amongst the bacteria that affects the poultry industry globally and has been reported for penicillinase production which is an important mechanism of resistance to penicillin-G.[4] The enzyme penicillinase is responsible for the hydrolysis and inactivation of penicillin-G.[5,6] By quantifying the hydrolysed/non-hydrolysed forms of penicillin-G in bacterial culture, one can determine whether a bacterial strain is resistant to that antibiotic.[6] In this study, we developed and used a reliable and fast liquid chromatography-mass spectrometry (LC-MS/MS) technique to detect penicillin-G-resistance and/or -susceptibility of *S. aureus* strains in a chicken bacteraemia model.

Materials and Methods

The sensitivity of the clinical isolate (V02-13-S03-003-007) was determined by acidimetric test and minimum inhibitory concentration (MIC) according to the Clinical and Laboratory Standards Institute (CLSI) guideline.[7] Twenty-one male 8-week-old specific pathogen-free (SPF) white Leghorn chickens were acclimated for 1 week, and then randomly divided into three (control, susceptible and resistant) groups. *S. aureus* bacteraemia was developed by inoculating resistant and quality control strains to chickens of resistant and susceptible groups, respectively, following a previously reported method.[8] No bacteria were inoculated to the control chickens. The animal experimental protocol was approved (Approval No. 2018–413) by the Institutional Animal Care and Use Committee of Animal and Plant Quarantine Agency, who follows the Animal Protection Act of the Republic of Korea as a guideline. The chickens were observed for signs and symptoms of infection such as fever by rectal temperature measurement (Rossmax TG120 thermometer, Taipei, Taiwan), movement frequency and salivation. The first sign of infection (fever) was observed after 4~5 h of bacterial inoculation in all chickens of resistant and susceptible groups. The solution of penicillin-G (10 mg/kg b.w., Sigma-Aldrich, St. Louis, MO, USA) was intravenously administered to chickens of all groups just after infection detection. The chickens infected by resistant *S. aureus* were later found with salivation, lethargy, inability of standing, and finally died within 7 to 10 days of the bacterial inoculation. After 5, 10, 20, 30, 60, 120 and 180 min of drug administration, blood samples were collected from the wings of the chicken and plasma were separated by centrifugation at 2000 ×g for 10 min at 4°C. Plasma samples were centrifuged in a Centrifugal Filter (Amicon® Ultra-15 10K, Merck Millipore Ltd., Carrigtwohill, Co. Cork, Ireland) at 4°C for 60 min at 3000 ×g to eliminate proteins and other endogenous constituents of plasma. The levels of penicillin-G and penicilloic acid of penicillin-G from the bacteria-free blood supernatant were quantified by LC-MS/MS (Shimadzu 8045, Nakagyo-ku, Kyoto, Japan) following a previously reported method.[5] Penicillin-G and penicilloic acid of penicillin-G (LGC Standards, Teddington, UK) were spiked into the blood of control chickens to validate the method before analyzing the blood samples of the bacteraemia model. The linearity was determined for a concentration range of 0, 1, 5, 10, and 20 μg/mL. Regression coefficients (r^2) >0.99 were considered to be acceptable. The limit of

Table 1 Antimicrobial Susceptibility Profile of 2 Strains of *Staphylococcus aureus* to Penicillin-G

Strain ID	Source	Acidimetric Test	MIC (μg/mL)	CLSI Breakpoint
ATCC 25,923	QC Strain	-	0.125	Susceptible
V02-13-S03-003-007	Chicken carcass	+	512	Resistant

Abbreviations: MIC, minimum inhibitory concentration; CLSI, clinical and laboratory standards institute; QC, quality control.

Table 2 Multiple Reaction Monitoring (MRM) Parameters for Penicillin-G and Plasma Biomarker (Penicilloic Acid of Penicillin-G), and Validation Data for Developed Analytical Method

Substance	MRM Transition (m/z)	RT (min)	R^2 (at 5 points)	CV% (n=3)	LOD (ng/mL)	LOQ (ng/mL)
Penicillin-G	335.1>160.2, 176.1	5.77	0.99	4.2	21	68
Penicilloic acid of penicillin-G	353.1>309.2, 174.1	4.39	0.99	8.5	37	122

Abbreviations: MRM, multiple reaction monitoring; RT, retention time; R^2, regression coefficient; CV%, percentages of coefficients of variation; LOD, limit of detection; LOQ, limit of quantitation.

detection (LOD) and limit of quantitation (LOQ) were calculated by means of the standard deviation (SD) of the responses and the slope (S) of the calibration curve, using the formulas LOD = 3.3×(SD/S) and LOQ = 10×(SD/S). The SD of the response was determined based on the standard deviation of *y*-intercepts of the calibration curves. We used LC-MS/MS systems (Shimadzu 8045, Kyoto, Japan) to evaluate the blood samples of the bacteraemia model after evaluating the mass shift response by the liquid chromatography quadrupole time-of-flight mass spectrometry (LC/Q-TOF/MS; Agilent 6545, Santa Clara, CA, USA). The LC-MS /MS systems are quite convenient and more available than LC/Q-TOF/MS, and are easily applicable in the clinical field. The strains were considered susceptible and resistant to penicillin-G when there were, respectively, <18 Da and >18 Da mass-shifts of peaks, respectively, in the sample spectrum compared to the spectrum of the reference standard.[6] Finally, the antibacterial resistance/susceptible status of *S. aureus* strain was reconfirmed by antimicrobial resistance index (ARI) values which were calculated by our previously

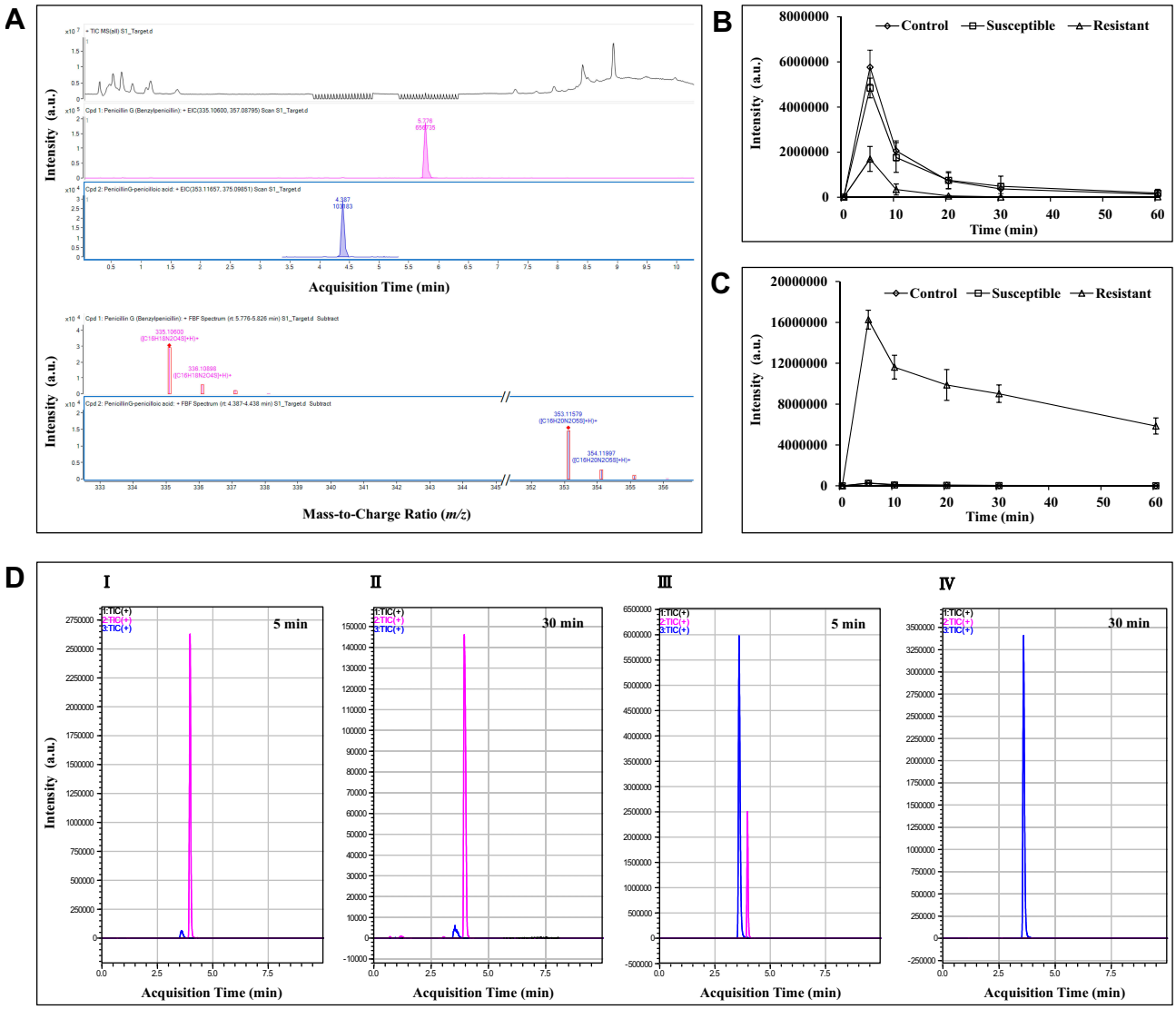

Figure 1 Representative chromatograms and intensities of active penicillin-G and hydrolyzed penicillin-G in chicken blood. (**A**) Liquid chromatography quadrupole time-of-flight mass spectrometry (LC/Q-TOF/MS) chromatograms of penicillin-G and penicilloic acid of penicillin-G extracted from antibiotic-spiked blood samples of chicken (in vitro). Changes in the signal intensities (mean±standard deviation) of penicillin-G (**B**) and the hydrolyzed form of penicillin-G (**C**) in the blood samples of infected or non-infected chickens at different time intervals. (**D**) Representative chromatograms obtained by liquid chromatography–mass spectrometry (LC-MS/MS) show the changes of intensities of active penicillin-G (pink-colored picks) and hydrolyzed penicillin-G (blue-colored picks) in chicken blood in presence of (**I, II**) susceptible and (**III, IV**) resistant *Staphylococcus aureus*. The chromatograms (**I** and **III**) were obtained from the blood sample collected after 5 min of drug administration, and the chromatograms (**II** and **IV**) were collected after 30 min of drug administration.

Abbreviations: CPD, compound; EIC, extracted ion chromatogram; FBF, finding by formula; TIC, total ion chromatogram.

reported formula using the concentrations of penicillin-G and hydrolyzed products of penicillin-G.[5]

$$\text{ARI} = (\text{Combined concentration of all hydrolyzed products of penicillin} - \text{G} / \text{Concentration of active penicillin} - \text{G})$$

If the value of the ARI is below 1, then the strain is considered to be susceptible, whereas if the ARI value is greater than 1, then the strain is considered to be resistant as mentioned earlier.[9]

Results and Discussion

The proposed rapid detection method revealed that the signal intensity of inactive/hydrolysed penicillin-G was several-fold higher than that of the active penicillin-G in the blood sample of chicken infected with *S. aureus* clinical strain (V02-13-S03-003-007) and treated with penicillin-G. Thus, the ARI value found in this clinical isolate was higher than the neutral ARI value "1" indicating the penicillin-G-resistance pattern of that strain. Conversely, in the blood sample of chicken infected with *S. aureus* QC strain and treated with penicillin-G, the signal intensity of active penicillin-G was several-fold higher than that of the inactive/hydrolysed penicillin-G and hence, the ARI value in this strain was lower than the neutral ARI value demonstrating the penicillin-G susceptibility of that strain. Similarly, the clinical isolate of *S. aureus* was found to be resistant to penicillin-G by conventional acidimetric test (Table 1). The MIC of penicillin-G against clinical isolate was 512 µg/mL, which was higher than the MIC against quality control strain (0.125 µg/mL) also indicates the resistant status of the clinical isolate (Table 1).

This optimized analytical method ensures that the measured signals come from the desired compounds and that there is no interference from diluents or mobile phase or from endogenous compounds of the chicken blood. The validation results of this LC-MS/MS method for penicillin-G and hydrolysed penicillin-G are shown in Table 2. The regression coefficient (r^2) values for both the compounds in this study were >0.99. The data obtained in this study demonstrate that the proposed method can be utilized for evaluating susceptibility to β-lactam antimicrobials.

Bloodstream infections by *S. aureus* are often associated with severe sepsis and septic shock. The models of bacteraemia (sepsis) are well established in different animal species and provide a clear understanding of the progression of diseases and bacterial population dynamics.[10] The bacteraemia model in this study is reproducible and easy to set up, and the severity of the infection is easily adjusted. Incubation of a bacterium inside the chicken body in the presence of a β-lactam antibiotic leads to the hydrolysis of that antibiotic, if the organism becomes antibiotic-resistant by gaining the β-lactamase gene and expressing the enzyme.[10] The strains can be considered susceptible and resistant to penicillin-G when there were <18 Da and >18 Da mass-shifts of peaks, respectively, in the sample spectrum compared to the spectrum of the reference standard.[6] The penicillinase enzyme generated by *S. aureus* inside the chicken body transforms the active penicillin-G to an inactive metabolite by hydrolysis, which is evident by the mass shift from 335.10600 to 353.11579 Da (Figure 1A) as quantified using LC/Q-TOF/MS. Differences in signal intensities of penicillin-G and its penicilloic acid in presence of susceptible and resistant strains of *S. aureus* in white Leghorn chickens are shown in Supplementary Table 1, and Figure 1B–D. The ratio of signal intensities of hydrolyzed penicillin-G and active penicillin-G at different time intervals after injecting penicillin-G to infected/non-infected chickens are shown in Table 3. The signal intensity of active penicillin-G is several-fold greater than that of the inactive/hydrolysed penicillin-G in the susceptible strain by LC-MS/MS. Conversely, the signal intensity of active penicillin-G is several-fold lower than that of inactive/hydrolysed penicillin-G in the resistant strain. The active penicillin-G is

Table 3 Ratio of Signal Intensities of Hydrolyzed Penicillin-G and Active Penicillin-G at Different Time Intervals After Injecting Penicillin-G to Infected/Non-Infected Chickens

	Time (min)					
	0	5	10	20	30	60
Control	0.04±0.00	0.05±0.00	0.06±0.00	0.08±0.00	0.11±0.00	0.13±0.00
Susceptible	0.46±0.03	0.05±0.00	0.04±0.00	0.06±0.00	0.06±0.00	0.05±0.00
Resistant	0.46±0.05	3.46±0.15*	33.53±4.55*	149.04±17.51*	544.48±52.23*	197.52±10.45*

Notes: Results are shown as mean±standard deviation of intensities obtained from blood of seven chickens in each group. *Indicates significant difference (p <0.05) among different groups at each time point.

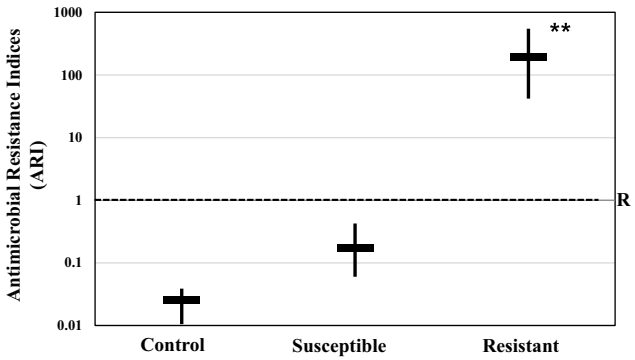

Figure 2 Antimicrobial resistance indices (ARI) of susceptible and resistant *Staphylococcus aureus* cells in blood samples (30 min) of chicken bacteremia model. ARI values less than "1" and greater than "1" indicate that the bacterial cells inside the patient are susceptible and resistant to penicillin-G, respectively. Results are shown as mean±SD from blood samples of seven chickens in each group. **Indicates significant difference (p <0.05) than other groups. "R" indicates the critical ARI value for susceptibility/resistance.

hydrolysed gradually with time in the resistant strain-induced bacteraemia model, which is evident by the differences in signal intensities at different time points. In the resistant strain-induced bacteraemia model, negligible or no signal intensity of active penicillin-G was found in the sample collected after 30 min of drug administration indicating that the active penicillin-G can be completely degraded within 30 min. Even though the active penicillin-G in resistant strain-induced bacteria model was completely degraded within 30 min, we further investigated the intensities of active penicillin-G and hydrolysed penicillin-G in blood samples collected after 60 min of drug application. As no noticeable intensities of active penicillin-G were detected in samples collected after 30–60 min of drug administration, blood samples collected from the chicken after 120 and 180 min of drug application were not analyzed. The ARI is used to determine the prevalence and degree of resistance of a bacterial strain to a particular antibacterial. The ARI value of clinical isolate was 192.47 which is extremely higher than the neutral ARI value "1", demonstrating the penicillin-G-resistance pattern of that strain (Figure 2). The total time required to analyze a sample, including the collection, preparation and extraction of the antibiotic-containing blood sample, was 1.5 h. This method dramatically decreases the antibacterial susceptibility testing time compared to that of the conventional ASTs and may thus permit an earlier de-escalation of antibacterial treatment. In fact, all the chickens of resistant *S. aureus*-inoculated group died within 7 to 10 days of the bacterial inoculation, whereas all chickens except 1 in susceptible *S. aureus*-inoculated group were alive up to 10 days of the observational period in our study. In conclusion, rapid ASTs are exposed to decrease the analysis time; to diminish the usage of antibiotics and, therefore, the costs of therapeutics; and to aid in preventing the spread of antibacterial resistance. This newly developed method could be complementary with existing AST methods which are usually practicing in the evaluation of β-lactam antibiotic resistance and may help in promptly selecting an appropriate β-lactam antibiotic in a critical clinical situation to quicken the treatment process.

Ethics Approval

All procedures performed in this study were approved by the ethics committee of Animal and Plant Quarantine Agency, Republic of Korea.

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Disclosure

The authors declare that they have no conflicts of interest.

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