

Received: 2014.10.07
Accepted: 2014.10.30
Published: 2014.11.12

Matrix-Assisted Laser Desorption Ionization: Time of Flight Mass Spectrometry-Identified Models for Detection of ESBL-Producing Bacterial Strains

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ACDEG 1,2 **Bo Li***
CDF 2 **Tongsheng Guo***
A 2 **Fen Qu**
CDF 2 **Boan Li**
CDF 2 **Haibin Wang**
BCD 2 **Zhiqiang Sun**
BCD 2 **Xiaohan Li**
BC 2 **Zhiqiang Gao**
DF 2 **Chunmei Bao**
B 2 **Chenglong Zhang**
B 2 **Xiaoxi Li**
AG 2 **Yuanli Mao**

1 Graduate Student Team, Chinese PLA Postgraduate Medical School, Beijing China
2 Center for Clinical Laboratory, 302 Hospital of PLA, Beijing, China

* These authors contributed equally to this work

Corresponding Author:

Yuanli Mao, e-mail: pipi780816@aliyun.com

Source of support:

The National Science and Technology Major Project of the Ministry of Science and Technology of China (2013ZX09J13106)

Background: The increase in the amount of extended spectrum beta-lactamases (ESBL)-producing gram-negative bacteria is seriously threatening human health in recent years. Therefore, it is necessary to develop a rapid and reliable method for identification of ESBLs. The purpose of this study was to establish a novel method to discriminate between ESBL-producing and non-ESBL-producing bacteria by using the matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) technique.

Material/Methods: We detected hydrolyzed production of cefotaxime after incubation with 69 gram-negative bacteria by using MALDI-TOF-MS. Then we established genetic algorithm (GA), supervised neural networks (SNN), and quick classifier (QC) models using several peaks to identify ESBL-producing strains. To confirm the clinical applicability of the models established, a blinded validation test was performed in 34 clinical isolated strains.

Results: Using ClinPro Tools software, we identified 4 peaks (456 Da, 396 Da, 370 Da, and 371 Da) in mass spectra of cefotaxime solution that have high enough specificity to discriminate ESBL-producing from non-ESBL-producing strains. Recognition capability of models established were 97.5% (GA), 92.5% (SNN), and 92.5% (QC), and cross validation rates were 90.15% (GA), 97.62 (SNN), and 97.62% (QC). The accuracy rates of the blinded validation test were 82.4% (GA), 88.2% (SNN), and 82.4% (QC).

Conclusions: Our results demonstrate that identification of ESBLs strains by MALDI-TOF-MS has potential clinical value and could be widely used in the future as a routine test in clinical microbiology laboratories.

MeSH Keywords: **beta-Lactamases • Drug Resistance, Bacterial • Mass Spectrometry • Spectrometry, Mass, Matrix-Assisted Laser Desorption-Ionization**

Full-text PDF: <http://www.basic.medscimonit.com/abstract/index/idArt/892670>



1983



4



6



25



Background

Antibiotic resistance is a critical public health problem around the world, especially the spread of multi-drug-resistant organisms (MDRO), and has led to increased infected patient mortality. Many of these increases are due to deficiency of available antibiotics. The number of reported cases of MDRO nearly quadrupled in the past decade and the World Health Organization has declared that MDRO is among the top 3 threats to human health [1–3]. Extended-spectrum beta-lactamases (ESBL)-producing gram-negative bacteria are the most common MDRO and can hydrolyze beta-lactam antibiotics such as penicillin, cephalosporin, and monobactams. ESBL enzymes are mainly mediated by the conjugative plasmids and transferred between bacteria. So far, more than 100 groups of ESBL have been identified around the world and 3 of the major groups are TEM, SHV, and CTX-M. These enzymes are most commonly produced by *Klebsiella* spp. and *Escherichia coli*. They have also been found in other Enterobacteriaceae bacteria and some non-fermenters [4,5].

The infection rate of ESBL-producing bacteria is probably underestimated because these bacteria often remain undetected by routine testing methods. Kirby-Bauer disk diffusion method is most commonly used to detect ESBL in many clinical microbiology laboratories. However, ESBL strains might show a false sensitive zone of inhibition in this method, which is time-consuming and cannot provide timely diagnostic information for anti-infective therapy [6]. Another common method for ESBL detection is a molecular test, which can provide faster results than culture, but its use is limited by high cost [7–9].

In recent years, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) has already been used in clinical diagnosis and medical research fields, including discovery of cancer biomarkers, diagnosis of bacterial infection, identification of mutations and genotypes of viruses, and detection of antibiotic resistance [10–20]. MALDI-TOF-MS technique is more precise, rapid, and cost-effective than the traditional methods, and at present the main application of this technology is bacterial identification in clinical microbiology laboratories [18,21]. Recently, MALDI-TOF-MS has emerged as a fast and accurate technology for the detection of antibiotic resistance. In this study, we assessed the feasibility of the use of mass spectrometry for detection of antibiotic-resistant strains, and found some specific mass peaks to set up algorithm models to discriminate ESBL-producing and non-ESBL-producing strains.

Material and Methods

Bacterial strains

All bacterial strains were isolated from clinical samples from 302 People's Liberation Army (PLA) hospitals in Beijing, China. A total of 69 clinical isolates were tested by MALDI-TOF-MS, including 44 *E. coli* species (18 ESBL-producing and 26 non-ESBL-producing) and 25 *K. pneumoniae* species (15 ESBL-producing and 10 non-ESBL-producing). The distribution of the sources of the samples is shown in Table 1. Another 34 isolates (12 ESBL-producing and 22 non-ESBL-producing) were tested as blinded validation samples to confirm the clinical applicability of the methods. All bacterial strains were identified by the Vitek2 system and ESBLs were confirmed by Kirby-Bauer disk diffusion method. The bacteria were incubated on Columbia blood agar plates (BioMerieux, France) overnight at 37°C.

Sample preparation

Cefotaxime (purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) was dissolved in distilled water to final concentration of 0.5 mg/ml [11]. Five bacterial colonies were picked and resuspended in 10 µl of antibiotic solution. Subsequently, the solutions were incubated at 37°C under agitation for 3 h and then centrifuged for 2 min at 12 000 g at room temperature. The supernatant was removed for analysis by MALDI-TOF-MS. To confirm the presence of ESBLs, clavulanic acid at 0.05 mg/ml concentration was added to antibiotic solutions in another parallel test.

MALDI-TOF MS analysis

We transferred 1 µl of supernatant from the incubated solution onto a 384 polished steel target plate and then samples were air-dried under a biosafety cabinet. Every dried sample was mixed with 1 µl of MALDI matrix (10 mg/ml of α -cyano-4-hydroxycinnamic acid (HCCA) dissolved in 50% acetonitrile and 2.5% trifluoroacetic acid (TFA); Bruker Daltonics, Bremen, Germany). Measurements were performed using an Autoflex MALDI-TOF MS (Bruker Daltonics, Germany) instrument. The parameter settings were: positive reflector mode (RP) in the mass range of 200 Da to 1500Da; ion source 1:20kV; ion source 2:17.5 kV; pulsed ion extraction: 120 ns; laser wavelength: 337 nm; laser frequency: 67–100 Hz; lens voltage: 6.5 kV; detector gain: 2650 V.

Data analysis and model generation

Data analysis was performed with ClinPro Tools software (v3.0). Peaks with signal-to-noise ratio (S/N) >5 were picked out and performed the statistical analysis. Normally distributed data were analyzed with Student's t tests and non-normally distributed data were analyzed by the Wilcoxon test. To distinguish

Table 1. Distribution of the various sources of ESBL producing and non- ESBL producing strains.

Source of specimens	<i>E. coli</i> strains			<i>K. pneumoniae</i> strains		
	ESBL	Non-ESBL	Total	ESBL	Non-ESBL (-)	Total
Blood	9	14	23	6	4	10
Ascites	2	1	3	1	2	3
Hydrothorax	1	2	3	1	1	2
Sputum	0	0	0	4	1	5
Throat swab	0	0	0	1	0	1
Midstream urine	4	5	9	0	2	2
Drainage	1	3	4	2	0	2
Secretion	1	1	2	0	0	0
Total	18	26	44	15	10	25

Table 2. ClinProTools peak statistics for the 4 peaks between ESBL and non-ESBL groups.

Index	Mass	DAve	PTTA	PWKW	PAD	Ave (non-ESBL)	Ave (ESBL)	SD (non-ESBL)	SD (ESBL)	CV (non-ESBL)	CV (ESBL)
148	370.12	31.6	<0.000001	<0.000001	0.00167	35.66	67.26	8.24	11.15	23.11	16.57
149	371.14	11.52	<0.000001	<0.000001	0.000053	7.03	18.55	1.53	5.23	21.81	28.18
166	396.11	30.37	<0.000001	<0.000001	0.0316	107.22	76.85	12.88	15.89	12.01	20.67
201	456.22	34.77	<0.000001	<0.000001	0.0365	127.63	92.86	10.7	16.09	8.38	17.33

ESBL and non-ESBL strains, 3 different machine-learning algorithms were used: genetic algorithm (GA), supervised neural network (SNN), and quick classifier (QC).

Blinded validation of clinical samples

To confirm the clinical applicability of the models established, a blinded validation test was conducted following the completion of the statistical analysis. A total of 34 clinical bacterial strains were collected and detected ESBLs by 2 methods (Mass spectrometry models and Kirby-Bauer disk diffusion method) simultaneously. K-B method results served as the criterion standard. Accuracy, sensitivity, specificity, positive and negative predictive values (PPV and NPV), and Youden’s index were calculated to assess the performances of the models.

Results

Discrepancy analysis of mass spectra

The hydrolysis of the cefotaxime by ESBL-producing and non-ESBL-producing strains was analyzed. The molecular peaks of

cefotaxime (456 Da and 396Da) were found in the spectra. Incubation of cefotaxime with resistant strains resulted in the decrease of the molecular peaks at 456 Da and 396Da, but the peaks did not disappear completely. In addition, all spectra derived from resistant bacteria revealed increased peaks at 370 Da and 371 Da, corresponding to the hydrolyzed form of cefotaxime. Using ClinPro Tools software, the discrepancy analysis of the 4 peaks showed that all of them were significantly different ($p < 0.05$) between ESBL and non-ESBL strains. The statistical results of the 4 peaks are shown in Table 2. To demonstrate the differences visually, statistical plots of the 4 peaks are shown in Figures 1–4. The spectra peaks distribution maps (Figure 1) of to the 4 peaks showed that 2 groups of spots were completely separate and we could easily discriminate the ESBL from the non-ESBL strains. From the simulated 2-dimensional gel electrophoresis map and whole mass spectra map (Figures 2–4), peak intensities at 370 Da and 371 Da increased significantly in resistant strains. In contrast, the peaks at 456 Da and 396 Da were clearly reduced in resistant strains. The area under the curve (AUCs) of the ROCs for peaks 370 Da, 371 Da, 456 Da, and 396 Da were 1.00, 1.00, 0.97, and 0.93, respectively (Figure 5). To rule out the possibility of the presence of non-ESBL mechanisms, clavulanic acid was added

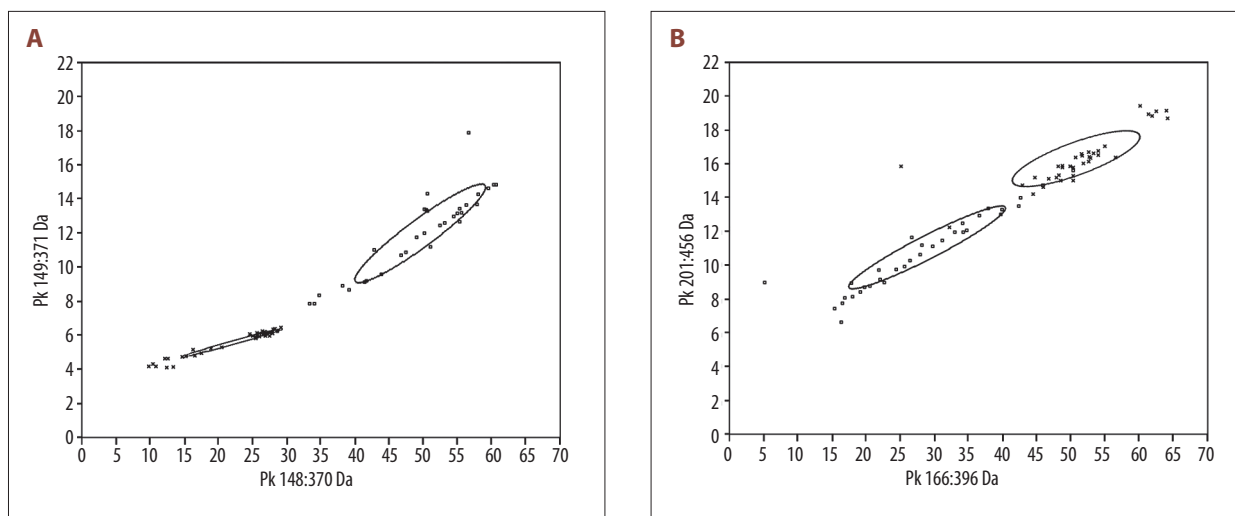


Figure 1. (A) The distribution maps of ESBL-producing strains and non-ESBL-producing strains (circle, ESBL-producing strains; cross, non-ESBL-producing strains) with 2 peaks (370 Da and 371 Da); (B) The distribution maps of ESBL-producing strains and non-ESBL-producing strains (circle, ESBL-producing strains; cross, non-ESBL-producing strains) with 2 peaks (456 Da and 396 Da).

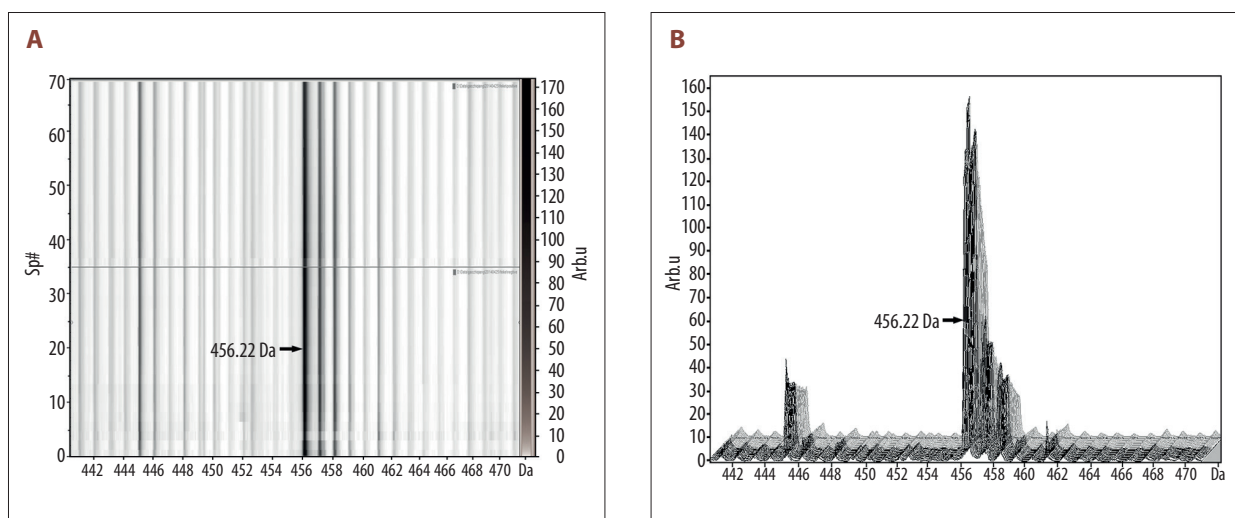


Figure 2. Representative spectra of the 456 Da peak: (A) The simulated 2-dimensional gel electrophoresis results (the upload: ESBL-producing strains, the download: non-ESBL-producing strains); (B) The whole mass spectra (black, non-ESBL-producing strains, gray, ESBL-producing strains).

to antibiotic solutions in another parallel test. Figure 6 demonstrates that the peaks at 370 Da and 371 Da were clearly reduced in the ESBL group in the presence of clavulanic acid, but peaks at 456 Da and 396 Da were clearly increased.

Establishment of ESBL strains identified models

We used ClinPro Tools software to analyze the data and establish 3 identified models. Four individual peaks (370 Da, 371 Da, 456 Da, and 396 Da) were combined to establish the GA, SNN, and QC models. Results of identification for ESBLs by the 3 models were: GA model with a cross-validation of 90.15% and a recognition capability of 97.5%, QC model with

a cross-validation of 97.62% and a recognition capability of 92.5%, and SNN model with a cross-validation of 97.62% and a recognition capability of 92.5%. From the results in Table 3 we infer that by using the 4 peaks to construct algorithm models, we could achieve around 95% accuracy rate for detecting ESBL strains. Therefore, a blinded validation study with more clinical samples should be done with the follow-up research.

Blinded validation of clinical samples

A total of 34 clinical isolated bacterial strains, including 12 ESBL-producing and 22 non-ESBL-producing strains, were successfully analyzed as validation samples by MALDI-TOF MS models.

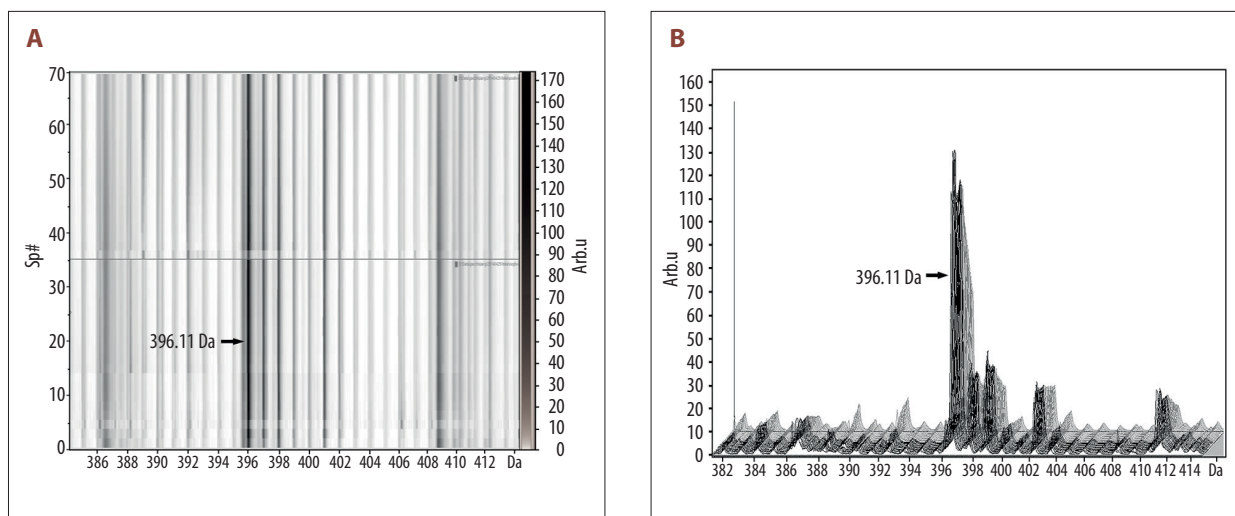


Figure 3. Representative spectra of the 396 Da peak: (A) The simulated 2-dimensional gel electrophoresis results (the upload: ESBL-producing strains, the download: non-ESBL-producing strains); (B) The whole mass spectra (black, non-ESBL-producing strains, gray, ESBL-producing strains).

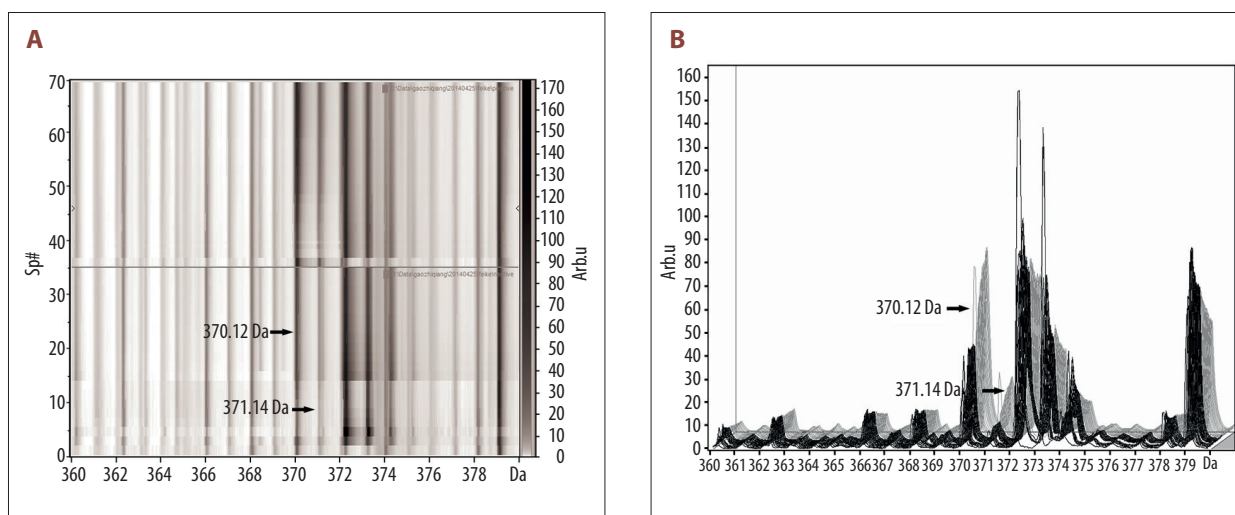


Figure 4. Representative spectra of the 370 Da and 371 Da peaks: (A) The simulated 2-dimensional gel electrophoresis results (the upload: ESBL-producing strains, the download: non-ESBL-producing strains); (B) The whole mass spectra (black, non-ESBL-producing strains, gray, ESBL-producing strains).

Among these 34 samples, 28 (82.4%) were correctly identified by GA models with 4 false-negatives and 2 false-positives, giving a sensitivity of 71.4% and a specificity of 90.0%. We correctly identified 30 (88.2%) samples by SNN models with 2 false-negatives and 2 false-positives, giving a sensitivity of 83.3% and a specificity of 90.9%. For QC model, the number of correctly identified samples was 28 (82.4%), with 3 false-negatives and 3 false-positives, resulting in a sensitivity of 75.0% and a specificity of 86.4%. The diagnostic performances of 3 algorithm models are listed in Table 4.

Discussion

MALDI-TOF-MS is a powerful tool for the detection and identification of proteins, peptides, polysaccharides, nucleic acids, and other biological molecules. It has been used in clinical diagnosis and medical research successfully since the 1980s. In recent years MALDI-TOF-MS has become a routine method for bacterial identification in clinical microbiology laboratories because of its advantages of rapid and high throughput. However, the development of antibiotic resistance research using mass spectrometry lags far behind the identification of bacteria; therefore, many bacteriologists have focused their attention on the use of mass spectrometry for investigating antibiotic resistance. Many

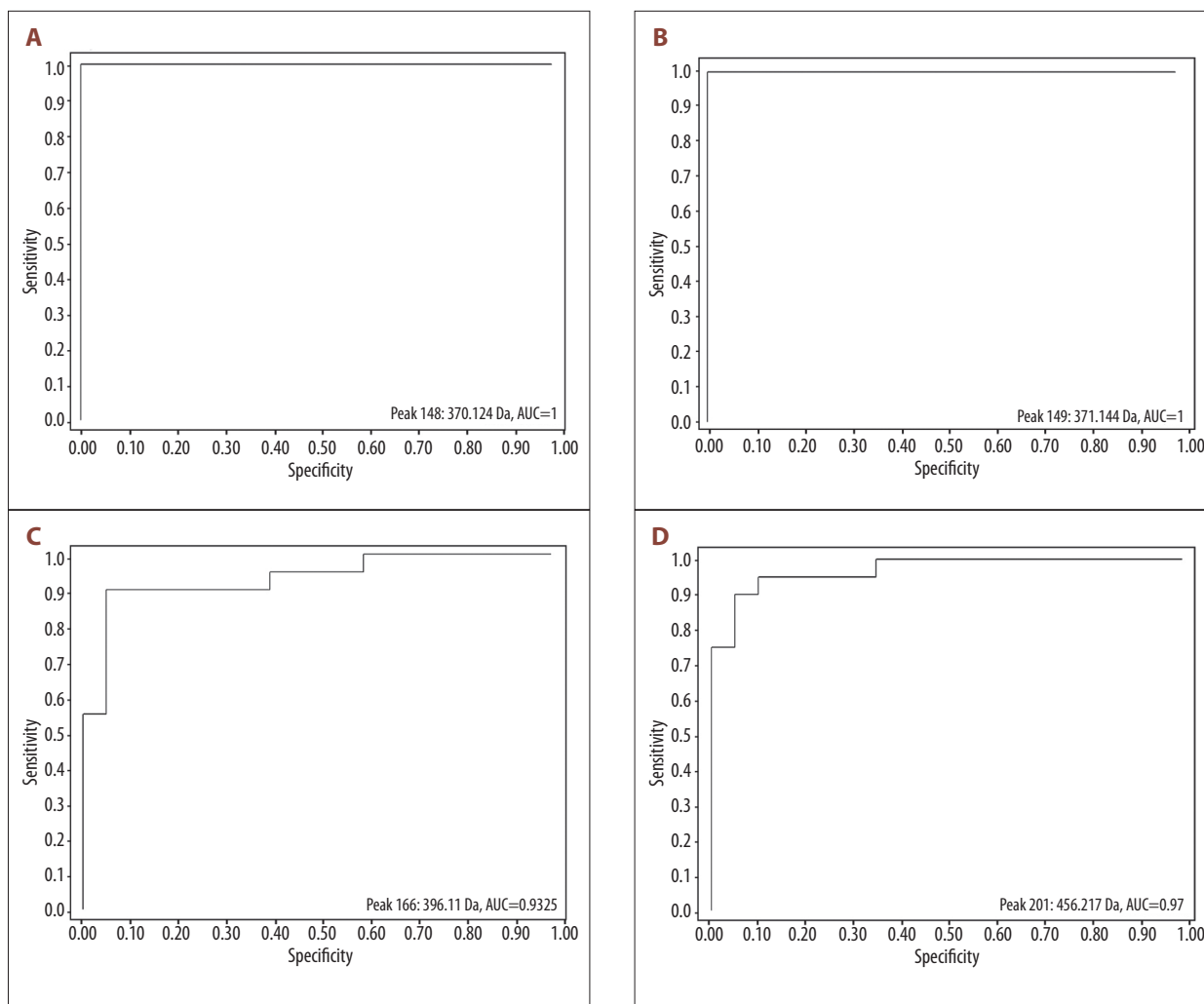


Figure 5. Receiver operating characteristic (ROC) curves for differential peaks. (A) peak 370 Da; (B) peak 371 Da; (C) peak 396 Da; (D) peak 456 Da.

studies have attempted to find the differences in the spectral patterns between resistant strains and non-resistant strains, but there are still no reliable “biomarkers” for identifying resistant strains [14,22–25]. In 2012, Sparbier K. et al. [11] created a novel approach to identify resistant bacteria strains by using MALDI-TOF-MS. The principle of the method is: β -lactam antibiotics were incubated with the sensitive strains and resistant strains and hydrolysis of antibiotics was detected by using MALDI-TOF-MS after incubation. The hydrolysis of the antibiotics leads to a molecular mass change, which can be easily detected by mass spectrometry. Therefore, resistant strains can be identified according to the changes in the mass spectra profiles. However, it is difficult to determine the standardization of assay evaluation.

In this study, ClinPro Tools software was used to obtain information about the changes in the mass spectra and establish mathematical models to identify ESBL strains automatically, thereby avoiding the problem of standardization of assay evaluation.

We evaluated the performance of the MALDI-TOF-MS system for the detection of ESBLs from the clinical isolated gram-negative strains, including 25 *K. pneumoniae* strains and 44 *E. coli* strains. Several beta-lactam antibiotics at different concentrations were tested with 69 clinical isolates (data not shown). Cefotaxime at a concentration of 0.5 mg/ml obtained the best effect in discriminating ESBL-producing from non-ESBL-producing strains. A total of 4 molecular peaks in cefotaxime were selected to continue our data analysis – they were molecular peaks of cefotaxime (456 Da and 396 Da) and a hydrolyzed form of cefotaxime (370 Da and 371 Da). All spectra derived from resistant bacteria revealed a clear reduction of molecular peaks of cefotaxime (456 Da and 396 Da) and an increase of the peaks of the hydrolyzed form of cefotaxime (370 Da and 371 Da).

The identified models based on the 4 peaks showed similar performance and yielded a cross-validation and recognition rate of about 95% in identifying ESBL strains and can meet the needs of

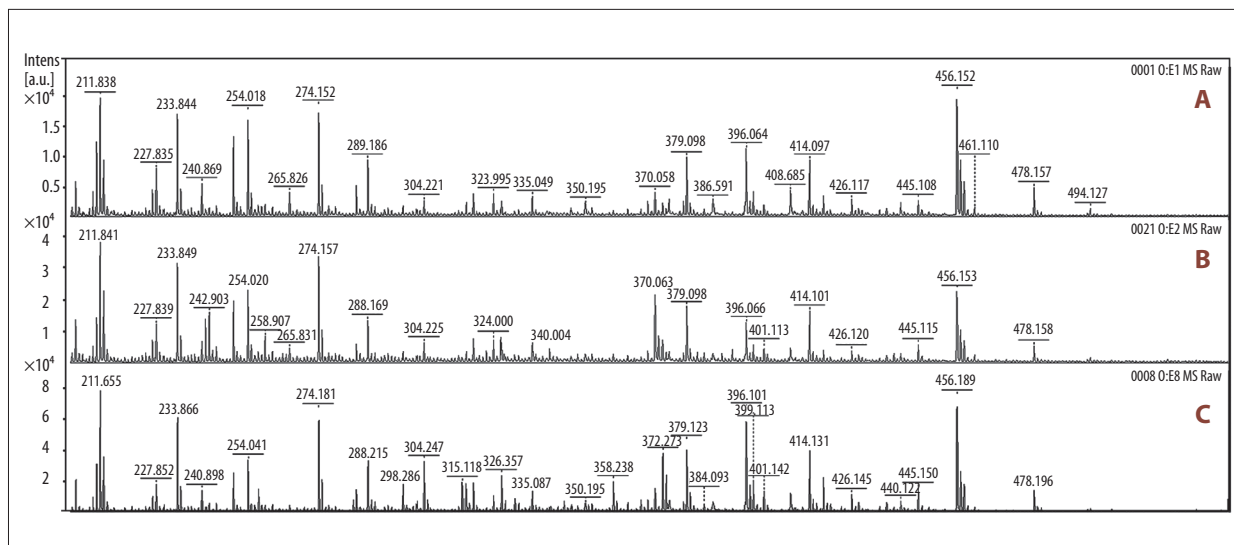


Figure 6. (A) Mass spectra of cefotaxime after incubation with the non-ESBL-producing strain; (B) Mass spectra of cefotaxime after incubation with the ESBL-producing strain; (C) Mass spectra of cefotaxime plus clavulanic acid after incubation with the ESBL-producing strain.

Table 3. Validation results of three ESBL classified models.

	Recognition rate (%)			Cross-validation rate (%)		
	ESBL	Non-ESBL	Total	ESBL	Non-ESBL	Total
GA	95	100	97.5	91.67	88.64	90.15
SNN	85	100	92.5	95.24	100.00	97.62
QC	100	85	92.5	100.00	95.24	97.62

Table 4. Diagnostic performances of 3 algorithms models with blinded validation samples.

	GA	SNN	QC
Accuracy (%)	82.4	88.2	82.4
Sensitivity (%)	71.4	83.3	75.0
Specificity (%)	90.0	90.9	86.4
Positive predictive values (%)	83.3	83.3	75.0
Negative predictive values (%)	81.8	90.9	86.4
Positive likelihood ratio	7.14	9.17	5.50
Negative likelihood ratio	0.32	0.18	0.29
Youden's index	0.61	0.74	0.61

clinical application after further confirmation. However, despite the previous experiments showing that the cross-validation rate and recognition rate of the models for identifying ESBL strains were very high, the results of blinded validation are still not satisfactory because these models have imperfect clinical applicability at present and their accuracy rate is still only about 85%.

Conclusions

The numbers of samples tested in our study limits the reliability of the models, and results of the blinded validation study are not yet reliable enough for use in routine clinical diagnosis. Despite this, we still found some specific peaks to discriminate

ESBL-producing from non- ESBL-producing strains. We established 3 identified models of ESBL-producing strains, and the results of validation were very good. Therefore, we believe that

MALDI-TOF-MS should be regarded as a promising tool for the detection of ESBL-producing bacteria and will become widely used in clinical work in the future.

References:

1. Bassetti M, Ginocchio F, Mikulska M: New treatment options against gram-negative organisms. *Crit Care*, 2011; 15: 215
2. Worthington RJ, Melander C: Combination approaches to combat multi-drug-resistant bacteria. *Trends Biotechnol*, 2013; 31: 177–84
3. Balkhair A, Al-Farsi YM, Al-Muharrmi Z et al: Epidemiology of multi-drug resistant organisms in a teaching hospital in oman: a one-year hospital-based study. *Scientific World Journal*, 2014; 2014: 157102
4. Demirel I, Kinnunen A, Onnberg A et al: Comparison of host response mechanisms evoked by extended spectrum beta lactamase (ESBL)- and non-ESBL-producing uropathogenic *E. coli*. *BMC microbiology* 2013;13.
5. Kim JS Kim SJ, Jeon SE et al: Characterization of CTX-M-type extended-spectrum beta-lactamase-producing diarrheagenic *Escherichia coli* isolates in the Republic of Korea during 2008–2011. *J Microbiol Biotechnol*, 2014; 24: 421–26
6. Kumar D, Singh AK, Ali MR, Chander Y: Antimicrobial Susceptibility Profile of Extended Spectrum beta-Lactamase (ESBL) Producing *Escherichia coli* from Various Clinical Samples. *Infect Dis*, 2014; 7: 1–8
7. Grohs P, Tillecovidin B, Caumont-Prim A et al: Comparison of five media for detection of extended-spectrum Beta-lactamase by use of the wasp instrument for automated specimen processing. *J Clin Microbiol*, 2013; 51: 2713–16
8. Garrec H, Drieux-Rouzet L, Golmard JL et al: Comparison of nine phenotypic methods for detection of extended-spectrum beta-lactamase production by Enterobacteriaceae. *J Clin Microbiol*, 2011; 49: 1048–57
9. Gazin M, Paasch F, Goossens H et al: Current trends in culture-based and molecular detection of extended-spectrum-beta-lactamase-harboring and carbapenem-resistant Enterobacteriaceae. *J Clin Microbiol*, 2012; 50: 1140–46
10. Niyompanich S, Jaresitthikunchai J, Srisanga K et al: Source-Identifying Biomarker Ions between Environmental and Clinical Burkholderia pseudomallei Using Whole-Cell Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). *PLoS One*, 2014; 9: e99160
11. Sparbier K, Schubert S, Weller U et al: Matrix-assisted laser desorption ionization-time of flight mass spectrometry-based functional assay for rapid detection of resistance against beta-lactam antibiotics. *J Clin Microbiol*, 2012; 50: 927–37
12. Taneja S, Ahmad I, Sen S et al: Plasma peptidome profiling of acute hepatitis E patients by MALDI-TOF/TOF. *Proteome Sci*, 2011; 9: 5
13. Wang J, Wang X, Lin S et al: Identification of kininogen-1 as a serum biomarker for the early detection of advanced colorectal adenoma and colorectal cancer. *PLoS One*, 2013; 8: e70519
14. Matsumura Y, Yamamoto M, Nagao M et al: Detection of extended-spectrum-beta-lactamase-producing *Escherichia coli* ST131 and ST405 clonal groups by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol*, 2014; 52: 1034–40
15. Khot PD, Fisher MA: Novel approach for differentiating *Shigella* species and *Escherichia coli* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol*, 2013; 51: 3711–16
16. Huijbers A, Velstra B, Dekker TJ et al: Proteomic serum biomarkers and their potential application in cancer screening programs. *Int J Mol Sci*, 2010; 11: 4175–93
17. Griffin PM, Price GR, Schooneveldt JM et al: Use of matrix-assisted laser desorption ionization-time of flight mass spectrometry to identify vancomycin-resistant enterococci and investigate the epidemiology of an outbreak. *J Clin Microbiol*, 2012; 50: 2918–31
18. Ayyadurai S, Flaudrops C, Raoult D, Drancourt M: Rapid identification and typing of *Yersinia pestis* and other *Yersinia* species by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. *BMC Microbiol*, 2010; 10: 285
19. Cobo F: Application of maldi-tof mass spectrometry in clinical virology: a review. *Open Virol J*, 2013; 27: 84–90
20. Qiu F, Gao YH, Jiang CG et al: Serum proteomic profile analysis for endometrial carcinoma detection with MALDI-TOF MS. *Arch Med Sci*, 2010; 6: 245–52
21. Lista F, Reubsaet FA, De Santis R et al: Reliable identification at the species level of *Brucella* isolates with MALDI-TOF-MS. *BMC Microbiol*, 2011; 11: 267
22. Schaumann R, Knoop N, Genzel GH: A step towards the discrimination of beta-lactamase-producing clinical isolates of Enterobacteriaceae and *Pseudomonas aeruginosa* by MALDI-TOF mass spectrometry. *Med Sci Monit*, 2012; 18(9): MT71–77.
23. Camara JE, Hays FA: Discrimination between wild-type and ampicillin-resistant *Escherichia coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal Bioanal Chem*, 2007; 389: 1633–38
24. Ikryannikova LN, Shitikov EA, Zhivankova DG et al: A MALDI TOF MS-based minisequencing method for rapid detection of TEM-type extended-spectrum beta-lactamases in clinical strains of Enterobacteriaceae. *J Microbiol Methods*, 2008; 75: 385–91
25. Burckhardt I, Zimmermann S: Using matrix-assisted laser desorption ionization-time of flight mass spectrometry to detect carbapenem resistance within 1 to 2.5 hours. *J Clin Microbiol*, 2011; 49: 3321–24