A rapid MALDI-TOF mass spectrometry-based method for colistin susceptibility testing in Escherichia coli

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Introduction
The increasing prevalence of antibiotic resistance has become a serious threat to public health because of the lack of effective clinical therapeutic anti-infective options. Some of the most concerning issues are the increasing emergence and rapid dissemination of multi-drug resistance bacteria, especially carbapenem-resistant Enterobacteriaceae (CRE) (Hansen, 2021). Colistin is an antibiotic of the polymixin class, recognized as a last-resort treatment option because of its broad-spectrum activity against multi-drug resistant Gram-negative bacteria including CRE. However, since the plasmid-mediated colistin-resistance gene mcr-1 emerged in China in 2016 (Liu et al., 2016), the mcr-1 gene has been reported globally as a great challenge to the efficacy of colistin because of transferability between strains and species (Yang et al., 2017). To date, 21 mcr-1 variants (mcr-1.2 to mcr-1.22) have been identified according to GenBank records.

The broth microdilution (BMD) method is the gold standard technique for colistin susceptibility testing by the Clinical Laboratory Standard Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). It was chosen as the unique reference method for antimicrobial susceptibility over other classical methods including agar dilution, disc diffusion and E-test methods (Poirel et al., 2017; Jayol et al., 2018). However, the BMD method is time-consuming for routine laboratory test because it required to wait 18–24 h for reading the results. Automated dilution systems including MicroScan, Vitek 2 and BD Phoenix can be applied in laboratories for screening of colistin-resistance, but positive results need to be confirmed by the BMD method because of the low accuracy of these systems (Chew et al., 2017b, 2017a). MCR-1-mediated colistin-resistance is still identified by PCR based on the confirmed colistin-resistance phenotype through the above-mentioned methods. Therefore, a test that enables both rapid and reliable identification and
antimicrobial susceptibility testing for MCR-1-mediated colistin-resistance is urgently needed.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been increasingly reported as an alternative method to rapid identify microorganisms including directly from positive blood cultures as well as to rapidly detect carbapenem-resistant (Sakarikou et al., 2017; Yu et al., 2018) and colistin-resistant (Dortet et al., 2018) strains through antibiotic hydrolysis assays. Herein, a semi-quantitative MALDI-TOF MS-based approach is presented, and its applicability to the detection of resistance of E. coli against colistin is demonstrated.

**Results**

**MIC and PCR results of verification strains**

We chose 128 E. coli strains for verification; the colistin MIC values were from ≤ 0.25 to 16 µg ml⁻¹. The PCR results showed 50 E. coli isolates were mcr-1-negative, and 78 isolates were mcr-1-positive. The MIC and PCR results of 11 isolates were internally coherent, MIC values of two strains were 1 µg ml⁻¹ but mcr-1 were positive, and nine strains were 2 µg ml⁻¹ also mcr-1-positive (Table 1).

**Optimization of the analysis protocol and creation of the model**

In the first hour, the difference in the mass spectra between the sensitive (ATCC 25922) and resistant strain (E218-2) with colistin was not significant. The distinction between two strains was already visible after 2 h of incubation with 64 µg ml⁻¹ colistin (Fig. 1), and therefore, the optimal incubation time was 2 h for this study. The incubations in the BHI only (Fig. 1C and D) show the familiar peak pattern already known from the mass spectrometric identification for the two strains plus internal standard peaks. In contrast, the incubations in the presence of 64 µg ml⁻¹ colistin gave different results. The resistant strain (Fig. 1B) showed a spectrum equivalent to that obtained from in the BHI only. The susceptible strain spectrum (Fig. 1A) contained only the peaks of the internal standard, and a few very small peaks derived from the bacterium. Direct examination of spectra is very time-consuming and not applicable in routine settings. Therefore, it was also necessary to achieve a reliable separation of the peaks from sensitive and resistant strains using the R software assay (Yang Xu, personal communication).

As use of an automated software tool is more practical for larger data analysis, a prototype algorithm was developed using R software. Figure 2 shows the RG values for ATCC25922 and E218-2 incubated in the presence or absence of colistin (64 µg ml⁻¹) on five different days repeated three times. In all the analyses, the RG values of ATCC 25922 were 0.1 to 0.35, and thus, all were < 0.4, and the RG values for E218-2 were all approximately 1.5. Thus, a clear distinction between the sensitive and resistant strains was identified.

E2525-1-2, E3647-2 and five mcr-1-negative E. coli strains were incubated with increasing colistin concentrations (from 0.25 to 64 µg ml⁻¹), and the complete titration curve for the seven strains with increasing concentrations of colistin after 2 h of incubation is shown in Fig. 3. The best separation between the sensitive and resistant strains was achieved at a concentration of 4 µg ml⁻¹ and an RG rate cut-off value of 0.6. An RG rate of > 0.6 was interpreted as indicating resistance.

**Analysis of clinical isolates**

For the subsequent experiments, a 2 h incubation time and a breakpoint concentration of 4 µg ml⁻¹ of colistin were applied for the clinical isolates. In total, 128 E. coli strains were analysed using the semi-quantitative MS-based approach. An RG value of < 0.6 indicated sensitive to colistin and an RG value of > 0.6 indicated

| MS No. | Original No. | Origin | MIC of colistin (µg ml⁻¹) | mcr-1 gene |
|--------|--------------|--------|---------------------------|------------|
| E218-2 | ATCC25922 | Reported in (Shen et al., 2018) | 4 | Positive |
| 1      | 3919        | The Children’s Hospital of Zhejiang University School of Medicine | ≤ 0.25 | Negative |
| 2      | 3964        | The Children’s Hospital of Zhejiang University School of Medicine | ≤ 0.25 | Negative |
| 3      | 3566        | The Children’s Hospital of Zhejiang University School of Medicine | ≤ 0.25 | Negative |
| 4      | 3980        | The Children’s Hospital of Zhejiang University School of Medicine | 2 | Negative |
| 6      | E2525-1-2   | Reported in (Shen et al., 2018) | 4 | Positive |
| 7      | E3647-2     | Reported in (Shen et al., 2018) | 4 | Positive |
The MS approach confirmed the routine classification in most cases, with six false-positive results and two false-negative results obtained compared with the MIC method, although the reason for this divergent behaviour was unclear. Visual inspection of the spectra confirmed they were in concordance with the RG values (Table 1). Disregarding the repeated tests, a sensitivity of 97.4% and a specificity of 88% were obtained for the MS-based approach, the positive predict value was 92.59% and the negative predict value was 94.74%.

Discussion

To date, the colistin-resistance determinant MCR-1 has been reported globally in over 50 countries on five continents from animals, environment and human samples. Although colistin is a last-resort treatment for multi-drug resistant bacterial infections, the \textit{mcr-1} gene has been increasingly identified in carbapenemase-producing \textit{Enterobacteriaceae} (CPE) isolates (Shen \textit{et al.,} 2018). The widespread nature of \textit{mcr-1} in CPE is likely to pose a serious public health crisis. At present, all commercial diagnostics for colistin-resistance can be grouped into phenotypic or molecular tests, but molecular tests are costly and require a skilled user, which is why they are rarely used in the clinical laboratory. The phenotypic tests of colistin remain a challenge for clinical laboratories to implement because of difficulties in the performance, reproducibility and accuracy of available methods (Maxson \textit{et al.,} 2017). Both CLSI and EUCAST...
currently recommend BMD as the only reliable method for colistin susceptibility testing, but few laboratories have the capacity to perform BMD (Lutgring et al., 2019). Disc diffusion, the E-test and commercial methods such as Vitek 2 are not reliable in determining polymyxin susceptibility. Ka Lip Chew et al. (2017b) compared three products using BMD, and the rates of essential agreement of colistin test results among BMD and Vitek 2, Sensititre, and E-test were 93.4%, 89.5% and 75.0% respectively. Some reports showed even strains that were colistin susceptible were detected by BMD, although they carried the mcr gene (Chew et al., 2017b,2017a). This study showed when the MIC values were 1 or 2 \( \mu g \) ml\(^{-1}\), but mcr-1 were positive. In our previous publication used the electrospray ionization mass spectrometry (ESI-MS, MALDI SYNAPT Q-TOF MS, Water Corp, Milford, MA, USA) detecting the Lipid A by the modified Bligh-Dyer method (Liu et al., 2016), whilst the extraction process in this report was complicated, not suitable for rapid clinical detection. Additionally, the rapid polymyxin NP test, developed as a biochemical test, can visually detect colistin-resistance within 2 h based on a colour change. The limitation of this method is the high cost for the necessary consumable materials. Laurent Dortet et al. (2018) developed a rapid MALDIxin test to detect polymyxin resistance that requires mass spectrometers equipped with reflectron mode. At present, most MALDI-TOF MS instruments in microbiology laboratories are Bruker MBT or Mérieux Vitek machines, and operate in linear mode, and thus, are not suitable for this method. Therefore, we investigated a MS-based method that relies on microbiology laboratories using their existing mass spectrometers to rapidly confirm colistin susceptibility and provide different ideas for clinical laboratories.

MALDI-TOF MS is a useful tool for the rapid detection of antimicrobial resistance mechanisms. One quick method for detecting resistance by MALDI-TOF MS is direct analysis of the mass peak profiles of bacteria as susceptible and resistant microorganisms of the same species can be differentiated on the basis of their spectra. For example, MRSA has a small peptide called PSM-mec, and the presence of this peptide can be detected in the MALDI-TOF spectra as a peak at 2,415 Da. However, Rhoads et al. reported that the sensitivities of the 2,415-Da peak for mecA carriage in Staphylococcus aureus and Staphylococcus epidermidis were low (37% and 6%, respectively), whereas the specificities were high (≥ 98%) (Rhoads et al., 2016). A 11 109 Da mass peak was used as a biomarker to identify KPC-producing Klebsiella pneumoniae with excellent results (Youn et al., 2016), but this biomarker has not yet been reported in China. Another MALDI-TOF MS method of detecting resistance employs hydrolysis whereby the antibiotic structure was modified by bacterial enzymes, and then, the newly formed mass peaks can be identified in the MALDI-TOF spectrum. Jung et al. (2014) validated a procedure for detecting \( \beta \)-lactam resistance in 2.5 h in Enterobacteriaceae in positive blood cultures. MALDI Biotyper (MBT) has a STAR-BL module and Compass software (Bruker Daltonik) for qualitative interpretation of spectra for detecting \( \beta \)-lactamase resistance (ESBLs and AmpC) by MALDI-TOF MS (Bruker Daltonik) (Oviano et al., 2017). The
main advantage of a hydrolysis method is its potential to be used to detect all types of hydrolases, even the rare ones. One shortcoming of the hydrolysis method was its lower sensitivity for detecting low-level activity hydrolases like OXA-48 producers. Another shortcoming of hydrolysis is that other resistance mechanism, such as the efflux pump, membrane porins and permeability, cannot be detected.

A semi-quantitative MALDI-TOF MS method is more sensitive than most optical or visual approaches and shortens the time required to obtain results. R software provides an algorithm to allow automated evaluation of mass spectra results. Katrin Sparbier et al. presented a semi-quantitative MALDI-TOF MS-based approach for the detection of resistance in different species against various antibiotics, such as cefotaxime-\textit{E. coli}, meropenem-\textit{K. pneumoniae} and meropenem-\textit{P. aeruginosa} to name a few (Sparbier et al., 2016). This method was also used to test \textit{C. glabrata} antifungal susceptibility against echinocandins and was shown to have a high sensitivity (80%) and specificity (95%) compared with the microdilution method (Vatanshenassan et al., 2019). Tucker Maxson et al. (2017) used 35 strains of \textit{S. aureus} and four antibiotics to optimize and test the assay, resulting in an overall accuracy rate of 95%. To the best of our knowledge, this is the first study where a method has been developed for determining \textit{E. coli} susceptibility against colistin and provides a promising approach for obtaining rapid results with high accuracy compared with the molecular and standard microdilution methods.

The semi-quantitative MALDI-TOF MS method described herein provided a sensitivity of 97.4% and a specificity of 88% for rapid testing of colistin susceptibility. The development of this model required only few colistin-resistant or colistin-sensitive stains to determine the optimal incubation time, breakpoint concentration and cut-off value. This method should be easily extended to other bacterial species and antibiotics and does not require specialized media or instrumentation. In addition, the internal standard used in this study was RNase A, which is more economical when compared to the standard RNase B described previously (Lange et al., 2014). For clinically verified strains, the pre-processing time was only 2.5 h. This study was limited by the fact that only \textit{E. coli} samples that were \textit{mcr}-positive or \textit{mcr}-negative were investigated. By far, the \textit{mcr} gene is the main reason to cause colistin-resistance in \textit{E. coli}. This gene encodes a plasmid-borne phosphoethanolamine transferase that has been reported in \textit{E. coli} isolates from animal, food, environment and human samples worldwide (Al-Tawfiq et al., 2017; Faccone et al., 2020). Further studies should include investigation of other resistance mechanisms of colistin-resistant bacterial species other than \textit{E. coli}. A shorter turn-around time (TAT) of susceptibility results is essential to optimize antimicrobial treatment in patients, especially for those who are critical. As shortening the TAT is more valuable for critical patients, such as those with sepsis, the direct used of this method for screening of positive blood cultures or sterile body fluids should be examined in the future. Nevertheless, this MS-based approach is simple to set-up, employs a short incubation time, and provides excellent outcomes with respect to the sensitivity and specificity.

Experimental procedures

\textbf{Bacterial strains}

A total of three \textit{mcr}-1-positive and six \textit{mcr}-1-negative \textit{E. coli} strains were used for establishing the model. The three \textit{mcr}-1-positive \textit{E. coli} samples (E218-2, E2525-1-2 and E3647-2) were collected from stool specimens as described previously (Shen et al., 2018). One of the six \textit{mcr}-1-negative \textit{E. coli} samples was ATCC 25922, and the other five strains (MS Number from 1 to 5) were collected from stool specimens from healthy individuals in Zhejiang in September 2016 and had colistin minimal inhibitory concentration (MIC) values from $\leq 0.25$ to $2 \mu g ml^{-1}$ (Table 2). Verification strains were collected from healthy individuals in Zhejiang. All isolates were inoculated onto Columbia agar supplemented with 5% sheep blood (Oxoid, Cambridge, UK) and then incubated at 35$^\circ$C. Fresh overnight cultures were used for the tests.

\textbf{PCR amplifications and sequencing}

All collected verification strains were screened for the presence of \textit{mcr}-1 positive strains using PCR with the primers \textit{mcr}-1-forward (5'-GCTCGGTCAGTCCGTTTG-3') and \textit{mcr}-1-reverse (5'-GAATGCGGTGCGGTCTTT-3').

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
MIC of colistin (\(\mu g\) ml\(^{-1}\)) & \textit{mcr}-1 gene & MS-based approach \\
\hline
\textbf{Negative} & \textbf{Positive} & \textbf{S} & \textbf{R} \\
\hline
0.25 & 9 & – & 9 & – \\
0.25 & 16 & – & 16 & 3 \\
0.5 & 13 & – & 13 & 1 \\
1 & 11 & 2 & 11 & 2\(^a\) \\
2 & – & 9 & 9 & – \\
4 & – & 39 & 1 & 39 \\
8 & – & 26 & 1 & 26 \\
16 & – & 2 & – & 2 \\
\hline
\end{tabular}
\caption{Number of MIC, PCR and MS-based approach in 128 verification \textit{E. coli} strains.}
\end{table}

\(^a\)Two strains showed R and \textit{mcr}-1 gene was positive.
The amplicons were subsequently sequenced by Sanger sequencing (Quan et al., 2017).

**Antimicrobial susceptibility testing**

BMD method was used to MIC of colistin (Shen et al., 2018). Strains that show colistin MIC ≤ 2 µg ml⁻¹ is interpreted as susceptible and MIC > 2 µg ml⁻¹ as resistant according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints v9.0. The quality control strain was *E. coli* ATCC 25922.

**MALDI-TOF MS semi-quantitative resistance profiling assay**

To determine the optimal incubation time for the resistance profiling assay, fresh bacterial isolates were incubated in brain–heart infusion (BHI) medium (Binhe, China) at a cell density of 0.5 McFarland standard. For each strain, two different 200 µl mixtures, one containing no antibiotics and one with 64 µg ml⁻¹ colistin (colistin thate sodium, China National Institutes for Drug Control, China), were prepared. The preparations were incubated in a 37°C water bath in a ThermoMixer (Cambridge, Grant GD100-P5, UK) for 1, 2 and 3 h. After incubation, cells were centrifuged and washed once with 300 µl 75% ethanol solution. Subsequently, bacteria were lysed according to the MALDI Biotyper standard protocol (Nagy et al., 2012) with 25 µl 70% formic acid and 25 µl acetonitrile containing RNase A (40 mg l⁻¹; Sigma-Aldrich, St Louis, MO, USA) as an internal standard. To obtain the breakpoint concentration of colistin for each sample, fresh bacterial isolates were incubated for the optimal incubation time at concentrations ranging from 0.025 to 64 µg ml⁻¹ of colistin. For clinical strains, fresh bacterial isolates were incubated in BHI medium at a cell density of 0.5 McFarland standard. Two incubation mixtures (200 µl), one without colistin and the other containing the breakpoint concentration of colistin, were prepared for each strain. Subsequently, bacterial cell lysis was performed as described above. One microlitre of the cell lysates was directly spotted onto a steel target. Dried spots were overlaid with 1 µL of matrix (10 mg ml⁻¹ of α-cyano-4-hydroxy-cinnamic acid [HCCA] in 50% acetonitrile-2.5% trifluoroacetic acid; Bruker Daltonik, Bremen, Germany). After drying, MALDI-TOF MS measurements were performed with a Microflex LT/SH mass spectrometer (Bruker Daltonik) equipped with a 60 Hz laser frequency. The parameters for the data collection included a mass range between 2000 and 20 000 Da, 240 laser shots and an intensity measurement (arbitrary units) of ≥ 10⁴ for at least one peak. An external calibration standard (bacterial test standard [BTS]; Bruker Daltonik) was used for the instrument calibration.

**Data evaluation**

Pseudogel view representations were performed using the gel view display of the MALDI Biotyper Compass Explorer software (Bruker Daltonik). Automated analyses of the spectra were performed with a prototype software tool written with the freely available software package R (Lange et al., 2014). The software package R software individually calculated the area under the curve (AUC) for each set-up. Finally, the relative growth (RG) value was calculated as the ratio of the AUCs of identical samples incubated with and without colistin:

\[ RG = \frac{AUC_{\text{BHI}-\text{colistin}}}{AUC_{\text{BHI}}}. \]

The RG values for ATCC25922 and E218-2 incubated in the presence or absence of colistin (64 µg ml⁻¹) on five different days repeated three times. Box plots would evaluated the RG values for ATCC25922 and E218-2. One box shows the three-time evaluation on five days of results. The minima and maxima are indicated by whiskers, and the 95% confidence intervals are indicated by boxes.

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**Conflict of interest**

None declared.

**Ethical approval**

Ethical approval was given by The Second Affiliated Hospital of Zhejiang University, School of Medicine, (2017-099). Individual consent forms were translated into Mandarin, and consent was obtained for all healthy volunteers before sampling.

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