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RESEARCH

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Rapid detection of ceftriaxone-resistant *Salmonella* by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry combined with the ratio of optical density

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Abstract

Background The increased resistance rate of *Salmonella* to third-generation cephalosporins represented by ceftriaxone (CRO) may result in the failure of the empirical use of third-generation cephalosporins for the treatment of *Salmonella* infection in children. The present study was conducted to evaluate a novel method for the rapid detection of CRO-resistant Salmonella (CRS).

Methods We introduced the concept of the ratio of optical density (ROD) with and without CRO and combined it with matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) to establish a new protocol for the rapid detection of CRS.

Results The optimal incubation time and CRO concentration determined by the model strain test were 2 h and 8 μg/ml, respectively. We then conducted confirmatory tests on 120 clinical strains. According to the receiver operating characteristic curve analysis, the ROD cutoff value for distinguishing CRS and non-CRS strains was 0.818 [area under the curve: 1.000; 95% confidence interval: 0.970–1.000; sensitivity: 100.00%; specificity: 100%; $P < 10^{-3}$].

Conclusions In conclusion, the protocol for the combined ROD and MALDI-TOF MS represents a rapid, accurate, and economical method for the detection of CRS.

Keywords Ceftriaxone, Drug Resistance, *Salmonella*, MALDI-TOF MS, Rapid diagnostic tests

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Background

Salmonella (Enterobacteriaceae) is a facultative anaerobic and dynamic gram-negative bacterium that can cause various infections, and it is usually classified as typhoid, paratyphoid, and nontyphoidal *Salmonella* (NTS) [1]. *Salmonella* (mainly NTS) is an important cause of human bacterial enteritis, and it can also cause serious, life-threatening, invasive infections in some patients [2]. This bacterium causes 93.8 million cases of gastroenteritis worldwide each year, including 155,000 deaths, and is a major public health problem worldwide; in addition, it imposes a crucial economic burden on health systems [3, 4]. *Salmonella* infection is usually self-limiting in children with normal immunity; however, immunocompromised, younger infants or children with suspected aggressive infections require effective antibiotic treatment [5, 6]. Third-generation cephalosporins and fluoroquinolones are the most important classes of antibiotics for the treatment of *Salmonella* infections, especially invasive cases [7]. The use of fluoroquinolones in children is avoided due to potential serious adverse reactions [8]. As a result, third-generation cephalosporins, which are represented by ceftriaxone (CRO), have become the drug of choice for the treatment of *Salmonella* infections in children, especially when aggressive infection is suspected [9].

Despite the extensive use of antibiotics, *Salmonella* strains resistant to third-generation cephalosporins continue to appear, and a growing trend is being observed. In an epidemiological study from Taiwan, China, the rate of resistance to CRO from the NTS isolated from samples collected from children in the area increased from 4.1% in 2012 to 14.3% in 2019 [10]. Another research from Ningbo, China, revealed that the NTS strains derived from children in the area were more than 30% resistant to CRO [11]. The situation of *Salmonella* resistance to CRO is a serious concern, which may lead to the possible failure of the empirical use of third-generation cephalosporins in the treatment of *Salmonella* infections in children (especially invasive ones) and more serious consequences [12]. Thus, adverse prognosis of *Salmonella* infection in children must be avoided through the rapid and accurate identification of CRO-resistant *Salmonella* (CRS) and timely adjustment of antibiotic treatment strategies. However, traditional biochemical identification and drug susceptibility tests often last for 3–4 days or even longer, which fails to meet the needs of clinical treatment. Therefore, a rapid, accurate, reliable, and economical method for the identification of CRS must be developed urgently. The increased application of matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) in clinical microbiology is a possible solution to this problem.

This study was conducted from the perspective of a previous idea of realizing the rapid detection of antibiotic sensitivity to pathogenic microorganisms through a short-term culture method based on MALDI-TOF MS. The concept of the ratio of optical density (ROD) was innovatively introduced to reflect the differences in bacterial growth rate between strains with or without CRO. Finally, we combined the ROD and MALDI-TOF MS to develop a rapid, accurate, and economical method for the detection of CRS.

Methods

Bacterial strains

Three CRS and three non-CRS strains were used to establish a model. One non-CRS strain was ATCC14028, and the remaining two non-CRS strains and three CRS strains were isolated from clinical specimens sent to the Department of Clinical Laboratory, Children's Hospital, Zhejiang University School of Medicine, between August 2019 and July 2020. The minimal inhibitory concentrations (MICs) for all strains were confirmed via E-test (Bio-Kont, Wenzhou, China). The outlined procedure consists of the following steps: Initially, fresh overnight cultures of the bacteria to be tested were utilized to create a 0.5 McFarland standard bacterial suspension, which was subsequently evenly spread onto Mueller-Hinton agar (MHA, Biocell, Zhengzhou, China). Following this, an E-test strip was applied to the MHA and the culture was allowed to incubate overnight at 35 °C. Finally, the minimum inhibitory concentrations (MICs) were determined and interpreted based on the Clinical and Laboratory Standards Institute breakpoints published annually [13]. Whole-genome sequencing (WGS) was performed to determine the genotypes of the three CRS strains utilized in this study, employing the Illumina HiSeq X-Ten platform (Illumina, San Diego, USA). Furthermore, a total of 120 *Salmonella* strains isolated from clinical specimens received by the laboratory from August 2019 to July 2020 were randomly selected in a 1:3 ratio (R vs. S) to validate the efficacy of the rapid identification protocol introduced in this research. All strains were cultured overnight at 35 °C on Columbia agar containing 5% sheep blood (Biocell, Zhengzhou, China), and fresh overnight cultures were used for the tests. E-test was performed to confirm the MICs of the 120 *Salmonella* strains. In addition, MALDI-TOF MS (Bruker Daltonik, Bremen, Germany) was used to reconfirm all *Salmonella* strains at the genus level included in this study. The process mainly included the following: ① the fresh overnight cultures were transferred to a cleaned MALDI target; ② carefully covering each sample with 1 μl 70% (V/V) formic acid and 1 μl matrix solution; ③ drying of samples at room temperature; ④ loading of the plate into the IVD MALDI Biotyper System (Bruker Daltonics, Bremen, Germany) for analysis.

Model strain testing

Determination of optimal incubation time

① The experimental model strains were cultured at 35 °C on Columbia agar containing 5% sheep blood for 16–18 h. ②The single colonies (colony diameter ≈ 1.5 mm) were selected using inoculation rings, placed in sterile test tubes containing 2 ml brain–heart infusion (BHI) medium (Binhe, Hangzhou, China), and thoroughly mixed. ③ The bacterial suspension was dispensed into two wells of a 96-well plate, with each well containing 200 µl. One well was added with CRO for a final concentration of 32 µg/ml, and the other had no CRO added to it. The final concentration was established through a thorough review of relevant literature and the MICs of the model strains [14]. ④ The 96-well plate was incubated in an enzyme-labeled instrument (Tecan, Grödig, Austria), and the OD was measured at 37 °C and 620 nm wavelength, with reading performed every 5 min for 180 min. ⑤ The data were exported, and the ROD ($ROD = OD_{BHI + CRO}/OD_{BHI}$) for each point in time was calculated. The optimal incubation time point (the shortest time needed to observe a significant difference in ROD) was determined through the comparison of the difference in the ROD between the sensitive and drug-resistant groups at each time point.

Determining the optimal concentration of CRO during incubation

① This step was the same as ① and ② in Determination of optimal incubation time. ② The bacterial suspension was distributed into eight wells of the 96-well plate, with each well containing 200 µl. A specific concentration of CRO suspension was added to seven of the wells (at final concentrations of 0.5, 1, 2, 4, 8, 16, and 32 µg/ml), and no CRO was added to the remaining one. ③ The 96-well plate was incubated in an enzyme-labeled instrument. The OD of bacterial suspension in each well was determined at the optimal incubation time point determined in Step 2.2.1 (37 °C, wavelength: 620 nm), and the ROD under different CRO concentrations was calculated. ④ The optimal CRO concentration during incubation was determined through a comparison of the difference in the ROD between the sensitive and drug-resistant groups after incubation at different CRO concentrations (the minimum CRO concentration showing a significant difference in ROD).

Clinical strain testing

A total of 120 clinical strains collected were validated after the model strain test. The optimal concentration of CRO and the short-term incubation time was determined by the model strain test. The other testing procedures are the same as the model testing procedures, and the flow is shown in Fig. 1.

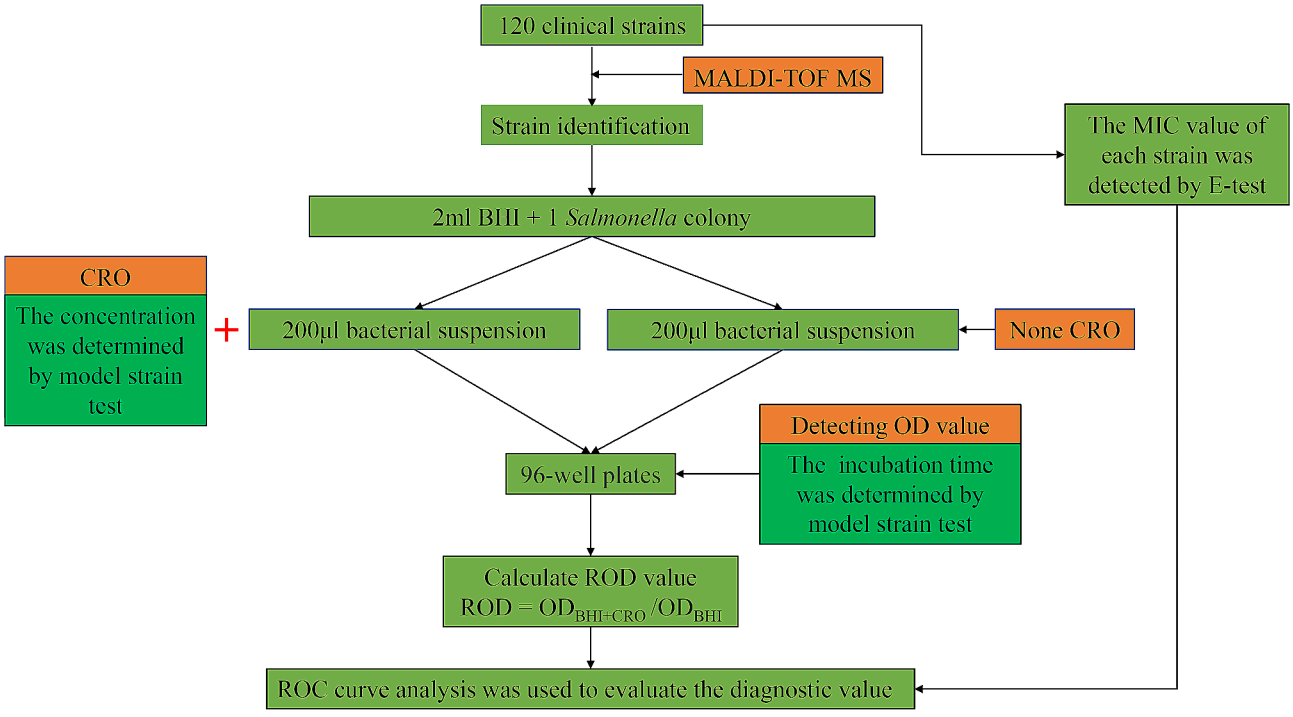

Fig. 1 Flow chart of the rapid detection of 120 clinical strains used in this study

Table 1 Information on model strains used in this study

Original No.	Origin	Bacterial species	MIC of ceftriaxone (μg/ml)	Genotype
ATCC14028	QC	*Salmonella typhimurium*	0.064	/
19CR6047	The Children's Hospital of Zhejiang University School of Medicine	*Salmonella typhimurium*	0.064	/
20CR6031	The Children's Hospital of Zhejiang University School of Medicine	*Salmonella typhimurium*	0.064	/
19CR6025	The Children's Hospital of Zhejiang University School of Medicine	*Salmonella typhimurium*	> 256	*CTX-M-55, TEM-1*
19CR6048	The Children's Hospital of Zhejiang University School of Medicine	*Salmonella typhimurium*	> 256	*CTX-M-55*
20CR6050	The Children's Hospital of Zhejiang University School of Medicine	*Salmonella typhimurium*	> 256	*CTX-M-55, TEM-1*

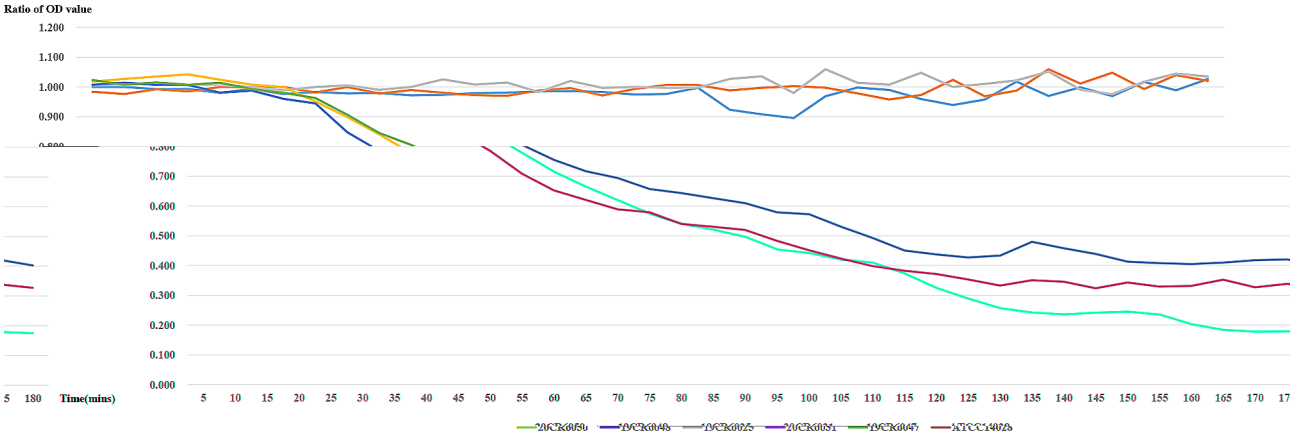

Fig. 2 Ratio of optical density value of the model strains at different time nodes

Data analysis

Quantitative variables were expressed as the median and interquartile range (25th and 75th percentiles). The Mann–Whitney U test was used to compare differences between continuous variables. The ROD is calculated as follows:

$$\mathrm{ROD} = \mathrm{OD}_{\mathrm{BHI+CRO}}/\mathrm{OD}_{\mathrm{BHI}}$$

Receiver operating characteristic (ROC) curve analysis was conducted to evaluate the diagnostic value of the proposed protocol for the detection of CRS strains in this study. The maximum value of the calculated Youden index was used to determine the best cutoff value. Sensitivity and specificity were determined to evaluate the diagnostic efficacy of the rapid identification protocol. The area under the curve (AUC) was interpreted as follows: AUC < 0.7, low accuracy; 0.9 ≥ AUC ≥ 0.7, moderate accuracy; AUC > 0.9, high accuracy. The statistical software MedCalc version 19.1 (MedCalc Software Ltd, Ostend, Belgium) was used for data analysis. Statistical significance was set at $P < 0.05$, and all tests of significance were two-sided.

Results

Results of model strain testing

The model strains comprised six strains of *Salmonella typhimurium*. Three model strains (ATCC14028, 19CR6047, and 20CR6031) exhibited sensitivity to CRO (MIC = 0.064 μg/ml), and the remaining three (19CR6025, 19CR6048, and 20CR6050) presented resistance (MIC ≥ 256 μg/ml). WGS was conducted to determine the genotypes of the three CRS strains. Table 1 shows the information on the model strains used in this study. The results of time echelon analysis of the model strains revealed that the minimum ROD difference between the CRS and non-CRS strains was more than 0.5 after incubation for 2 h, and the difference tended to be stable after incubation for 2 h. Therefore, the short-term incubation period was set at 2 h. Figure 2 displays the ROD of the model strains at different time nodes. The results of CRO-concentration echelon analysis of the model strains indicated that the minimum ROD difference between the CRS and non-CRS strains was more than 0.5 at the CRO concentration of 8 μg/ml. When the CRO concentration was less than 8 μg/ml, the minimum ROD difference was less than 0.5. However, at the CRO concentration greater than 8 μg/ml, the minimum ROD difference remained at 0.5–0.6 and did not increase significantly. Therefore, we set the optimal concentration of CRO during incubation to 8 μg/ml. Figure 3 shows the RODs of the model strains at different CRO concentrations.

Distribution of clinical strains

A total of 120 *Salmonella* strains isolated from stool samples of infants with diarrhea were included in the

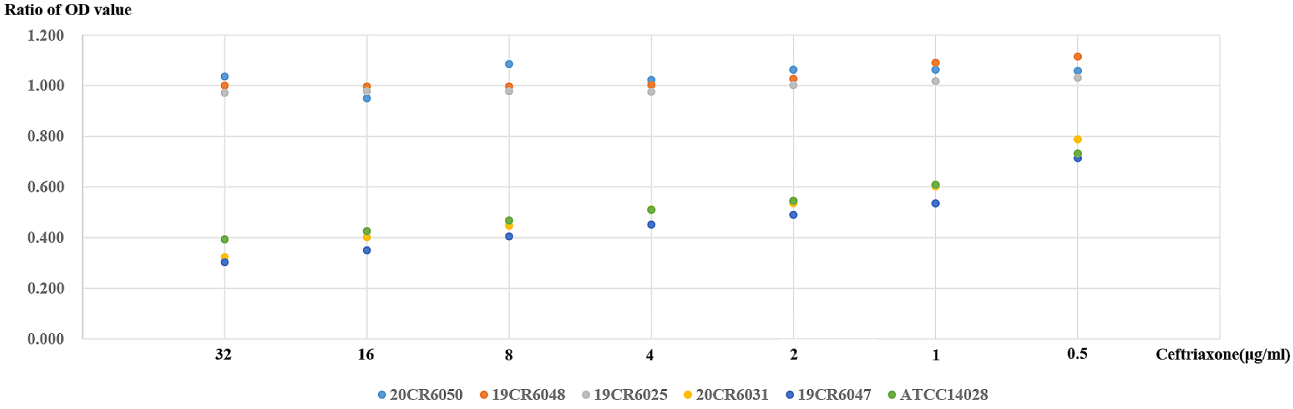

Fig. 3 Ratio of optical density value of the model strains at different ceftriaxone concentrations

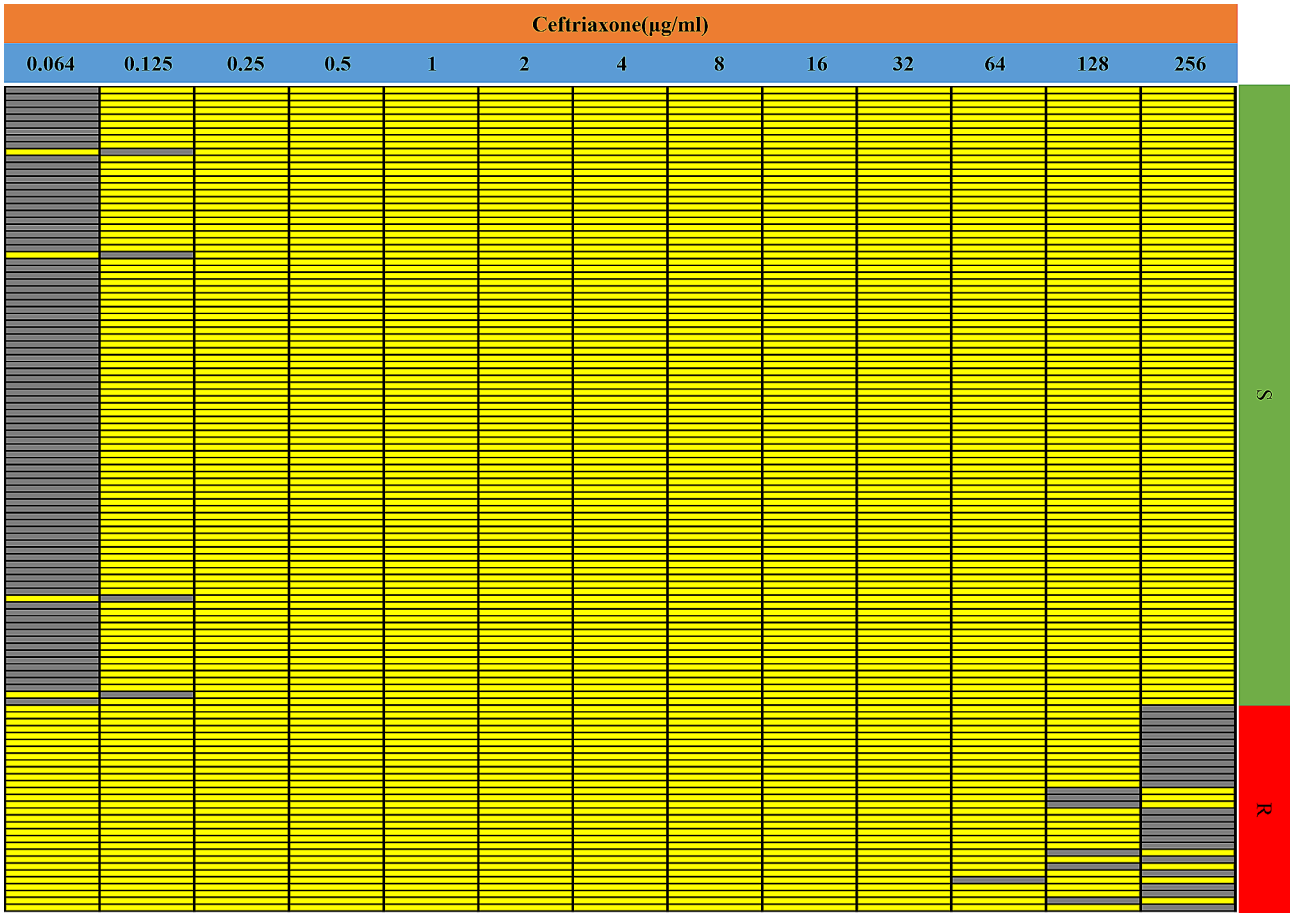

Fig. 4 Distribution of minimum inhibitory concentrations (MICs) of 120 clinical strains included in this study

confirmation tests for the proposed rapid identification protocol. These strains included 80 strains of *Salmonella* group B (66.7%), 15 strains of *Salmonella* group D (12.5%), 12 strains of *Salmonella* group C1 (10.0%), and 13 strains belonging to other serogroups (10.8%). Out of the 120 strains, 30 (25.0%) were CRS, and the remaining 90 (75.0%) were non-CRS strains. Figure 4 shows the distribution of MICs of the 120 clinical strains included in this study.

Results of clinical strain testing

Confirmatory tests were performed on the 120 clinical strains based on the optimal short-term incubation time (2 h) and optimal concentration of CRO (8 μg/ml) determined by the model strains. The 30 CRS strains and

90 non-CRS strains had median RODs of 0.989 (0.963–1.014) and 0.447 (0.265–0.587), respectively. A significant difference in the ROD was observed between the CRS and non-CRS strains according to the Mann–Whitney U test ($P<10^{-3}$). Figure 5 displays the scatter diagram of the ROD for the clinical strains after 2 h of incubation. The ROD showed a significant AUC of 1.000 (95% confidence interval (CI), 0.970–1.000, $P<10^{-3}$) during the detection of CRS strains via the rapid identification protocol proposed in this study. The optimal ROD cutoff value was 0.818. The sensitivity was 100.00%, and the specificity was 100.00%. Figure 6 shows the ROC curve for the performance of the rapid detection method introduced in this study for clinical isolates.

Discussion

The introduction of MALDI-TOF MS into clinical microbiology laboratories over the past decade has revolutionized the manner of identification of pathogenic microorganisms [15]. The research on MALDI-TOF MS in the rapid detection of antibiotic sensitivity has enabled the rapid identification and determination of the drug sensitivity of pathogenic microorganisms in clinical microbiology laboratories [16].

Three ways can be used to realize the rapid detection of the antibiotic sensitivity of pathogenic microorganisms based on MALDI-TOF MS. The first is the use of machine learning in the analysis of mass spectrograms for the rapid detection of antibiotic sensitivity of pathogenic microorganisms [17–19]. This method only needs to analyze the mass spectrogram of different strains without additional operations, but it requires proficiency in complex machine learning algorithms and special analysis software. The second method enables the rapid detection of antibiotic sensitivity of pathogenic microorganisms by measuring the marker peaks associated with drug resistance [20–24]. This method does not require complex mass spectrogram processing, but the scope of its application is limited, and the rapid detection of drug-resistant bacteria and drug-resistant mechanisms cannot be realized without drug-resistant marker peaks. The third method attains the rapid detection of antibiotic sensitivity to pathogenic microorganisms through short-term culture methods. The principle of this method is the

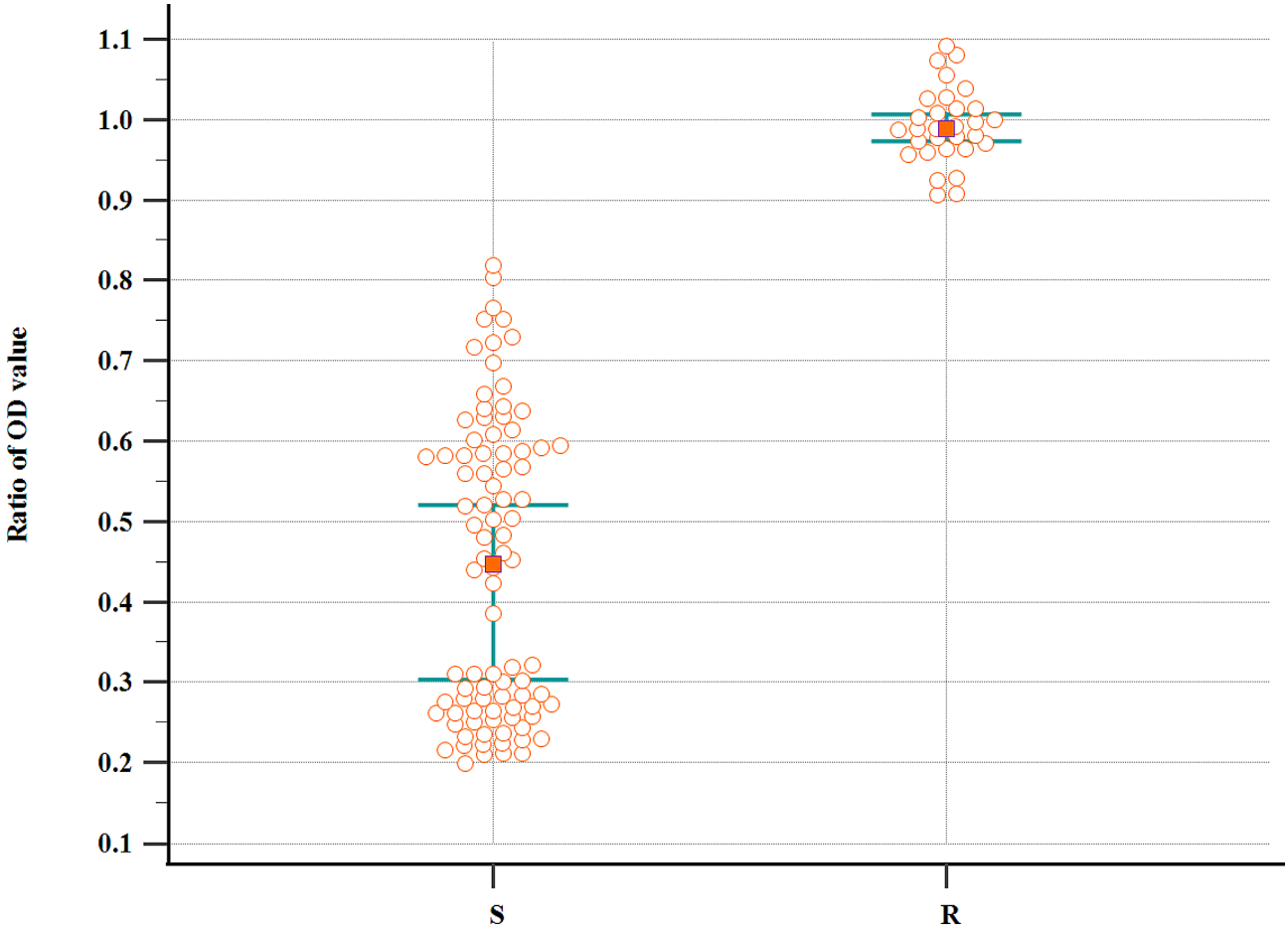

Fig. 5 Scatter diagram of the ratio of optical density of the clinical strains after 2 h of incubation

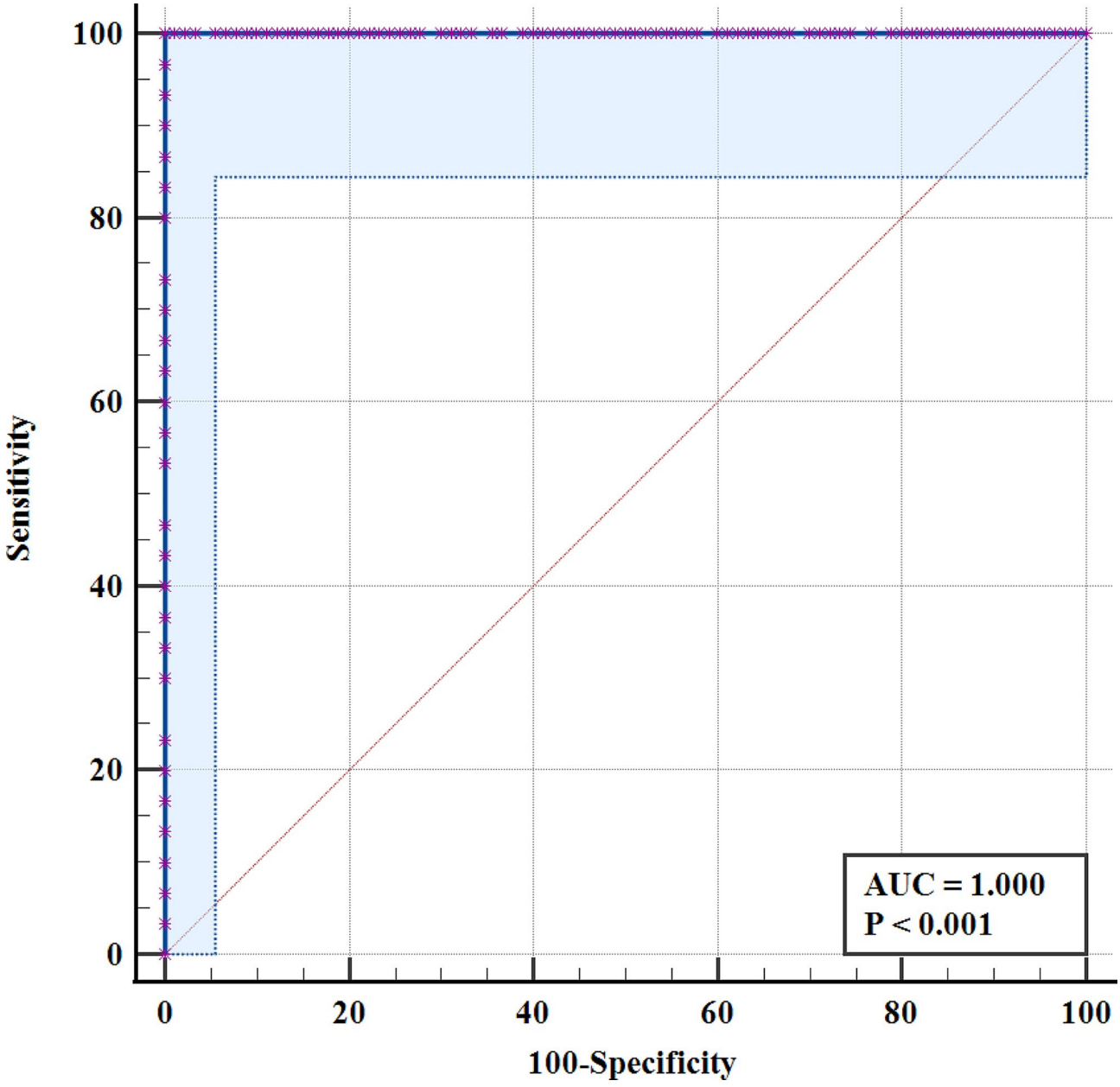

Fig. 6 Receiver operating characteristic curve of the performance of the rapid detection method introduced in this study for clinical isolates

short-time culture of pathogenic microorganisms with or without specific antibiotics, followed by the collection and analysis of culture products via MS. Two main methods are used in the subsequent MS stage: The first method involves performing MS on the collected short-term culture products, the use of R software for a series of processing of mass spectra, the calculation of the AUC of each peak map, and the calculation of the relative growth (RG) value from the AUC ($RG = AUC_{BHI + antibiotic}/AUC_{BHI}$) [14, 25]. Finally, the difference in RG value is used for the rapid detection of antibiotic sensitivity. Despite its simple operation, the method requires specialized software to perform complex analysis of mass spectrum data during data processing. The second method uses a microsystem for short-term culture and identifies culture products via MS. Reliable identification results (score ≥ 1.7) with or without a specific antibiotic indicate resistance to a specific antibiotic. A reliable identification obtained in the absence of a specific antibiotic (score ≥ 1.7) but not in the presence of a specific antibiotic (score < 1.7) indicates no resistance to a specific antibiotic [26]. Although this method is simple, rapid drug sensitivity results depend on the score of MS identification, and any factors that can affect such score may have adverse

effects on drug sensitivity results. Therefore, the reliability of the second method is questionable, especially in the identification of a large number of clinical strains.

In this study, we used the first method from the short-term culture protocol and innovatively introduced the concept of ROD to reflect differences in bacterial growth rates between *Salmonella* strains with or without CRO. Then, we combined MALDI-TOF MS and ROD to construct a novel method for the rapid detection of CRS. First, model strains were used to determine the two main parameters, namely, the optimal incubation time and optimal concentration of CRO during incubation. Under ideal conditions, the difference in the ROD between sensitive and resistant strains after short-term culture should be between 0 and 1. Therefore, when the ROD difference exceeded 0.5, a relatively significant difference was considered. As shown in Fig. 2, the minimum ROD difference between the CRS and non-CRS strains was more than 0.5 after incubation for 2 h, and the difference tended to plateau afterward. Therefore, the optimal incubation time was 2 h. As shown in Fig. 3, the model strains revealed that the minimum ROD difference between the CRS and non-CRS strains was more than 0.5 at the CRO concentration of 8 μg/ml. When the CRO concentration was less than 8 μg/ml, the minimum ROD difference was less than 0.5. Therefore, the optimal concentration of CRO during incubation was 8 μg/ml. Then, confirmatory tests were performed on 120 clinical strains based on the optimal short-term incubation period (2 h) and optimal concentration of CRO (8 μg/ml) determined using the model strains. The selected *Salmonella* strains showed a distribution that was close to that of clinical isolates (the main strain was *Salmonella* group B, and CRO-resistant strains accounted for 25%). According to the results of ROC analysis of clinical *Salmonella* detection, the rapid identification protocol exhibited a nearly perfect detection capability for clinical CRS strains (AUC: 1.000; 95% CI: 0.970–1.000; sensitivity: 100.00%; specificity: 100.00%; $P<10^{-3}$). Thus, this rapid identification protocol is not only accurate and reliable but also greatly reduces the detection time compared with the conventional clinical method.

Finally, two limitations of the study must be noted: ① This work is single-center research, and the detection parameters of the proposed rapid identification protocol may be inapplicable to other regions due to variations in epidemic strains. Therefore, the detection parameters must be redetermined when applying this rapid identification protocol in other regions. ② Given the small number of strains, *Salmonella* strains for CRO intermediate susceptibility were excluded in the confirmatory testing of clinical strains in this study. Therefore, the effectiveness of detection for these strains is unknown. However, given the small number of *Salmonella* strains for CRO intermediation, their influence on the effectiveness of the introduced rapid identification protocol is limited.

Conclusions

In summary, the protocol combining ROD and MALDI-TOF MS is a rapid, accurate, and economical method for CRS detection.

Abbreviations

AUC	Area under the curve
BHI	Brain–heart infusion
CRO	Ceftriaxone
CRS	CRO-resistant Salmonella
MALDI-TOF MS	Matrix-assisted laser desorption–ionization time-of-flight mass spectrometry
MHA	Mueller-Hinton agar
MICs	Minimal inhibitory concentrations
NTS	Nontyphoidal *Salmonella*
ROD	Ratio of optical density
WGS	Whole-genome sequencing

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None reported.

Author contributions

C.F. takes responsibility for the conception and design of the study. Z.Z. takes responsibility for analysis of data and drafting the article. M.Z. contributed to acquisition and analysis and interpretation of data. J.L. contributed to review and editing the manuscript. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The study was approved by the ethics committee of the Children's Hospital, Zhejiang University School of Medicine, China(2021-IRB-031).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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