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Evaluation of the Diagnostic Value of Direct Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry for Identifying Candida Species in Oral Candidiasis Patients

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Abstract

Oral Candidiasis is one of the most common oral mucosal infectious diseases in clinic. Accurate and rapid identification of yeasts is important to initiate appropriate treatment. In the first phase of the present study, 175 candida isolates were collected from non-stimulated whole saliva of 170 patients with oral candidiasis, and cultured on Sabouraud's agar. Rapid Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) was compared to classical testing for identifying these Candida isolates. The accuracy was 94.3% and 84.0%, respectively, for *C. albicans* and non *C. albicans* isolates. In order to shortening the test time, 57 participants were enrolled in the second phase. Clinical candida isolates were collected from gargle specimens, cultured in liquid medium for less than 48 hours. The optimal accuracy of MALDI-TOF MS for gargle specimens was 76.1%, which were cultured in Sabouraud's medium for 24 hours. MALDI-TOF MS is effective and rapid (12.7-24.7 h for turnaround time, 5.5 min for hands-on time per sample) for Candida spp. identification, and could be used as optional methods for rapid detection and diagnosis of oral candidiasis patients in clinic.

Keywords: MALDI-TOF MS; *Candida*; Saliva; Liquid culture; Effective/rapid analysis

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Introduction

Oral candidiasis is one of the most common opportunistic mycosis in humans, being a fungal infection of the oral mucous membranes caused by *Candida* species [1]. Such infection may cause pain, burning sensation, xerostomia, and changes in taste perception. Patients with hyperplastic candidiasis exhibit a risk of malignant change [2]. With the increasing number of immunosuppressed patients, such as cancer, transplant, and acquired immunodeficiency syndrome (AIDS) patients, as well as older individuals wearing dentures, the prevalence of Candidiasis is also increasing [3].

Candida albicans is the major species that causes oral infection, but non-*albicans Candida* species have become common in recent years [4]. Some such species, including *C. glabrata* and *C. krusei*, are more resistant to antifungal agents than is *C. albicans* [5]. Accurate and rapid identification of yeasts is important to initiate appropriate treatment. However, classical methods used for identification, such as phenotypic and chromogenic

media-based culture approaches, are time-consuming and labor-intensive [6]. Various molecular techniques, including PCR and peptide-nucleic acid fluorescent *in situ* hybridization, have been developed to accelerate identifications. However, these methods are too expensive and technically demanding to be implemented as routine clinical laboratory techniques [7,8].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently emerged as a rapid and powerful tool for identifying microbial species [9-12]. Since 2011, direct identification of *Candida* from blood culture media has been possible, and has markedly lowered test times [13]. However, no study has yet described the use of MALDI-TOF MS for direct identification of fungal pathogens in liquid cultures of saliva samples. In the present study, we assessed the accuracy of MALDI-TOF for identifying clinical fungal pathogens isolated from patients with oral candidiasis, and conducted a preliminary study on the utility of MALDI-TOF MS-based methodologies for direct identification of fungal pathogens from liquid cultures of saliva. We compared the method to the classical CHROM agar test and PCR-based sequencing in terms of both accuracy and cost.

Materials and Methods

Study design

This study was divided into two phases. In the first phase, 170 patients were enrolled, and Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) detection was carried out based on a Sabouraud's agar cultured method. In the second phase, in order to simplify the method and shorten the test time, specimens cultured in liquid agar were used in the MALDI-TOF MS detection.

Patients and samples

This clinical trial has been registered at www.clinicaltrial.gov (ChiCTR-DDT-13003991). The study protocol has been approved by The Institutional Review Board of the Peking University School and Hospital of Stomatology (approval number PKUSSIRB-2013021). In the first phase of the study, 170 patients with oral candidiasis were recruited from the Department of Oral Medicine at the Peking University School of Stomatology and the Department of Oral Medicine of the Capital Medical University of Beijing (Stomatological Hospital). Non-stimulated saliva samples (10 mL) were collected from each subject. In the second phase, 57 participants, 42 with oral candidiasis and 15 healthy participants (negative controls), were recruited from the Department of Oral Medicine of the Peking University School of Stomatolog. Saliva and mouthwash samples were collected.

Sample selection

In the first phase, 5 mL non-stimulated saliva was collected from each subject. In the second phase, non-stimulated saliva, cotton swabs and gargle specimen were collected from 5 participants for a pilot study, and only gargle specimen were collected from the rest participants. Cotton swabs were applied to the dorsum, double buccal mucosa, and palate, and placed in 5 mL sterile saline. Each participant was required to wash the mouth (gargle) with 15 mL sterile saline. Gargle specimens were centrifuged at 2,500 rpm and concentrated to 5 mL volumes. Two trained clinicians performed collecting procedures. All samples were transported to the laboratory at room temperature within 2 hours. The identification processes were carried out in a blinded manner via MALDI-TOF MS, sequencing, and CHROM agar culture.

Isolation of *Candida* from clinical samples

In the first study phase, 1 mL aliquot of saliva from each sample was spread on Sabouraud's agar and incubated at 37°C for 48 h. Three to five colonies were suspended in 400 µL deionized water and shaken for 10 minutes. In the second study phase, 1 mL aliquot of garble specimen was suspended in different liquid medium for less than 48 hours. All *Candida* isolates were identified using internal transcribed spacer (ITS) sequencing, and those from the first experiment were also identified via MALDI-TOF MS.

Polymerase chain reaction

The primers ITS1 and ITS4 were used for PCR. All sequencing reactions were performed on a standard thermocycler using 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s. The PCR products were stored at 4°C until they were sent to the BGI Tech Company (Beijing, China) for sequencing. The resulting sequences were compared to those in the National Center of Biotechnology Information (NCBI) database using BLAST searches; data with confidence levels >97% were considered to reflect correct identifications.

Mass-mapping using MALDI-TOF

In the first phase of the experiment, 300 µL yeast suspension, prepared as described above, was added to 900 µL anhydrous ethanol and centrifuged at 13,000 rpm for 10 min at 4°C. TROPICALIShe supernatants were removed and the pellets were resuspended in 50 µL 70% (v/v) formic acid (Sigma-Aldrich, St. Louis, MO). Then 50 µL acetonitrile (Sigma-Aldrich) was added, and the suspensions were again centrifuged at 13,000 rpm for 10 min at 4°C. ALBICANSliquots (1 µL) of supernatants were spotted in triplicate onto a ground-steel target (Bioyong Technologies Inc., Beijing, China) and air-dried for 10 min. Each spot was overlaid with 1 µL matrix solution (alpha-cyano-4-hydroxycinnamic acid in 50% [v/v] acetonitrile/0.1% [v/v] trifluoroacetic acid; both from Sigma-Aldrich) and allowed to co-crystallize for 1 min at room temperature. Spectra were acquired using a MALDI-TOF MS (Bioyong) operating in the linear positive mode (laser frequency, 20 Hz; acceleration voltage, 20 kV; pulser voltage, 1.9 kV; Einzel voltage 5 kV; delayed extension focus mass, 10,000; and mass range, 2,000–13,000 Da).

The test spectra were identified using BioExplorer software to compare them to reference mass spectrum profiles (MSPs). The identification process began with pre-processing of test spectra and the generation of peak lists that were compared and aligned with those of the MSPs. Then the test spectra were calibrated using the MSPs. In this step, an initial mass error can be selected within the identification parameters (parameter Max. Mass Error

of the raw spectrum). BioExplorer attempts to align the highest peaks of each spectrum within the confines of the initial mass error; then the peaks are aligned and the spectra are calibrated with the quadratic calibration held constant. The aim is to maximize homology.

The test spectra were matched to MSPs, based on dedicated score values. To this end, MSP peak information was transformed to a maximum accessible score (point) value. A peak frequency reflects the occurrence of that peak within spectra used to generate the MSPs. A score ≥ 10.0 was considered to reflect a high-confidence identification. Three replicates of each specimen were assayed, and would be identified if two tests yielded scores ≥ 10.

In the second experiment, we tried to simplify the method and shorten the test time. Clinical specimens were cultured in yeast extract peptone dextrose medium (YPD), yeast malt medium (YM), Sabouraud's medium (SAB), and brain heart infusion (BHI) broth for 6, 12, 24, and 48 h at 37°C **(Figure 1)**. Suspensions (1 ml) were added to 1 mL PBS, vortexed, and centrifuged at 3,000 rpm for 5 min; then the supernatants were removed. This procedure was repeated three times and the samples were tested via MALDI-TOF MS as described above. All isolates and specimens were also evaluated by growth on CHROM agar. The PCR sequence data served as the gold standard for identification.

Results

Identification of non-stimulated whole saliva clinical isolates cultured on sabouraud's agar

Totally, 175 clinical *Candida* isolates were isolated from 170 saliva samples: 120 *C. albicans*, 16 *C. glabrata*, 14 *C. tropicalis*, 11 *C. krusei*, 11 *C. parapsilosis*, 1 *C. dubliniensis*, 1 *C. rugosa*, and 1 *C. lusitaniae* **(Table 1a).** Statistical analysis showed that the sensitivity and specificity of the MALDI-TOF MS system were 93.3% and 96.4%, respectively, for identifying *C. albicans*, and 78.2% and 86.7%, respectively, for identifying non-*C. albicans* species **(Table 1b).** The MALDI-TOF MS system correctly identified 89.6% of all *Candida* strains.

Direct identification of *Candida* from liquid medium

The pilot experiment showed that the positive identification rates were higher from gargle and cotton swab samples than from saliva samples **(Table 2)**, possibly attributable to effects of salivary proteins. In addition, gargle samples were more convenient to collect than cotton swab samples. Thus we used gargle samples in subsequent analyses.

Sequencing data revealed that 55 clinical *Candida* isolates were isolated from 42 mouthwash samples: 31 *C. albicans*, 11 *C. glabrata*, 7 *C. tropicalis*, 1 *C. krusei*, 1 *C. parapsilosis*, 1 *C. dubliniensis*, and 1 *C. rugosa*. Samples from nine patients yielded two *Candida* spp., and samples from two patients contained three *Candida* spp. Finally, no *Candida* spp. was found in any of the 15 healthy controls.

Upon MALDI-TOF MS analysis, the results from all four liquid media were negative after 6 h; however, after culture for 12 h, the detection rates from YPD, YM, SAB, and BHI media were 52.2%, 39.1%, 47.8%, and 13%, respectively. After culture for 24 h, the rates of detection were 58.1%, 64.5%, 71%, and 25.8%, respectively. After culture for 48 h, the rates of detection were 56.3%, 68.8%, 75%, and 18.8%, respectively **(Figure 2).** In contrast, all 15 healthy control samples remained negative after 48 h.

With the exception of 11 samples from individuals infected with multiple *Candida* spp., gargle samples yielded the best results after culture in SAB liquid medium for 24 h. Under these conditions, the sensitivity, specificity, and coincidence rates for *C. albicans* were 66.7%, 90%, and 74.2%, and for non-*C. albicans* species were 65.2%, 83.9%, and 60%, respectively **(Figure 3a and 3b).** In contrast, the 11 cases of infection with multiple *Candida* spp. yielded optimal coincidence rates of only 29.8% via MALDI-TOF MS.

With the exception of samples from the 11 individuals infected with multiple *Candida* spp., MALDI-TOF MS correctly identified 76.1% of all *Candida* strains. In contrast, the identification rate using CHROM agar culture was 96.3%; this difference was significant ($P<0.05$).

Finally, we performed a cost/benefit analysis to determine whether the MALDI-TOF MS system was economically feasible for routine use. MALDI-TOF MS yielded the best results in terms of total turnaround time and reagent cost; these were 12.7–24.7 h and 3.5 CNY, respectively.

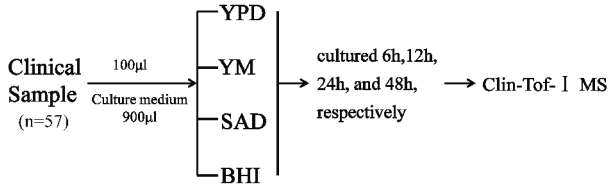

Figure 1 Flow diagram for direct MALDI-TOF analysis of liquid cultures to identify *Candida* species.

Table 1a Performance of the MALDI-TOF MS system for identifying *Candida* spp. from Sabouraud's agar cultures.

Candida species	No. of samples tested	No. of isolates identified using MALDI-TOF MS	No. of correct identifications via MALDI-TOF MS
C. albicans	120	114	112
C. glabrata	16	19	13
C. tropicalis	14	16	12
C. krusei	11	11	9
C. parapsilosis	11	10	8
C. dubliniensis	1	3	1
C. rugosa	1	0	0
C. lusitaniae	1	0	0
Total	175	173	155

Table 1b Performance of the MALDI-TOF MS system for identifying *Candida* spp. from Sabouraud's agar cultures.

Candida species	Sen% (95% CI)	Spe% (95% CI)	PPV% (95% CI)	NPV% (95% CI)	+LR	−LR
C. albicans	93.3 (± 4.5)	96.4 (± 4.9)	98.2 (± 2.4)	86.9 (± 8.5)	25.7	0.07
C. glabrata	81.3 (± 19.1)	96.2 (± 3.0)	68.4 (± 20.9)	98.1 (± 2.2)	21.5	0.19
C. tropicalis	85.7 (± 18.3)	97.5 (± 2.4)	75.0 (± 21.2)	98.7 (± 1.7)	34.5	0.15
C. krusei	81.8 (± 22.8)	98.8 (± 1.7)	81.8 (± 22.8)	98.8 (± 1.7)	67.1	0.18
C. parapsilosis	72.7 (± 26.3)	98.8 (± 1.7)	80.0 (± 24.8)	98.2 (± 2.0)	59.6	0.28
C. dubliniensi[s] a	–	–	–	–	–	–
C. rugosa [a]	–	–	–	–	–	–
C. lusitaniae [a]						

[a]Not tested. Statistical analysis was not performed when fewer than five isolates were identified. Notes: Species include *Candida albicans* (*C. albicans*), *Candida glabrata* (*C. glabrata*), *Candida tropicalis* (*C. tropicalis*), *Candida krusei* (*C. krusei*), *Candida parapsilosis* (*C. parapsilosis*), *Candida dubliniensis* (*C. dubliniensis*), *Candida rugosa* (*C. rugosa*), *Candida lusitaniae* (*C. lusitaniae*);
Sen: Sensitivity; Spe: Specificity; PPV: Positive Predictive Value; NPV: Negative Predictive Value; ± LR, positive/negative likelihood ratio; CR, concordance rate; 95% CI: 95% confidence interval

Table 2 The highest positive detection rates (% values) of the MALDI-TOF MS system used for direct identification of *Candida* spp. from three types of clinical samples (*n*=5).

Sample	Positive detection rate (%)			
	YPD	YM	SAD	BHI
Saliva	0	0	20	0
Mouthwash	20	20	100	40
Cotton swab	20	20	100	40

Notes: YPD: Yeast extract Peptone Dextrose medium; YM: Yeast Malt Medium; SAB, Sabouraud's medium; BHI: Brain Heart Infusion Broth.

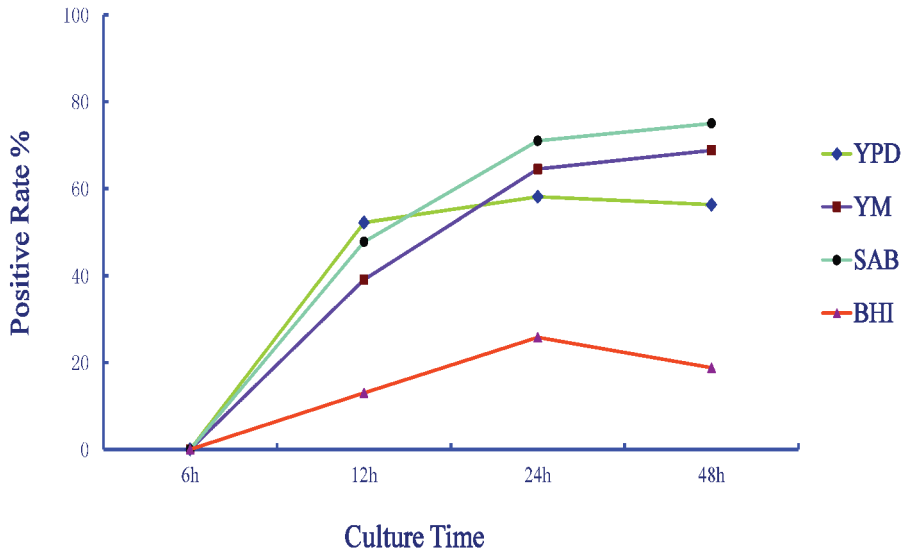

Figure 2 Positive detection rates (% values) using the MALDI-TOF MS system for direct identification of *Candida* spp. from four different liquid media.

Discussion

We evaluated the utility of MALDI-TOF MS for identifying *Candida* species, and attempted to reduce the turnaround time using liquid culture samples. With a mean hands-on time of 5.5 min, the accuracy of identification of samples containing a single strain was 76.1% after 24 h of culture in liquid medium and 89.6% after 48 h culture on Sabouraud's agar. Thus, the method is an optional tool for rapid, direct identification of yeasts from liquid culture media.

More than 150 *Candida* species are known, each of which exhibits a unique antifungal susceptibility profile [4]. Therefore, rapid and accurate identification of species is important for effective treatment of oral candidiasis. The current methods of identification used in clinical laboratories, such as culture on chromogenic media and rapid commercial systems, require 2-5 days of culture after collection of saliva or gargle specimens. In addition, the results are not very reliable. Although DNA sequencing is highly accurate, use of this method also requires

Fig 3a

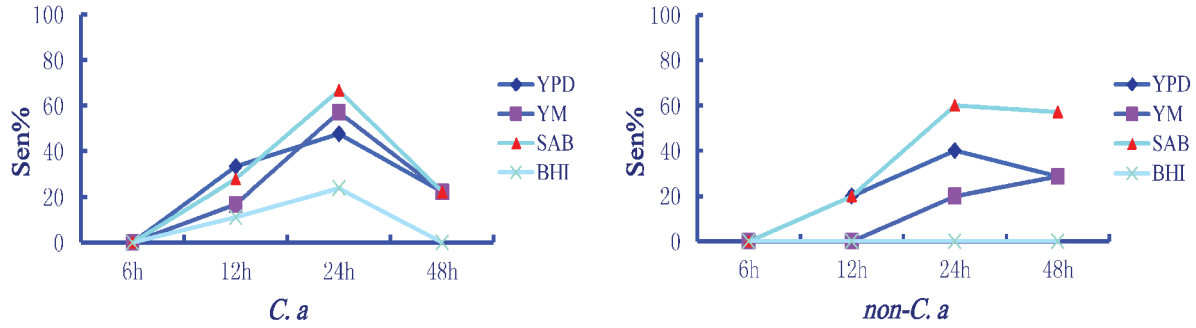

Fig 3b

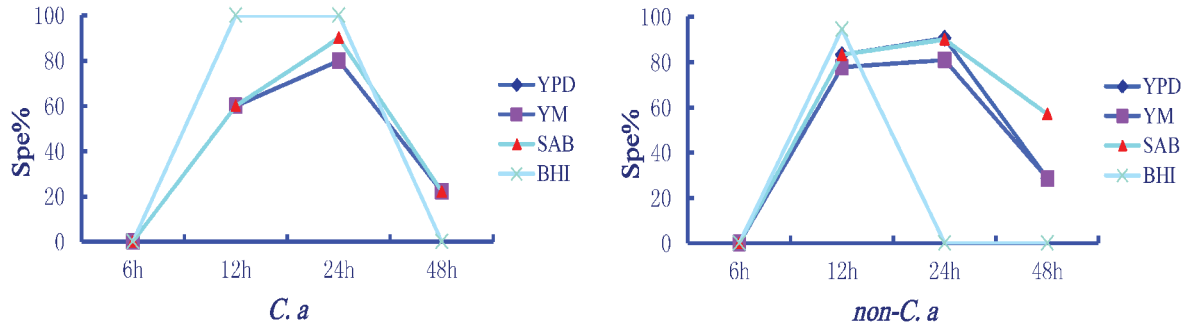

Figure 3 **a:** Sensitivity of the MALDI-TOF MS system for identifying *Candida* spp. from four different liquid media at culture durations of (a) 12 h, (b) 24 h, and (c) 48 h; * = 0. **3b:** The specificity of the MALDI-TOF MS system for identifying *Candida* spp. from four different liquid media at culture durations of (a) 12 h, (b) 24 h, and (c) 48 h.

2-4 days after sample collection because of the need to isolate and purify fungal colonies. In addition, the high cost of the method and the requirement for specific laboratory equipment limit its application in routine clinical testing. MALDI-TOF MS-based identification has the potential to reduce test times, thus substantially reducing delays in initiation or adjustment of treatment, minimizing adverse effects and reducing the emergence of resistant strains [14].

In the first phase of the present study, 94.3% of *C. albicans* and 84.0% of non-*C. albicans* isolates were correctly identified respectively, which is consistent with previous studies [7,15,16]. Earlier works that identified yeasts directly from blood culture specimens via MALDI-TOF MS-based techniques reported promising findings [13,17,18]. These studies involved culture of fungi in complex liquid media that included proteins and blood cells. Proteins and peptides derived from blood cells and serum can yield strong signals upon MS, hampering interpretations of specific peaks [18]. To prevent interference by such materials and to facilitate detection of microbial proteins, additional reagents such as Tween 80 [13], a MALDI sepsityper lysis solution [18], saponin [19], or sodium dodecyl sulfate [20] can be added to samples, increasing sensitivities to 87.7-97.3%.

In the second phase of our present study, gargle samples eluted three times with PBS were used to reduce the effects of salivary proteins and culture media. The pretreatment increased the sensitivity to 66.7%, thus 20% higher than that of saliva samples alone.

The sensitivity of MALDI-TOF MS used to identify organisms in blood depends on the size of the inoculum. In a previous study [21], spiking with *Staphylococcus aureus* and *Escherichia coli* suggested that bacteria at concentrations of 10^7 to 10^8 CFU/mL were identified correctly, whereas levels of 10^6 CFU/mL yielded signals indistinguishable from those of negative controls; this suggests that at least 10^6 CFU must be deposited on an MALDI target plate to obtain an identification spectrum. Each inoculum must also contain sufficient cells to overcome background peaks derived from the medium and other microorganisms present, including *Streptococcus mutans* and *Lactobacillus* spp. As reported previously, use of different culture media, and varying culture duration, may also affect spectral quality [22,23]. In the present study, culture in SAB medium for 24 h yielded the best spectra. Under these conditions, the sensitivity and specificity for *C. albicans* and non-*C. albicans* were 66.7% and 90.0%, and 60.0 and 95.2%, respectively. These differences might be associated with the presence of chloramphenicol in SAB; this inhibits bacterial growth. Also, the number of *Candida* cells may be inadequate for identification after only 6 h of culture. In addition, fungal cultures enter the stationary phase and decline in cell number after 48 h.

Finally, we compared the cost/benefit of MALDI-TOF to those of DNA sequencing and CHROM agar culture. MALDI-TOF was associated with a reagent cost of 3.5 RMB, a mean hands-on time of 5.5 min, and a total turnaround time of 12.7-24.7 h per sample **(Table 3)**. These values are lower than those of conventional molecular testing, and are consistent with previous reports [14,24]. The cost of the instrument and software is comparable to that of a gene sequencing platform [7]. The MALDI-TOF MS system can also be used to identify other microorganisms, polypeptides, proteins, and saccharides; therefore, such a system may be used as an optional methods for analysing unusual isolates, yielding more cost-effective and streamlined workflows.

In conclusion, we evaluated and confirmed that the MALDI-TOF system is a promising method that may used as an optional method for identificating *Candida* spp. Furthermore, identification results are available within 24 h of sample collection in most cases, when direct assays of liquid cultures are used. Such rapid data acquisition is currently impossible when conventional phenotypic testing or chromogenic media-based culture is used. Nevertheless, the method has several disadvantages, including a relatively sparse database and no current consensus on how to optimize the culture conditions. More studies with larger sample sizes by using MALDI-TOF system for the identification of *Candida* spp in saliva are needed in future to prove the above results.

Table 3 Time and cost estimates per isolate for routine identification methods vs. MALDI-TOF MS when liquid culture samples were used.

Method	Hands-on time per specimen (min)	Total turnaround time per specimen (h)	Cost of materials per specimen (RMB)
PCR	14.5	26–51.5	125–130
CHROMagar	3	48–96	9.5–19
MALDI-TOF MS	5.5	12.7–24.7	3.5

PCR: Polymerase Chain Reaction; MALDI-TOF MS: Matrix-Assisted Laser Desorption /Ionization Time-of-Flight Mass Spectrometry.

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