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A rapid two-step algorithm detects and identifies clinical macrolide and beta-lactam antibiotic resistance in clinical bacterial isolates

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ABSTRACT

Purpose: Aiming to identify macrolide and beta-lactam resistance in clinical bacterial isolates rapidly and accurately, a two-step algorithm was developed based on detection of eight antibiotic resistance genes.
Methods: Targeting at genes linked to bacterial macrolide (*msrA*, *ermA*, *ermB*, and *ermC*) and beta-lactam (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$) antibiotic resistances, this method includes a multiplex real-time PCR, a melting temperature profile analysis as well as a liquid bead microarray assay. Liquid bead microarray assay is applied only when indistinguishable T_m profile is observed.
Results: The clinical validity of this method was assessed on clinical bacterial isolates. Among the total 580 isolates that were determined by our diagnostic method, 75% of them were identified by the multiplex real-time PCR with melting temperature analysis alone, while the remaining 25% required both multiplex real-time PCR with melting temperature analysis and liquid bead microarray assay for identification. Compared with the traditional phenotypic antibiotic susceptibility test, an overall agreement of 81.2% (kappa = 0.614, 95% CI = 0.550–0.679) was observed, with a sensitivity and specificity of 87.7% and 73% respectively. Besides, the average test turn-around time is 3.9 h, which is much shorter in comparison with more than 24 h for the traditional phenotypic tests.
Conclusions: Having the advantages of the shorter operating time and comparable high sensitivity and specificity with the traditional phenotypic test, our two-step algorithm provides an efficient tool for rapid determination of macrolide and beta-lactam antibiotic resistances in clinical bacterial isolates.

1. Introduction

Antibiotic resistance has rapidly grown into a worldwide problem that threatens the effective treatment of a plethora of diseases. However, the rise of antibiotic resistant bacteria is not unexpected because organisms genetically adapt to selective pressures through evolution. The antibiotic misuse in clinical treatments during the past several decades has accelerated the development of antibiotic resistance in pathogenic bacteria (Buckwold and Ronald, 1979; Ringertz et al., 1990).

Traditional laboratory methods for detection and identification of antibiotic susceptibility of clinical bacterial isolates are time consuming. Starting with a pure culture, the turnaround time can be one to two days and sometimes weeks. Consequently, in the treatment of infections caused by bacterial pathogens, broad-spectrum empiric antibiotic therapy is often given to patients before the laboratory reporting. However, a previous study (Munson et al., 2003) has shown that the effect of notifications on antibiotic usage within 24 h is statistically much greater than when antibiotic susceptibility testing (AST) results are released days later. The longer it takes, the less important roles AST appears to play in the fine tuning of the antibiotic therapy. To reduce the increase in patient morbidity and mortality, as well as the transmission of drug resistant strains that are driven by inappropriate antibiotic regimens, the development of molecular diagnosis techniques for fast and accurate bacterial susceptibility testing is therefore urgently needed.

The mechanisms of antibiotic resistance are multifarious: bacteria may acquire resistance by mutations or by accepting antibiotic resistance genes from other organisms (Tenover, 2006). To date, hundreds of resistance genes have been reported to link with antibiotic resistance in both Gram-positive and Gram-negative bacteria (Liu and Pop, 2009). Among the Gram-negative bacteria, the production of extended-spectrum-beta-lactamase (ESBL) has emerged as an important mechanism of resistance to beta-lactam drugs. ESBLs are the most deleterious β-lactamases that can hydrolyze the commonly used beta-lactam antibiotics, such as penicillins and cephalosporins, and render them ineffective for treatment (Bush, 2013). The vast majority of ESBLs have been reported belonging to the TEM, SHV and CTX-M types, which are encoded by plasmid-located genes (Eftekhar et al., 2012; Labia et al., 1988). *erm* genes and *msr*(*A*) genes, which encode

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ribosomal methylases and drug efflux transporters respectively (Eady et al., 1993; Reynolds et al., 2003; Ross et al., 1996), are found to play important roles in most cases of macrolide-resistant Gram-positive bacteria, such as *Staphylococci*. Therefore, these antibiotic resistance genes can be used as targets for detecting antibiotic susceptibility of bacterial pathogens in clinical diagnostic laboratories.

In this study, we developed a two-step algorithm for identification of bacterial phenotypes by detecting eight resistance genes that are prevalent in macrolide and beta-lactam resistance in bacteria. The resistance genes were tested firstly by multiplex real-time PCR (mRT-PCR) followed by PCR product melting temperature (T_m) analyses; liquid bead microarray assays (LBMAs) were applied at the end only when indistinguishable melting temperature profiles were observed. After a pilot study in 17 antibiotic-resistant genotypes from bacteria with known species identities, the mRT-PCR–LBMA method was applied to 580 phenotypically characterized clinical bacterial isolates.

2. Materials and methods

2.1. Bacterial strains and drug susceptibility testing

17 bacterial isolates were selected for the pilot study. They are *Staphylococcus aureus* and *Staphylococcus haemolyticus* that carry macrolides resistance genes *msrA*, *ermA*, *ermB*, and/or *ermC*; *Escherichia coli* and *Klebsiella pneumoniae* that carry resistance genes bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$, and/or $bla_{CTX\text{-}M\text{-}9}$. Their antibiotic-resistant genotypes were confirmed by PCR amplification and DNA sequencing. Seven macrolide-resistant *S. aureus* and *S. haemolyticus* isolates were tested for susceptibility to oxacillin, gentamicin, erythromycin, lincomycin, tetracycline, vancomycin, clindamycin, rifampicin, norfloxacin and furantoin. Ten beta-lactam- resistant *E. coli* and *K. pneumoniae* isolates were tested for susceptibility to penicillin, ampicillin, ampicillin + clavulanate, tazocin, ticarcillin + clavulanate, cefalotin, cefotaxime, ceftazidime, imipenem and ciprofloxacin. A total of 580 clinical isolates, which includes 175 *Staphylococci*, 157 *E. coli*, 129 *K. pneumoniae* and 119 *P. aeruginosa*, were recovered in Shenzhen Futian Hospital between 2004 and 2012. Sources of these isolates included urine, blood, wounds, abscesses, sputum and feces. Antibiotic susceptibility tests were performed using the standard MIC methods as the instruction of the manufacturer (VITEK® 2 Compact, bioMérieux). All the tests were conducted following the guideline of the Clinical and Laboratory Standards Institute (Institute, 2009).

2.2. DNA preparation and multiplex real-time PCR

DNA was extracted from each isolate by using QIAamp DNA Mini Kit (QIAGEN Gmbh) following the instruction of the manufacturer. The forward and reverse primers designed in this study were listed in Table 1. The reverse primers were labeled at the 5′ terminus with biotin serving as a reporter in hybridization. The mRT-PCR was performed as described in previous studies with modifications (Ross et al., 1990). 25 μl real-time PCR mixtures contained 12.5 μl 2× Multiplex PCR Master Mix (Qiagen), 1 μl 20× SYBR Green I (Invitrogen), 2.5 μl Primer mix (2 μM each primer), 1 μl templates and 8 μl RNase-free water. mRT-PCR was performed using a Roche Lightcycler 3.0 real-time system (Roche Diagnostics, Mannheim, Germany) under the following conditions: 1 cycle of 95 °C for 15 min, 30 cycles of 30 s at 94 °C, 1 min 30 s at 62 °C and 1 min 30 s at 72 °C, followed by a final extension step at 72 °C for 10 min. DNA from susceptible *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 4352, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as negative controls for antibiotic resistance genes, RNase-free water was added as PCR system negative control.

2.3. T_m curve analysis

Melting curve analysis was done after the last cycle of the amplification by heating mRT-PCR products to 95 °C for 15 s, cooling to 55 °C for 30 s, and heating to 98 °C at 0.2 °C per second with continuous fluorescence recording. Melting curves were then converted automatically into melting peaks by plotting the negative derivative of fluorescence versus temperature.

2.4. Liquid bead microarray assay

The sequences of the probes for detecting different antibiotic resistance genes are listed in Table 1. Probes were covalently coupled to Luminex bead sets (Luminex, Austin, TX) following the oligonucleotide coupling protocol (Eftekhar et al., 2012). Briefly, each set of beads and its amine-substituted probe were incubated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) at room temperature for 30 min, washed by 0.02% Tween-20 and then resuspended in Tris–EDTA buffer at 150 beads/μl. Hybridization reactions were performed in a solution of 1 × tetramethyl ammonium chloride by adding 3 μl biotin-labeled mRT-PCR products or negative control to 22 μl conjugated beads. The mixtures were denatured at 95 °C for

Table 1
the mRT-PCR primers and LBMA probes of macrolides and beta-lactam antibiotic resistance genes in this study.

	Names	PCR primers		LBMA probes	PCR product size and T_m (°C) [a]
Macrolide resistance genes	*ermA*	F	TACAGAGTCTACACTTGGCTTAGG	ATTAATGGTGGAGATGGATA	344 bp
		R	TAGAAATTGATGGAGGCTTATGT		79.98 ± 0.97
	ermB	F	TGGCTAAAATAAGTAAACAGGTAAC	TCTTGGTGAATTAAAGTGACA	235 bp
		R	AATCAGATAGATGTCAGACGC		78.37 ± 1.15
	ermC	F	GATAATATCTTTGAAATCGGCTC	ATGGCAGTTACGAAATTACA	289 bp
		R	CCACGATTAAATAAATCTCATCAG		79.49 ± 1.35
	msrA	F	ACAAGAAGATGGCACAATAAGAGT	GTTGTTGTTCTAACTGTTCTTG	421 bp
		R	TTTTTGACTTCCTTTAACCAATGT		82.59 ± 1.05
Beta-lactam resistance genes	bla_{TEM}	F	AGTGGGTTACATCGAR[b]CTGGAT	CGCTTGAGCAAATTAAACA	485 bp
		R	GCCGGGAAGCTAGAGTAAGTAG		86.62 ± 0.59
	bla_{SHV}	F	CCTGTGTATTATCTCCCTGTTAGCC	CAAGGATCTTACCGCTGTT	287 bp
		R	CGAGTAGTCCACCAGATCCTGC		90.29 ± 0.89
	$bla_{CTX\text{-}M\text{-}1}$	F	GTCACGCTGTTGTTAGGAAGT	GCTCTAATTCGGCAAGTT	371 bp
		R	TTATTCATCGCCACGTTATC		87.72 ± 0.71
	$bla_{CTX\text{-}M\text{-}9}$	F	AGAGTGCAACGGATGATGTT	CGCTTGAGCAAATTAAACA	631 bp
		R	TGAGCCACGTCACCAACTG		92.1 ± 0.89
Bacterial negative controls [b]	*16S r*RNA	F	TGGAGCATGTGGTTTAATTCGA		158 bp
		R	TGCGGGACTTAACCCAACA		85.02 ± 0.89

[a] Represents the mean ± standard deviation for twenty determinations.
[b] *Staphylococcus aureus* ATCC25923 and *E. coli* ATCC25922.

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