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Fluorescent PCR detection of mecA in drug resistant MRSA: a methodological study

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Methicillin-resistant staphylococcus aureus (MRSA) has become one of the important pathogens of hospital infection [1–3]. Chromosome-mediated production of penicillin binding protein (PBP2a) is the main cause of MRSA resistance. The *mecA* (methicillin determinant A) is the gene encoding PBP2a on the chromosome [4,5]. Since *mecA* is strongly linked with MRSA, detection of *mecA* is considered to be the 'gold' standard for MRSA identification. At present, the main methods for MRSA detection include disk diffusion method (K–B method), broth microdilution method, *E* test and agar dilution method [6–8]. Fluorescent PCR method is rapid, specific and highly sensitive [6,9–11]. A recent study has compared the value of three different commercial real-time PCR assays in the detection of total *Staphylococcus aureus* [11]. This study showed that the real-time PCR detection of MRSA has a more than 90% sensitivity and specificity [11]. However, the number of MRSA specimens seemed too small to draw a conclusion.

The present study was designed to evaluate the reliability of fluorescent PCR detection of the *mecA* of MRSA specimens. Periodic reviews of the specimens were performed to evaluate the concordance between the fluorescent PCR and phenotypic assays. Our purpose was to provide reference data for the rapid detection of specimens for clinical testing departments, particularly the clinical laboratories in hospitals.

This study was approved by the institutional review board of Liaocheng People's Hospital and written consent was obtained from all participants. This study was conducted according to the principles expressed in the Declaration of Helsinki. A total of 70 clinical strains were collected, including 22 MRSA strains, 48 non-MRSA strains (other strains of Staphylococcus, *Streptococcus*, *Enterococcus* and *Gram-negative bacilli*). Two hundred and sixteen specimens of various types, including 67 sputum samples, 66 blood samples, 49 urine samples and 34 sterile body fluid samples, were collected. The sputum specimen was collected in the morning. Blood samples were collected with EDTA anticoagulant tube. Five to ten millilitres of mid-stream first morning urine were collected into a sterile tube. Pleural effusion, ascites, cerebrospinal fluid and other sterile body fluid specimens were extracted by puncture or collected in surgery following aseptic operating procedures. The volume was about 5 ml for each sample.

The bacterial identification was performed using the Vitek compact automated system. Cefoxitin disk diffusion method was used for the detection of MRSA strains [12]. All the tests and identification procedures were performed according to the 2014 edition of CLSI document [13]. The *S. aureus* suspension of about 0.5 McFarland turbidity units was prepared using sterile saline. The suspension was coated with sterile cotton swab evenly on the surface of MH agar, and then the discs containing 30 mg/disc Cefoxitin and 1 mg/disc Oxacillin were placed on the surface. The plates were incubated at 35 °C for 24 h, and then the diameter of the inhibition zone was measured. The cefoxitin inhibition zone with a diameter less than 21 mm and oxacillin inhibition zone with a diameter less than 10 mm were judged as resistance. The quality control strain was *S. aureus* ATCC 25923.

Four times volume of 4% NaOH solution was added to the sputum specimens and shaken well. The mixture was placed at room temperature for 30 min for liquefaction. Nearly 0.5 ml of the specimen was loaded into a 1.5 ml microcentrifuge tube, and 0.5 ml of 4% NaOH was added. The tube was left to stand at room temperature for 10 min before centrifuged at 4500 g for 5 min. Then, 1 ml of sterile saline solution was added to the precipitate and fully mixed. The mixture was centrifuged at 4500 g for 5 min, and then washed again. The supernatant was discarded, and the precipitate was fully mixed with 100 ml of nucleic acid extract and heated in boiling

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water bath for 10 min. The tube was removed and centrifuged at 4500 g for 5 min. Four mililitres of supernatant was used for PCR reaction.

Blood sample (2 ml) containing anticoagulant was left to stand to stratify. The upper layer, plasma layer and middle layer were removed and centrifuged at 4500 g for 2 min. Three millilitres of pleural effusion, ascites, cerebrospinal fluid or urine specimens were collected and centrifuged at 4500 g for 2 min. Colonies were collected by conventional methods, inoculated on appropriate medium, and incubated at 37 °C for 18–24 h. Colonies were picked into a 1.5 ml centrifuge tube containing 1 ml of normal saline. The specimens were diluted to 0.5 McFarland units and different concentrations according to the requirements. The next extraction procedure was performed same as sputum samples. Finally, 4 ml of supernatant was used as templates for further PCR reaction.

$N \times 36$ µl mixed solution and $N \times 0.4$ µl enzyme (Taq + UNG) were taken from the kit (*N* was the number of reaction tubes) and mixed to prepare the reaction solution. The reaction system was 40 µl. Four mililitres of supernatant which acted as template was added into the PCR (Lightcycle 480 II, Roche) reaction mixture containing 36 µl of mixture and 0.4 µl of enzyme (Taq + UNG). *Sau* gene (conserved sequence of *S. aureus*) and *mecA* gene of the bacterial suspension were detected. The reaction conditions were: 37 °C 2 min; 94 °C 2 min; 93 °C 15 S, 60 °C 60 S, 40 cycles, the fluorescence detection was performed at 60 °C. Positive quality control and negative quality control (included in the kit) were set up for both genes in all amplifications.

Identified MRSA clinical strains were inoculated and cultured by conventional methods. Colonies were picked to suspend in normal saline. The specimens were diluted to 0.5 McFarland units, and counted as 1.5×10^8 colony forming units (cfu/ml). The mixture was further diluted to concentrations of 5×10^6, 5×10^5, 5×10^4, and 5×10^3 cfu/ml, respectively. The samples in each concentration were detected for 5 times, respectively, and the positive rates of *Sau* gene and *mecA* gene were calculated. The minimum concentration with 100% positive detection rates for both genes was considered to be limit of detection.

Real-time PCR fluorescence detection was performed on 22 identified MRSA and 48 non-MRSA strains, and the results were statistically analysed to demonstrate sensitivity and specificity of the developed assay, respectively. PCR detection and phenotypic assay were compared to evaluate the concordance rates by analysing clinical specimens, including 67 sputum samples, 66 blood samples, 49 urine samples and 34 sterile body fluid samples.

The concentration with 100% positive detection rate of both *Sau* and *mecA* was the lower limit, which was 5×10^4 cfu/ml in this study. The intra-batch CV value of *Sau* and *mecA* in 5×10^6 cfu /ml sample was 0.82 and 0.71%, respectively, and the intra-batch CV value of Sau and mecA in 5×10^4 cfu/m was 0.85 and 0.44%, respectively. The inter-batch CV value of Sau and mecA of the samples at 5×10^6 cfu/ml was 1.51 and 1.65%, respectively; the inter-batch CV value of Sau and mecA of the samples at 5×10^4 cfu/ml was 1.99% and 1.72%, respectively. Fluorescence PCR detection of Sau and mecA in 22 identified MRSA strains of various specimens were all positive. Therefore, the sensitivity was 100%.

Forty-eight identified non-MRSA strains were screened by the real-time PCR assay, including other strains of *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Gram-negative bacilli*. Sau and mecA were negative in all the strains (Table 1). Therefore, the specificity of PCR detection of mecA was 100%. A total of 67 sputum samples were tested by both PCR detection and phenotypic assay, and the results of 66 clinical samples were consistent. The rate of concordance was 98% (Table 2). The results were inconsistent in one sample, which was identified as MRSA by phenotype assay, but negative by PCR detection. Subsequently, we confirmed that sample was negative by retesting.

Sixty-six blood samples were tested by both PCR detection and phenotypic assay. No sample showed positive results in both gene detections by PCR assay. There was also no MRSA strain identified by phenotypic assay. In addition, no sample showed positive results in both genes by PCR assay and no MRSA was identified by phenotypic assay in 49 urine samples and 34 sterile body fluid samples (Table 2). Hence, the concordance rate of the two methods in blood, urine and other body fluid samples was 100%.

The annual infection rate of methicillin-resistant Staphylococcus aureus (MRSA) is increasing. The resistance of MRSA continues to rise, and MRSA has become

Table 1. PCR detection of 48 non-MRSA clinical strains identified by phenotypic assay.

Clinical strains	Numbers of strains	*Sau*	*mecA*
MSSA	8	+	–
Coagulase-negative *Staphylococci*	1	–	+
Staphylococcus epidermis	13	–	–
Staphylococcus haemolyticus	2	–	+
Staphylococcus auricularis	1	–	+
Staphylococcus sciuri	2	–	–
Streptococcus	4	–	–
Enterococcus	6	–	–
Gram-negative *bacilli*	11	–	–

Table 2. PCR detection and phenotypic assay of strains in sputum samples.

		Sau and *mecA* both positive (+)	*Sau* and/or *mecA* negative (–)	Total (cases)
Results of phenotypic assay	MRSA (cases)	37	1	38
	Non-MRSA (cases)	0	29	29
Total cases		37	30	67

one of the bacteria of hospital infection which are hard to be controlled. Correct and rapid detection of MRSA is key to the control of hospital infection [14]. At present, the commonly used method of MRSA detection is phenotypic assay, but its long detection time (usually 2–3 days) has become the bottleneck restricting the rapid diagnosis and early intervention [6–8]. The *mecA* and PBP2a protein detection are still the gold standard for MRSA identification, but the *mecA* expression is affected by factors such as genetic background and inducer. The acquired drug resistance level of *mecA* ranges from phenotypic sensitivity to highly resistant, which also brings some difficulties to the phenotypic detection of MRSA [15]. The real-time PCR method directly detects the *mecA* on the bacterial chromosome to determine whether the bacteria are methicillin-resistant strains. This method is not affected by the conditions of drug sensitivity test, so the PCR detection is highly specific. Most importantly, the detection time is greatly shortened (3–4 h) making possibilities of the rapid diagnosis and treatment for the clinicians.

Our assay proved that the concordance rate of clinical detection of sputum samples was 98% with only one sample showing negative result. The sputum samples used in this experiment were only one of the dozens of the clinical specimens that could influence the sensitivity to some extent because of the small sample size. There might be some components that inhibited the PCR reaction, which also affected the sensitivity of detection, in the crude template directly extracted from sputum specimen. In addition, the sputum culture may have been affected by the composition of the medium, the bacterial inoculum size, incubation time, incubation temperature or inhibited by the antibiotics already used. The bacteria were not well distributed in the sputum, and only a very small part of the specimen was used in the culture. Therefore, to detect *mecA* in sputum and determine whether the sputum contains MRSA and the MRSA has reached a certain amount is more meaningful. This experiment took only 3–4 h from sampling to obtain the results, so the results could be reported to the clinicians in one day. For patients with high-risk factors, detection of *mecA* in their sputum could provide a quick guidance for therapeutic interventions, which is more helpful than the time-consuming sputum culture and drug susceptibility test.

Although two target genes were amplified in this experiment, and the amplification efficiencies were different, the low intra-batch CV and inter-batch CV values of *Sau* and *mecA* showed that the tests had good repeatability, and could meet the requirements of clinical testing. At the same time, the clinical MRSA strains and almost all common non-MRSA strains which might affect the identification were tested to determine the sensitivity and specificity. In the end, real-time PCR detection showed a 100% sensitivity and specificity in detecting mecA and Sau. These results were supported by previous studies where more than 92% sensitivity and specificity were reported [11].

In summary, *mecA* of MRSA can be detected with fluorescent PCR. This assay is rapid, sensitive and specific for detection of *mecA*. Direct and rapid detection with fluorescent PCR seems advantageous over blood culture or cerebrospinal fluid culture which usually takes several days. This work represents an advance in biomedical science because it shows that fluorescent PCR detection of MRSA is feasible and reliable, which may help timely detection of MRSA infection.

Disclosure statement

No potential conflict of interest was reported by the authors.

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