Original Article

Evaluation of Rapid Detection of Nasopharyngeal Colonization with MRSA by Real-Time PCR

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Objective To investigate the clinical application of Real-Time PCR for rapid detection of methicillin-resistant *Staphylococcus aureus* (MRSA) directly from nasopharyngeal swab specimens.

Methods We collected the nasal and throat swab specimens from patients or medical staffs in 3 intensive care units, blood laminar flow ward and respiratory ward in Beijing Hospital, Ministry of Health from December 2010 to April 2011. Each sample was tested by RT-PCR and conventional culture-based method for the presence of MRSA.

Results The total number of the specimens was 206. Compared with the conventional culture-based method, we demonstrated the diagnostic values for Real-Time PCR were 96.4% sensitivity, 96.6% specificity, 81.8% positive predictive rate, and 99.4% negative predictive rate. And the limit of detection was 10²CFU/ml.

Conclusions This Real-Time PCR is a simple, rapid, sensitive and specific method. With the high negative predictive value, it can be used for the exclusion of MRSA colonization or infection. However, the application of its low positive predictive value should be further evaluated.

Key words: Real-Time PCR; MRSA; Nasopharyngeal swab

As one of the most important medical pathogens, *Staphylococcus aureus* is associated with a wide spectrum of infections, such as skin and soft-tissue infection, pneumonia, arthritis,¹ osteomyelitis, endocarditis, bacteremia, and so on. Recently, with the overusage of antibiotics, methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as an critical role in hospital- and community-acquired infection. MRSA infection can result in significant morbidity, mortality, longer hospital stays, and more costs.¹⁻³ Yamamoto et al.⁴ has reported in 2008 that a 16-month-old, previously healthy Japanese boy who got pneumonia caused by community-acquired methicillin-resistant staphylococcus aureus (CA-MRSA) and finally died in spite of treatment on day 10 after admission. Published reports show that nasal colonization with MRSA serves as the most common reservoir for self-infection and nosocomial transmission.⁵⁻⁷ Hence, screening for MRSA carrier on admission is crucial for effective antimicrobial therapy and infection control. However, nowadays the most common method for MRSA detection is culture-based, which is relatively complicated and typically takes at least 48 to 72 hours.⁸ Furthermore, the sensitivity of the method is often affected by a variety of factors, including the process of sample collection, transportation, the quality of the agar and so on. The

delayed results can cause the wide spread of MRSA in hospital or community. It also has a negative effect on infection control programs. So it has great significance in the development of the method for rapid and accurate MRSA detection. We will describe a new Real-Time PCR, which detects MRSA directly from swab specimens, and assess its diagnostic values with comparison to the conventional culture-based method.

MATERIAL AND METHODS

Specimen Collection

Total of 103 patients and medical staffs with high risk of MRSA infection in Beijing Hospital, Ministry of Health during December 2010 to April 2011 were selected for this study. All the 206 nasal and throat swab specimens were collected with Copan swabs according to National Guide To Clinical Laboratory Procedures (Third Edition) and processed within 1 hour.

Reagents

MRSA Real-Time PCR Kit (Shanghai ZJ Bio-Tech Co., Ltd.), cefoxitin (Oxiod, England), Columbia sheep blood plate (bioMérieux, France), Coagulase (bioMérieux, France), Catalase (homemade), VITEK2 GP Test Kit (bioMérieux, France).

Equipments

Slan Real-Time PCR System (Shanghai Hongshi

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Medical Technology Co., Ltd), VITEK2 Compact System (bioMérieux, France), SHELLAB CO₂ Incubator (SHELLAB, America).

Sample processing

Every nasal or throat swab specimen was suspended in 1ml of sterile sodium chloride and vortexed absolutely. Then removed the swab away after 5 min and plugged with sterile stopper tightly.

Culture-based method

Identification for *Staphylococcus aureus*

The bacterial suspension was plated onto Columbia blood agar and incubated at 35°C for 24 to 48 hours. Then isolated the suspected *Staphylococcus aureus* (according to Gram stain, hemolysis, colony morphology) for further identification, including Gram stain, catalase test and coagulase test, and so on. If the result was not typical, the sample would be detected again with VITEK2 Compact System. The ATCC29213 and ATCC12228 strains were tested as positive and negative control samples, respectively.

Antibiotic sensitivity test

The *Staphylococcus aureus* was then tested by antibiotic sensitivity test (cefoxitin) according to CLSI Standards (2009). Criteria were as follows: if the diameter of inhibition zone is less than 21 mm, the result is reported as cefoxitin resistant, and if it is more than 22 mm, the result is reported as sensitive. If there is any small colony in inhibition zone, the result is reported as resistant as well. The ATCC43300 and ATCC25923 strains were tested as positive and negative control samples, respectively.

Real-Time PCR

DNA extraction Samples

Vortexed the bacterial suspension for 10 s and transferred 1ml into a sterile centrifugation tube. Following centrifugation at 13000 rpm for 2 min and removal of the supernatant, 100 µl of cell lysis was added into the tube with absolute vortex. Incubated the mixture in boiling water for 10 min and then centrifuged at 13000 rpm for 5 min. Total of 4 µl supernatant was taken as the template for PCR reaction. Controls: 100 µl of the positive and negative controls were transferred into a sterile centrifugation tube with 100 µl cell lysis. Vortexed absolutely and incubated the mixture in boiling water for 10 min. After centrifugation at 13000 rpm for 5 min, transferred 4 µl supernatant for PCR reaction.

Reagent preparation

Mixed 36 µl of nucleic acid fluorescent PCR mixture with 0.4 µl of enzyme (Taq + UNG), and vortexed for few minutes and stored at 4°C.

Real-Time PCR

The PCR system was composed of 4 µl DNA extraction and 36 µl prepared reagents. Each tube was then

inserted into Slan Real-Time PCR System for PCR. The parameters of the cycle were set as follows: 37°C × 2 min and 94°C × 2 min for 1 cycle; 93°C × 15 s and 60°C × 60 s for 40 cycles with fluorescence detection at 60 °C. The whole cycle process took 54 min. Criteria for PCR were as follows: if the Ct value is more than 40 or showed as undetected, then the result is reported as negative; if the Ct value is less than 35, the result is reported as positive; if the Ct value is between 35 and 40, the result is reported as positive while the curve is typically S-shaped with repeating test, otherwise the result is reported as negative. Only when *mecA* and *nuc* genes were both positive, it can be reported as MRSA positive.

The limit of detection

Prepared bacterial suspension at 0.5 Maxwell concentration with standard strain (ATCC43300), which was equal to 10⁸ CFU/ml, then made a serial dilution of 1:10 to get a gradient concentration from 10⁷ to 10 CFU/ml. The dilutions of 10⁴, 10³, 10², and 10 CFU/ml were detected with conventional culture method and Real-Time PCR at the same time. We plated 50 µl of each dilution on Columbia blood agar as described above and incubated for 24 hours for colony counting. Each dilution was tested for 3 times in parallel. Then the results were compared to that of Real-Time PCR. Colony forming units per milliliter (CFU/ml) = the average counts of the three agar × dilution factor × 20.

Statistical analysis

The sensitivity and specificity for Real-Time PCR were calculated by gold standard -conventional culture method. Differences in performance between the test results were assessed by the χ^2 statistic. Sensitivity and specificity were then calculated as follows: a true-positive (TP) was defined as a sample with confirmed MRSA isolated from culture and a positive RT-PCR result, a false-positive (FP) was defined as a sample with a negative culture result and a positive RT-PCR result, a true-negative (TN) was defined as a sample with a negative culture result and a negative RT-PCR result, a false-negative (FN) was defined as a sample with a positive culture result and a negative RT-PCR result. Sensitivity = TP/(TP + FN), Specificity = TN/(TN + FP)

RESULTS

Conventional culture method

Among all 103 persons tested, 19 were positive for *Staphylococcus aureus* by conventional culture, 16 of which were MRSA and 3 were MSSA. The results were shown in Table 1, from which we could see that ACU had the highest prevalence of MRSA in all departments and ICU was just a little lower than ACU. Of all kinds of the studied subjects, the patients got the

Table 1. Results of all 103 persons detected by conventional culture method

Department	Numbers of MRSA positive in different subjects			Total
	Patient	Doctor or nurse	Carer	
ICU	4/9	0/5	1/2	5/16
RCU	1/6	0/7	1/4	2/17
ACU	9/12	0/4	0/3	9/19
LAF	0/10	0/6	0/1	0/17
Respiratory Department	0/23	0/6	0/1	0/30
Microbiology Department	/	0/4	/	0/4
Total	14/60	0/32	2/11	16/103

highest prevalence of MRSA and the doctors or nurses had lower prevalence than carers. Of the 3 cases with MSSA, 2 were carers from RCU and 1 was a carer from Respiratory Department.

Real-Time PCR

Total of 206 swab specimens were tested by Real-Time PCR in accordance with the manufacturer's instructions strictly. Each test run external positive and negative control samples for quality control. A comparison of Real-Time PCR with culture method for the detection of MRSA was shown in Table 2. Among the 206 swab specimens, 27 were Real-Time PCR (+) and culture (+), 6 were Real-Time PCR(+) and culture (-), 1 was Real-Time PCR(-) and culture (+), and 172 were Real-Time PCR(-) and culture (-). So we can conclude that Real-Time PCR had a sensitivity of 96.4% and a specificity of 81.8%, with a positive predictive value of 81.8% and negative predictive value of 99.4%.

Table 2. Comparison of Real-Time PCR with culture method for MRSA

Real-Time PCR	Culture method		Total
	Positive	Negative	
Positive	27	6	33
Negative	1	172	173
Total	28	178	206

We calculated the colony of the four serial dilution according to the fomula described above, the results were in turn as follows: 1.56×10^8 CFU/ml, 2.18×10^8 CFU/ml, 6.56×10^8 CFU/ml, 3.60×10^8 CFU/ml, all of which were 10^8 CFU/ml. On the other hand, as shown in Figure 1, 10^4 CFU/ml dilution was negative with all three times of Real-Time PCR, while others were all positive. That is, the limit of detection of Real-Time PCR was 10^2 CFU/ml.

Figure 1. The results of Real-Time PCR for different dilutions.

Comparison of nasal with throat swabs

It is defined that if any swab specimen of the same person was MRSA positive according to the culture method, then the result is reported as MRSA positive. So 16 persons were MRSA positive, of which 13 nasal swab specimens and 15 throat swab specimens were positive, respectively. We compared the sensitivity of the two kinds of swab specimens by χ^2 statistic which showed $P > 0.05$ and meant there was no significant difference in sensitivity for MRSA screening between the two swab specimens.

DISCUSSION

Nowadays, MRSA has become a major nosocomial and community pathogen.⁹ Screening for MRSA of persons on admission is an important part of effective control measures.¹⁰⁻¹² Some studies have shown that rapid identification of MRSA colonization in person on admission can reduce the incidence of MRSA from 13.89/1000 to 4/1000 per day.¹³ With the strategy of “discover-eliminate”, including screening MRSA for persons exposed to MRSA, the prevalence of MRSA can reduce to 1% in 6 years.¹⁴ However, the keypoint of the strategy is how to detect all MRSA carriers or infection rapidly and accurately. Otherwise, there is a possibility for over-and under-detection of MRSA, both of which will be no good for infection control programs and effective treatment. Therefore, it's really so important to develop a reliable, rapid and efficient technique to identify MRSA.¹⁵ Currently, there are many different methods for detection of MRSA rapidly, including chromogenic media, latex agglutination test, PCR, and so on. However, all of these methods must be applied with the pure colony, which means at least waiting for 2 days. The Real-Time PCR described in our study can rapidly detect MRSA directly from clinical swab specimens.

The principle of Real-Time PCR is to detect *mecA* and *nuc* genes in the sample at the same time. *MecA* gene is the major reason for methicillin resistance in *Staphylococcus aureus*, which encodes penicillin-binding protein 2a (PBP2a). *Nuc* gene is *Staphylococcus aureus* specific and responsible for the thermonuclease. There have been some reports in our country about the Real-Time PCR for MRSA, but studies about detection of MRSA directly from clinical samples have not been seen yet. The whole time for PCR in our study is just 54 minutes. In our study, the

Real-Time PCR demonstrated a 96.4% sensitivity and 96.6% specificity for detection of MRSA compared with culture method. The assay can be performed by a single technologist with appropriate training, and allows the same-day report, because one batch just needs about 2 hours. The Slan Real-Time PCR System used in our study consisted of a single unit that can run 48 samples at one time, however, we can detect 96 samples simultaneously with SLAN-96P.

Previous Real-Time PCR assays have reported for rapid detection of MRSA from pure colony. Elsayed et al¹⁶ also reported a duplex Real-Time PCR for *mecA* and *nuc* genes which has 100% sensitivity and specificity for detecting and differentiating *Staphylococcus* spp. from pure strain isolates. We consider there must be some substances in the sample that can interfere the PCR.

As described above, there were 7 discrepant results in our study. We detected the remanent DNA extraction and the separated pure colony from the agar with Real-Time PCR again, and then compared the result with that of culture method. Here are the discussions about the possible reasons for the discrepancy:

One nasal swab from an ACU patient was Real-Time PCR negative and culture-positive, while the throat swab of the same person were both positive with two methods. After 48 hours incubation, we could only find 2 small colonies on the blood agar. We detected the pure colony with Real-Time PCR again and the result turned to be positive. So we may conclude that Real-Time PCR was false-negative because of low concentration of DNA or the presence of interference in the sample, other than the possibility of other mechanism of methicillin resistance.

Instead, there were 6 Real-Time PCR positive and culture-negative results in our study, and one of them turned to MSSA with repeating Real-Time PCR from the remanent DNA extraction. The reason may be due to the cross-contamination during the PCR. Two of the 6 samples were methicillin-resistant coagulase-negative staphylococci (MRCNS) with culture method, whose repeating Real-Time PCR were both *mecA*-positive and *nuc*-negative with the pure colony. So we considered that there may be only a little MSSA in the sample which could not grow on the agar with the interference of MRCNS or the concentration of MSSA in sample which was too low for the culture method to detect. Other 2 cases showed no staphylococci at all with the culture method and the Ct value of PCR were about 35, which equaled to 10 CFU/ml. This may be due to the limitation of the culture method for detecting low concentration of MRSA in swab specimen, which resulted in the false-negative result. Kipp et al¹⁷ ever reported that the generation time for small-colony variants (SCVs) of *Staphylococcus aureus* was six- to nine-fold longer than that for normal *S. aureus*, which resulted in tiny colonies and may not be visible

before 48 to 72 hours of incubation. The SCVs also had the reduced hemolytic activity and low coagulase activity, which resulted in the reduction of sensitivity of the culture method. And with the directed antibiotic therapy, especially those used directly in nose or throat, the growth of MRSA could also be inhibited. The last one of the 6 discrepancy was complicated. We detected the pure colony from the agar with both methods for 3 times, all of the results were MRSA with Real-Time PCR assay and MSSA with culture method. The most probable reason for this phenomenon was that *mecA* gene of MRSA was lost after effective treatment, so that it turned to MSSA but still retained the binding site for the specific probes. So the PCR for MRSA could be performed successfully. Of course, as the Real-Time PCR in our study was a double-locus assay, they may not be able to differentiate MRSA from a mixture of MSSA and MRCNS in the sample, which could result in the false-positive result of Real-Time PCR. This was also a major disadvantage of the method.

The negative predictive value in our study was 99.4%, which means if the Real-Time PCR showed negative, we could report it as negative without waiting for the result of the culture method. But on the other hand, our positive predictive value was not so high. This may be related to the low prevalence of MRSA in our area.¹⁸ Some studies showed that with combination of the history of MRSA infection and the culture method, the sensitivity of PCR may be improved while that of the culture method may be reduced.¹⁹ So if the 4 cases were false negative with culture method, it could affect the positive predictive value of Real-Time PCR to some extent. Then the positive predictive value would be modified as 93.9%. However, we didn't trace the patients and had no sufficient evidence to prove that the results of culture method were false-negative. So the positive predictive value need to be further assessed.

Every subject enrolled in our study had one nasal and throat swab specimen for MRSA screen, respectively. It showed no significant difference in the sensitivity of MRSA screening between the two swab specimens with χ^2 statistic. The conclusion was different from that of Denis et al.²⁰ who considered the sensitivity of the nasal swab (90.6%) was higher than that of the throat swab (76.5%). Maybe it was due to some directed local treatment in our enrollments. We also demonstrated the limit of the detection as 10^2 CFU/ml, which was enough for clinical application.

Patients in ACU, ICU and RCU of Beijing Hospital had higher prevalence than that in other departments. We found the patients seemed more serious and had longer hospital stays through investigation. So they had relatively lower self-resistance and were sensitive to MRSA infection. And the prevalence in doctors or nurses was lower than that in carers, which may be due to the less education for the carers. Therefore we need

to make stronger publicity to the non-professionals about the infections and controls to reduce the cross-infection and the spread of MRSA.

It should be pointed out that the epidemiology in the area had a relationship to the type of MRSA,²¹ which could affect the sensitivity and specificity of the method. So the conclusion of the Real-Time PCR in our study should only be applicable for this area.

In summary, the Real-Time PCR is a sensitive and specific test for the detection of MRSA colonization or infection directly from a swab specimen without a need for an initial culture. It can be performed by one technologist with no additional training and allows the same-day report. This promises to improve the efficiency and effectiveness of measures to control the spread of MRSA throughout health care facilities. In addition, the assay has a high negative predictive value that can be used for exclusion of MRSA, but its positive predictive value is low and needs to be further assessed.

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