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Application of *ica D, agr, mec A,* and *mre B* Gene Testing in Early Diagnosis of Periprosthetic Joint Infection

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Objective: This study intends to explore the diagnostic efficiency and value of polymerase chain reaction (PCR) in the early diagnosis of periprosthetic joint infection (PJI) based on the testing of the 4 specific genes of PJI pathogens: *ica D*, *agr*, *mec A*, and *mre B*.

Methods: Forty-one samples of ultrasonic cleavage RNA eluate were extracted from the human joint model of PJI caused by the 5 most common PJI pathogens to detect the PCR of *ica D*, *agr*, *mec A*, and *mre B* genes. Based on the detection results, the sensitivity, specificity, positive and negative predictive value, and accuracy of the 4 genes were analyzed and compared.

Results: The *mec A* test had a high sensitivity (55.56%). However, more false-positive results affected the test specificity (56.25%). The specificity of *mre B* test was higher (76.92%) but had lower sensitivity (26.67%); the sensitivities of the *ica D* and *agr* tests were 4.17% and 0%, respectively; the clinical value was limited.

Conclusion: The testing of *mec A* and *mre B* genes has high value in the early diagnosis of PJI. The testing of *ica D* and *agr* genes plays an important role in preliminary screening and reference for *Staphylococcus*-related PJI. Reasonable design and sequential application of *ica D*, *agr*, *mec A*, and *mre B* would give full play to the testing value and be more beneficial to the early diagnosis of PJI.

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Key words: Periprosthetic joint infection – Early diagnosis – Sequential testing of genes – Polymerase chain reaction

Periprosthetic infection (PJI) is one of the most serious and destructive complications of artificial joint replacement.[1] The occurrence of PJI usually means subsequent revision surgery and the subsequent heavy medical and economic burden, as well as a significant increase in the probability of surgical failure.[2,3] Early diagnosis and identification of pathogens can help doctors optimize the operation plan and choose the most favorable anti-infective treatment when PJI occurs, which is important for a good prognosis.[4,5]

The pathogenesis of PJI is closely related to the formation of biofilm. The rapid formation of biofilm, the use of routine antibiotics during perioperative period, the cleaning of human immune system, and the existence of many pathogenic bacteria that cannot be obtained by traditional culture methods make it difficult to culture PJI pathogens from clinical specimens in the early stage.[6,7] The inflammatory markers commonly used in clinic lack specificity; even if the detection index is abnormal, it cannot be confirmed that PJI occurred.[8,9] Molecular biology techniques, including polymerase chain reaction (PCR) for detection of pathogenic bacteria DNA in recent years, have become a feasible method in clinical practice. Compared with the traditional PJI pathogenic bacteria culture methods, the molecular biological techniques, such as PCR, for the detection of pathogenic bacteria DNA not only have many advantages, such as high sensitivity, low cost, reliable results, ease of use, ease of popularizing, and so on, they also cause less trauma to patients in the sampling process, resulting in a lower risk of secondary infection. It is even possible to obtain real-time test results from on-the-spot sampling during the revision operation to determine whether the patient has PJI or aseptic loosening, and feedback from the test results guide doctors in choosing the most favorable and appropriate operation plan.

The study of PJI pathogens shows that the epidemiology of PJI pathogens in different parts has its own characteristics. However, *Staphylococcus aureus, Staphylococcus epidermidis*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Escherichia coli* are the most common pathogens of PJI.[10,11] Single or multiple infections of these pathogens lead to the vast majority of clinical PJI cases. This study intends to achieve the diagnosis of these kinds of pathogens, which can achieve the purpose of early diagnosis of most clinical PJI cases. *ica D* and *agr* genes are specific genes related to the formation of staphylococcal biofilm, which is the most common and main pathogen of PJI. *mec A* is the major resistance gene of MRSA, and *mre B* is a specific gene of non-cocci including *E coli*. Through PCR analysis of the above four specific genes, we explore the clinical value of detecting early PJI and lay a foundation for further exploration.

Materials and Methods

Reagents and equipment

The reagents and equipment included Centrifuge sigma3-16k (American Sigma, St Louis, Missouri), enzyme labeling instrument uQuant (American Biotek, Shoreline, Washington), water purifier MAXI-MA ultrapure (British Elga, Buckinghamshire, UK), cryogenic refrigerator U57085 (British New Brunswick Scientific, St Albans, UK), Merieux turbidimeter Densicheck PLUS (American bioMérieux, Inc, Durham, North Carolina), constant temperature shaker Forma Orbital Shaker (American Thermo Electron Corp, West Palm Beach, Florida), electrophoresis instrument Bio-RAD pac3000 (American Bio-RAD, Hercules, California), super clean worktable OPTIGEL-12 (ads luminaire, Aulnay Sous Bois, France), electronic balance HM-202 (Shanghai Fangrui Instrument Co., Shanghai, China), vacuum cryogenic freeze dryer Christ101042 (Bioblock Scientific, Illkirch, France), ultrasonic crusher U200S-control (IKA LabortechnikStaufen, Staufen Im Breisgau, Germany), ultrasonic cleaning machine KS-120EI (Kesheng,Ningbo, Zhejiang, China), pancreatic soy peptone medium TSB (UK Oxoid, Hampshire, UK), and tryptone plate TSA (UK Oxoid).

Acquisition, grouping, and culture of experimental strains

The strains used in the experiment, *S aureus* (ATCC25923), MRSA (ATCC43300), *E coli* (ATCC8739), *E coli* (ATCC25922), and *S epidermidis* (SE243), were examined and approved by the Ethics Committee of Shanghai Ninth People's Hospital affiliated to Shanghai Jiaotong University. Moreover, 8 patients with infection after artificial knee arthroplasty were isolated from Shanghai Ninth People's

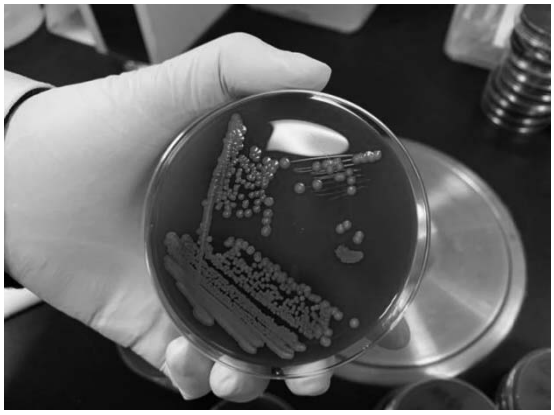

Fig. 1 The strain was inoculated on the blood plate, 37°C. Static culture 24 hours.

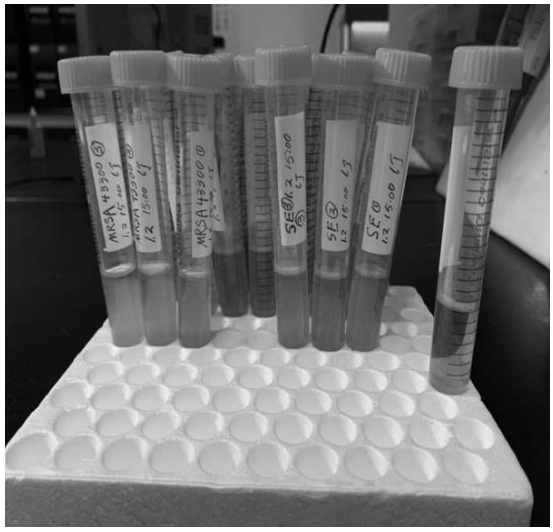

Fig. 2 Culture of monoclonal strain in sterile glass tube containg TSB.

Hospital affiliated to Shanghai Jiaotong University, including 5 men and 3 women, 48 to 62 years of age, with an average of 55.5 years of age. The patients were treated with antibiotics in time after the operation and achieved good clinical prognosis.

Experimental strain grouping, biofilm culture of 3 of the 5 most common PJI pathogens [*S aureus* (ATCC25923), MRSA (ATCC43300), and *E coli* (ATCC25922)], was divided into 3 groups, with 3 cover slides repeated in each group. In addition, the biofilm cultures of 2 kinds of *E coli* (ATCC8739) and *S epidermidis* (SE243) were divided into 2 groups with 3 cover slides in each group and 2 blank control samples. A total of 41 samples were collected, including 9 strains of *S aureus* (ATCC25923), 9 strains of MRSA (ATCC43300), 9 strains of *E coli* (ATCC25922), 6 strains of *E coli* (ATCC8739), 6 strains of *S epidermidis* (SE243), and 2 blank control samples.

Experimental strain culture

The monoclonal amplification was selected and frozen in tryptic soy broth (TSB) containing 20% glycerol at –80°C after the strain was revived. The cryopreserved strains were inoculated on the blood plate and cultured at 37°C for 24 hours (Fig. 1). The clones were inoculated into 10-mL aseptic glass tube containing 3 mL TSB (Fig. 2) and cultured in a shaker at 130 rpm, 37°C, and aerobic conditions for 48 hours (Figs. 3 and 4). The bacteria were dynamically cultured in fresh TSB medium 2 consecutive times. The bacterial suspension was put into a centrifuge tube and centrifuged at 8000*g* for 10 minutes. The 5 kinds of bacteria were diluted to 1.0×10^6 colony-forming units (CFUs)/mL with fresh TSB medium by McDonnell's turbidimetric method.

Identification of biofilm production ability of experimental strains

The standard flat-bottomed 96-well culture plate was selected, and the preceding samples containing 5 kinds of PJI pathogenic bacteria were diluted to 1.0×10^6 CFU/mL bacterial suspension with fresh TSB. The final volume of the solution in the hole was 200 μL and was statically cultured at 37°C for 24 hours. The culture medium was carefully poured out, the 0.01 mol/L PBS buffer (pH 7.4) was rinsed gently 3 times to eliminate free planktonic bacteria, which was dried in an oven at 60°C for 1 hour, and a 200-μL 0. 1% crystal violet solution was added and dyed for 5 minutes at room temperature. The dye solution was lightly washed with double distilled water 3 times to remove excess stains and dried at 37°C for 2 hours. Then, 200 μL 30% acetic acid solution was added to fully dissolve for 10 minutes and put into the enzyme meter to measure the absorbance value of the solution at a wavelength of 492 nm; the result is expressed as the OD492 value.

Preparation of ultrasonic concussion bacteria solution and real-time fluorescence quantitative PCR testing

The standard flat-bottomed cell culture bottle was taken, and the bacterial suspension containing the samples mentioned previously was added. In each cell culture bottle, 3 cover slides were added

Fig. 3 Bacterial solution containing cover glass before film formation of shaking table.

Fig. 4 Forty-eight-hour constant temperature water bath shaking table contains cover glass bacterial solution after film formation.

Fig. 5 RNA eluent.

(tweezers), 10 mL TSB (bacterial concentration: 1.0×10^6 CFU/mL) was added, which was dissolved in a constant temperature shaker at 37°C and 120 rpm, and the film was cultured for 48 hours (Figs. 3 and 4). We carefully poured out the culture medium and gently rinsed with 0.01 mol/L PBS buffer (pH 7.4) 3 times to eliminate free planktonic bacteria, and the washed slides were added to the 15-mL centrifuge tube. For each 15-mL centrifuge tube, the vortex oscillated for 30 seconds, then the 40 kHz oscillated for 50 minutes, and then the vortex oscillated for 30 seconds. The RNA eluent after ultrasonic shock cracking was collected (Fig. 5). The RNA eluent samples (including aseptic blank samples) were extracted and purified by DNA, *ica D*, *agr*, *mec A*, and *mre B* genes were extracted and purified by real-time quantitative PCR reaction and 1% agarose gel electrophoresis, and the gel electrophoresis results of each gene were photographed and analyzed (Fig. 6).

Calculated diagnostic efficiency of each gene

The results of PCR showed that the products that could be amplified were positive, and those without amplification were negative. The diagnostic efficiency of each gene was calculated according to whether the actual experimental results were consistent with the designed test results (Table 1).

Based on Table 1, the diagnostic results are true positive (A), false positive (B), true negative (C), and false negative (D). These 4 diagnostic results are represented by the letters A, B, C, and D, respectively. The sensitivity = A/(A + D), also known as the true-positive rate, and represents the ability of the test to find positive results, and the higher the sensitivity, the lower the missed diagnosis rate; the specificity = C/(B + C), also known as the true-negative rate, and represents the ability of the test to exclude positive results, and the higher the specificity, the lower the misdiagnosis rate. The higher the positive predictive value (PPV) = A/(A + B), the

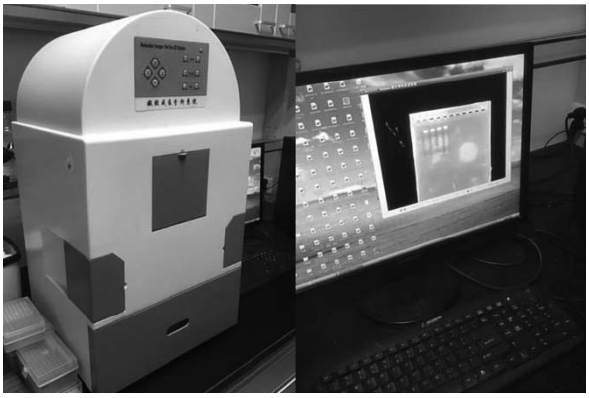

Fig. 6 Gel electrophoresis imaging analysis system.

more reliable the positive result. The higher the negative predictive value (NPV) = C/(C + D), the more reliable the negative results. The accuracy = (A + C)/(A + B + C + D), which is the overall evaluation of the diagnostic efficiency.

Results

Identification of biofilm production ability of experimental strains

Based on the binding characteristics of crystal violet and biofilm, the amount of bacterial biofilm growth can be calculated by measuring the change of absorbance. The results are as follows: the average OD492 value of TSB medium of the *S aureus* group (ATCC25923 group) was 0.482. In addition, the average OD492 value of TSB medium of the MRSA group (ATCC43300 group) was 0.495. The average OD492 values of TSB culture medium for the *E coli* group (ATCC8739 group), TSB culture medium for the *E. coli* group (ATCC25922 group), and TSB culture medium for the *S epidermidis* group (SE243 group) were 0.465, 0.485, and 0.471, respectively. As a result, these strains could form biofilm in this culture system. Moreover, the biofilm culture model of pathogenic strains related to periprosthetic infection after artificial joint replacement was successfully established.

Table 1 Determination of diagnostic efficiency

	Results of PCR testing of actual gene	Sample should display result based on experimental design
True positive A	Positive (+)	Positive (+)
False positive B	Positive (+)	Negative (−)
True negative C	Negative (−)	Negative (−)
False negative D	Negative (−)	Positive (+)

Testing sensitivity = A/(A + D), also known as true positive rate, and represents the ability of the test to find positive results. The higher the sensitivity, the lower the rate of missed diagnosis. The specificity of the test = C/(B + C), also known as the true negative rate, and represents the ability of the test to exclude positive results. The higher the specificity, the lower the misdiagnosis rate. Positive predictive value = A/(A + B). The higher the positive predictive value is, the more reliable the positive result is. Negative predictive value = C/(C + D). The higher the negative predictive value is, the more reliable the negative result is. Accuracy = (A + C)/(A + B + C + D), which is the overall evaluation of diagnostic efficiency.

Results of PCR testing of the ica D gene

Analysis of sensitivity and specificity of *ica D* gene PCR testing

The results of PCR testing of *ica D* gene showed that only 12 samples in the first group were positive, whereas none of the other samples were positive (Figs. 7–9). Based on the experimental design, 24 samples of staphylococci in 8 groups should be positive. The actual results of the *ica D* gene PCR test showed that 1 case was true positive and 23 cases were false negative. The sensitivity of calculated *ica D* gene testing was 4.17% (1/24). Because there were no false-positive results, there were 15 non–*S aureus* samples and 2 blank control samples in 5 groups. A total of 40 samples showed *ica D* negative, 17 cases were true negative, and 0 cases were false positive, so the specificity of *ica D* testing was 100% (17/17; Table 2).

As a result, *ica D* had low sensitivity and high specificity. The PCR testing of the *ica D* gene has high specificity, which is its advantage. However, its

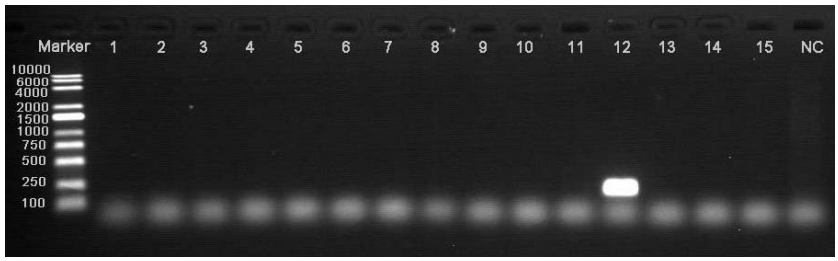

Fig. 7 *ica D* electrophoretic results of samples group 1.

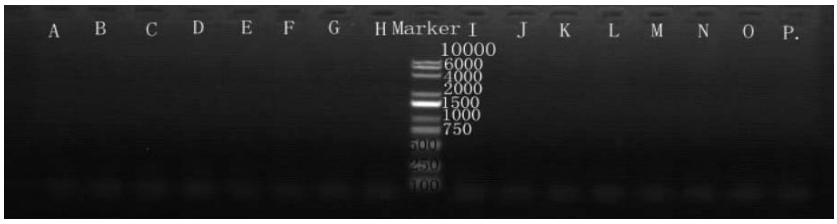

Fig. 8 *ica D* electrophoretic results of samples group 2.

sensitivity is relatively low, which limits its clinical application value.

Analysis of PPV, NPV, and accuracy of PCR testing of the *ica D* gene

The results of *ica D* gene PCR testing were true positive in 1 case, false positive in 0 cases, true negative in 17 cases, and false negative in 23 cases. The PPV and NPV of *ica D* gene PCR testing were 100% (1/1) and 42.5% (17/40), respectively, and the accuracy of *ica D* gene PCR testing was 43.9% (18/41; Table 2).

Results of PCR testing of the agr gene

The results of PCR testing of the *agr* gene showed that there was no expression in the tested samples. In addition, the expression of the positive control was normal (Figs. 10 and 11).

Analysis of sensitivity and specificity of *agr* gene PCR testing

Based on the experimental design, 24 samples of 8 groups of staphylococci should be positive. In the actual experiment, no positive results were detected in 24 staphylococcal infection samples in 8 groups, including the *S aureus* group, MRSA group, and *S epidermidis* group. The PCR results of the *agr* gene were true positive in 0 cases and false negative in 24 cases. The sensitivity of calculation was 0% (0/24; Table 3).

Table 2 Determination of diagnostic efficiency of ica D

	Actual *ica D* test results	Sample should display result based on experimental design
True positive A	Positive (+) 1	Positive (+) 24
False positive B	Positive (+) 0	Negative (−) 17
True negative C	Negative (−) 17	Negative (−) 17
False negative D	Negative (−) 23	Positive (+) 24

ica D testing sensitivity = A/(A + D) = 1/24 = 4.17%. *ica D* testing specificity = C/(B + C) = 17/17 = 100%. *ica D* PPV = A/(A + B) = 1/1 = 100%. *ica D* NPV = C/(C + D) = 17/40 = 42.5%. *ica D* testing accuracy = (A + C)/(A + B + C + D) = 18/41 = 43.9%.

According to the experimental results, there were no false-positive results in 15 *E coli* samples belonging to 2 different strains in 5 groups, and the results in the blank control group were also negative, which means that 17 cases of the *agr* gene PCR test results were true negative and 0 cases were false positive. The calculated specificity of *agr* gene PCR testing was 100% (17/17; Table 3). In the gene combinations tested, the *agr* gene and *ica D* gene are similar; both have the disadvantage of low sensitivity and high specificity.

Analysis of PPV, NPV, and accuracy of PCR testing of the *agr* gene

The results of the *agr* gene PCR test were true positive in 0 cases, false positive in 0 cases, true negative in 17 cases, and false negative in 24 cases. The PPV of the *agr* gene PCR test was 100% (0/0), and the NPV was 41.46% (17/41). The accuracy of *agr* gene PCR testing was 41.46% (17/41; Table 3).

Results of PCR testing of the mec A gene

The PCR test results of the *mec A* gene showed that electrophoresis results of samples 3, 8, and 11 in first group were negative, and the other samples (1, 2, 4, 5, 6, 7, 9, 10, 12, 13, 14, and 15) were all positive. The electrophoresis results of the second group showed that A, C, D, G, J, and L had positive expressions, and the electrophoresis results of the third group showed that B+ had positive expression (Figs. 12–14).

Fig. 9 *ica D* electrophoretic results of samples group 3.

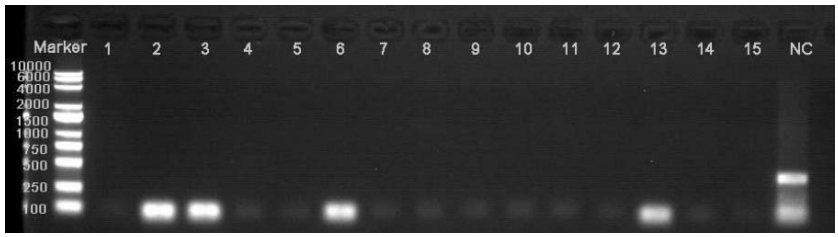

Fig. 10 *agr* electrophoretic results of samples group 1.

Fig. 11 *agr* electrophoretic results of samples groups 2 and 3.

Table 3 Determination of diagnostic efficiency of agr

	Actual *agr* test results	Sample should display result based on experimental design
True positive A	Positive (+) 0	Positive (+) 24
False positive B	Positive (+) 0	Negative (−) 17
True negative C	Negative (−) 17	Negative (−) 17
False negative D	Negative (−) 24	Positive (+) 24

agr testing sensitivity = A/(A + D) = 0/24 = 0%. *agr* testing specificity = C/(B + C) = 17/17 = 100%. *agr* PPV = A/(A + B) = 0/0 = 100%. *agr* NPV = C/(C + D) = 17/41 = 41.46%. *agr* testing accuracy = (A + C)/(A + B + C + D) = 17/41 = 41.46%.

Analysis of sensitivity and specificity of *mec A* gene PCR testing

Based on the experimental design, 9 samples of 3 groups of MRSA should be positive. In the actual experiment, the *mec A* gene PCR testing experiment showed that 5 cases of 9 samples in 3 groups of MRSA had positive results. The PCR test results of the *mec A* gene were true positive in 5 cases and false negative in 4 cases. The sensitivity testing of *mec A* gene was 55.56% (5/9; Table 4).

Based on the experimental design, another 30 bacteria samples in 10 groups and 2 samples in the control group should be negative for *mec A*. In the actual experiment, the *mec A* test was negative in the blank control group, whereas about 14 samples of false-positive results were tested in all the other 30 non-MRSA infection samples in 10 groups, including the *S aureus* group, *S epidermidis* group, and *E coli* group. The PCR test results of the *mec A* gene were false positive in 14 cases and true negative in 18 cases, and the specificity detection of the *mec A* gene was 56.25% (18/32; Table 4). This result means specificity of the *mec A* gene is a bit lower.

Analysis of PPV, NPV, and accuracy of PCR testing of the *mec A* gene

The *mec A* gene test results showed 5 true positives, 14 false positives, 18 true negatives, and 4 false negatives; the PPV of the *mec A* gene PCR test was 26.32% (5/19), and the NPV was 81.82% (18/22). The accuracy of the *mec A* gene test was 56% (23/41; Table 4).

According to our experiment results, *mec A* gene detection has the advantage of higher sensitivity and the disadvantage of lower specificity.

mec A is a structural gene encoding the penicillin-binding protein PBP2a, and it is the main resistance factor of MRSA. *S aureus* and other staphylococci could produce the *mecA* gene after they become resistant to methicillin, so generally a positive test of *mecA* would be used as hard proof for methicillin-resistant staphylococcus infection.

The phenomenon of more false-positive samples of the *mec A* gene test in the experiment may be caused by various reasons. First, because methicillin-sensitive *S aureus* (MSSA) may also carry the *mec A* gene, this may account for the occurrence of false positives in the *S aureus* group. For the same reason, *S epidermidis* also may carry the *mec A* gene, which was positive in the test. These are all possible factors for the positive detection of the *mec A* gene in the *S aureus* and *S epidermidis* groups, and these reasons lead to the high false-positive rate of *mec A* gene

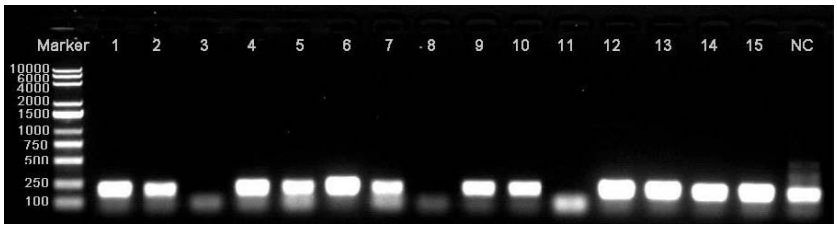

Fig. 12 *mec A* electrophoretic results of samples group 1.

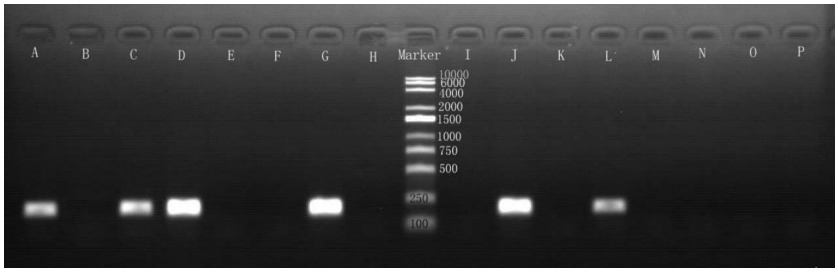

Fig. 13 *mec A* electrophoretic results of samples group 2.

detection. In clinical research, the positive rate of the *mec A* gene will remain high because of antibiotic resistance caused by antibiotic abuse in the perioperative period.

Results of PCR testing of the mre B gene

The PCR test results of the *mre B* gene showed that all pathogenic bacteria samples in the first group are negative, the positive control expression was positive, and the control group showed negative results. A, E, H, I, L, M, N, and P in the second group of the *mre B* test showed positive results, and the others were all negative. In the third group of experiments, H+ and I+ showed positive results. All other samples were negative (Figs. 15–17).

Analysis of sensitivity and specificity of *mre B* gene PCR testing

Based on the experimental design, a total of 15 samples of 5 groups of *E coli* are supposed to be positive in the *mre B* PCR detection test. However, actually, the *mre B* gene PCR test results showed that 4 cases of 15 samples in 5 groups of *E coli* had positive results. The *mre B* gene PCR test results show true positive in 4 cases and false negative in 11 cases, and the sensitivity of the *mre B* gene was about 26.67% (4/15; Table 5).

Based on the experimental design, besides the 5 groups of *E coli* with a total of 15 samples, another 8 groups of 24 bacteria samples and 2 blank control samples are supposed to be negative for *mre B* testing. The actual *mre B* gene PCR test experiment showed that the test results of the blank control group were negative, and 6 cases of false-positive results were tested in the other non–*E coli*–infected pathogen groups. The PCR test results of the *mre B* gene were false positive in 6 cases and true negative in 20 cases, and the specificity of *mre B* gene detection was 76.92% (20/26; Table 5). This result

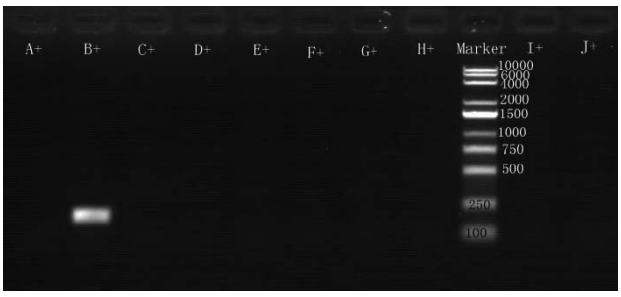

Fig. 14 *mec A* electrophoretic results of samples group 3.

Table 4 Determination of diagnostic efficiency of mec A

	Actual *mec A* test results	Sample should display result based on experimental design
True positive A	Positive (+) 5	Positive (+) 9
False positive B	Positive (+) 14	Negative (−) 32
True negative C	Negative (−) 1 8	Negative (−) 32
False negative D	Negative (−) 4	Positive (+) 9

mec A detection sensitivity = A/(A + D) = 5/9 = 55.56%. *mec A* detection specificity = C/(B + C) = 18/32 = 56.25%. *mec A* PPV = A/(A + B) = 5/19 = 26.32%. *mec A* NPV = C/(C + D) = 18/22 = 81.82%. *mec A* detection accuracy = (A + C)/(A + B + C + D) = 23/41 = 56%.

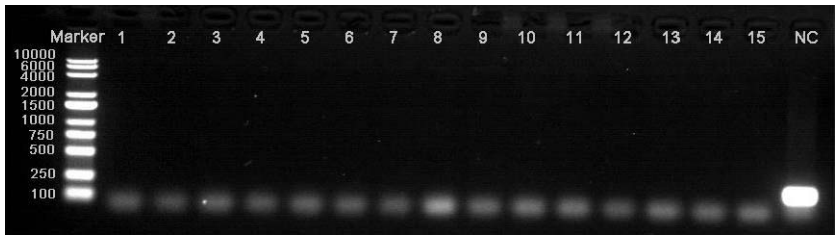

Fig. 15 *mre B* electrophoretic results of samples group 1.

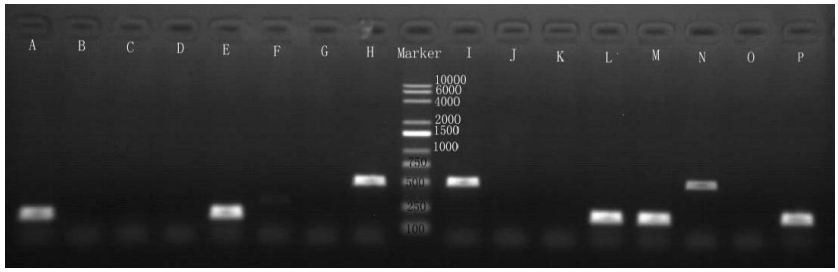

Fig. 16 *mre B* electrophoretic results of samples group 2.

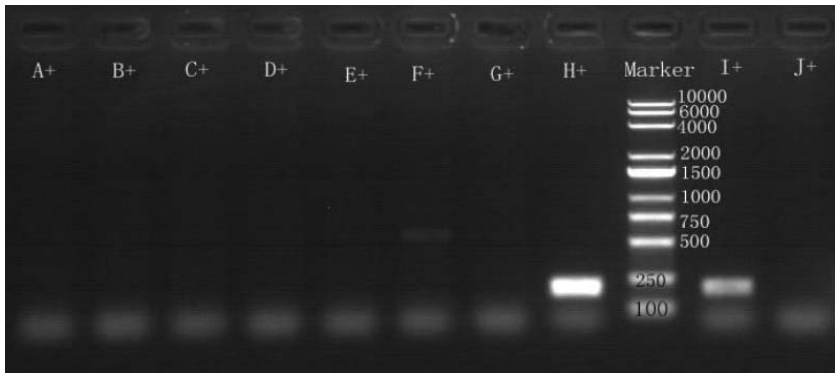

Fig. 17 *mre B* electrophoretic results of samples group 3.

means that the specificity of *mre B* gene detection is higher.

Analysis of PPV, NPV, and accuracy of PCR testing of the *mre B* gene

The *mre B* gene test results showed 4 true positives, 6 false positives, 20 true negatives, and 11 false negatives; the PPV of the *mre B* gene was about 40% (4/10), and the NPV was 64.5% (20/31). The test accuracy of the *mre B* gene was about 58.53% (24/41; Table 5).

The *mre B* gene could express actin-like protein, which would exist in all non-cocci. A positive result in the *mreB* gene PCR test also confirmed the diagnosis of PJI and suggests that the pathogenic bacterial would be non-cocci. In the actual experiment, 4 samples in the *E coli* group show positive results for *mre B* gene detection. The *mre B* gene existed only in the non-cocci (two e.coli groups, ATCC 8739 and ATCC 25922, in our experiment). According to design, the *mre B* gene should not be tested in cocci group including *S. aureus*, MRSA, and *S. epidermidis*, since six cases of false-positive *mre B* gene were tested in cocci bacteria groups, three in the MRSA(ATCC43300) group, three in the *S. aureus* (ATCC25923) group, and none in the *S. epidermidis* group (Table 6).

Discussion

Early diagnosis of PJI and identification of specific infection flora can help clinicians to take targeted treatment measures in time. In addition, it is necessary to test the characteristic genes of common pathogenic bacteria of PJI. It is reported in the literature that staphylococci, especially *S aureus* and *S epidermidis*, are the most common pathogenic bacteria in different parts of PJI, accounting for 65%–82% of the pathogens of PJI.[12] In addition, it is reported that staphylococci, especially *S aureus* and *S epidermidis*, are the most common pathogenic bacteria in different parts of PJI. The test rate of

Table 5 Determination of diagnostic efficiency of mre B

	Actual *mre B* test results	Sample should display result based on experimental design
True positive A	Positive (+) 4	Positive (+) 15
False positive B	Positive (+) 6	Negative (−) 26
True negative C	Negative (−) 20	Negative (−) 26
False negative D	Negative (−) 11	Positive (+) 15

mre B testing sensitivity = A/(A + D) = 4/15 = 26.67%. *mre B* testing specificity = C/(B + C) = 20/26 = 76.92%. *mre B* PPV = A/(A + B) = 4/10 = 40%. *mre B* NPV = C/(C + D) = 20/31 = 64.5%. *mre B* testing accuracy = (A + C)/(A + B + C + D) = 24/41 = 58.53%.

MRSA in PJI samples is also increasing with the use of antibiotics in the perioperative period.[13,14] The most common and main pathogens of PJI, including *S aureus, S epidermidis*, MRSA, and *E coli*, lead to the vast majority of PJI cases caused by single or multiple infections. *ica D* and *agr* are staphylococcal-specific genes that play a specific role in different stages of staphylococcal biofilm formation and are closely related to staphylococcal biofilm formation. Biofilm is formed by bacteria adhering to the surface of organisms or nonorganisms to adapt to the living environment, producing extracellular macromolecular polymers to wrap the bacteria, and can resist host immune defense mechanisms such as antibiotics, disinfectants, and interference phagocytosis. As a consequence, the biofilm of pathogenic bacteria plays an important role in the pathogenesis of PJI.[15] The *mec A* gene is one of the major factors of resistance in MRSA, and the *mre B* gene would express actin-like protein that would exist in all non-cocci, and these proteins with a spiral net structure could guide protein movement in cell wall biosynthesis; thus, *mre B* is a specific gene of non-cocci including *E coli*.[16,17] This study discusses the testing of specific genes of most common PJI pathogens, *ica D*, *agr*, *mec A*, and *mre B*, to diagnose pathogen infection in PJI samples, to evaluate the value of each gene in early diagnosis of PJI, and to design a reasonable gene test sequence and combination based on the diagnostic advantages of each gene.

Discussion on PCR testing results of the ica D gene

Biofilm formation is the main pathogenic factor of conditional pathogenic staphylococci. The main role of the *ica* gene is to regulate staphylococcal biofilm formation, including 4 kinds of *ica ADBC*, among which the detection rate of *ica D* is the highest.[19–21] As a consequence, we add *ica D* as a reference gene

Table 6 Calculation of diagnostic results of all target genes

Target testing gene	True positive A	False positive B	True negative C	False negative D
ica D	1	0	17	23
agr	0	0	17	24
mec A	5	14	18	4
mre B	4	6	20	11

to the gene combination, but the actual experimental results show that even the highest detection rate of *ica D* has a sensitivity of only 4.17%, which is a relatively low sensitivity for the 4 gene indicators in the combination. Moreover, the test results can only be used as a reference in the combination.

Cao *et al*[19] carried out grouped PCR testing of *ica A* and *ica D* genes in blood culture, nonblood culture, and environmental skin–derived *S epidermidis*. As a result, the testing identified rates of *ica A* and *ica D* genes in all samples were less than 25%, most of them were less than 20%, and the difference was not statistically significant. This is consistent with the low sensitivity of *ica D* among the 4 gene combinations, which confirms that the *ica* gene can only be used as a reference index in the detection of staphylococci because of its low sensitivity.

Jin *et al*[20] hold the opinion that the *ica* gene can be used as a virulence marker such as infectious *S epidermidis* to determine whether *S epidermidis* isolated from blood culture is pathogenic bacteria or contaminated bacteria. After all, *S epidermidis* as a pathogenic bacteria generally carry *ica* operons to form extracellular polysaccharides, so *ica* is often tested. In this study, both *ica A* and *ica B* alleles were used as indicators for PCR detection, and a high sensitivity of the *ica* test was obtained. This result is not consistent with our experimental results and with the report of Cao *et al*.[19] In the opinion of the authors, this is mainly because the pathogenicity of *S epidermidis* is not the most important factor in the infection of *S epidermidis*. In other words, the role of the *ica* gene in the pathogenicity of *S epidermidis* should not be overestimated. The results of Fei[21] also support this view. Fei[21] suggested that the expression of the *ica A* and *ica D* genes has an important effect on the synthesis of polysaccharide-intercellular-adhesion (PIA) in staphylococci, but not all PIA-synthesizing strains *ica A* and/or *ica D* genes are positive. In addition, it is also believed that the *ica A* and/or *ica D* genes are not the only factors affecting staphylococcal biofilm formation. This study is consistent with our experimental

results. Besides, the conclusion of Fei[21] can reasonably explain the low positive rate of *ica D* gene detection in our experiment.

Discussion on the results of PCR detection of the agr gene

The *agr* gene plays a role independent of *ica D* in the biofilm formation of PJI pathogenic bacteria.[18] The *agr* gene, which is similar to the *sar A* gene, is an auxiliary gene regulator in the biofilm formation process. The *agr* system encoded by the *agr* gene is not expressed in all samples when the positive control is normal. The normal positive control indicates that the experimental process of *agr* gene detection is correct, whereas the nonexpression of all samples means that it has low sensitivity. The detection rate is low, and there are 2 possibilities for this negative result. The first may be false negative; that is, the sample carries the gene but is not detected for various reasons. After all, we have a group of experiments specifically aimed at the *agr* gene, and the positive control results show normal. As a consequence, false negative is not very likely. The second is true negative, and there are many cases of true negative. First, it is possible that most staphylococci, including experimental samples, do not carry this gene, because the *agr* gene is not essential for staphylococcal survival and biofilm formation. As a consequence, there is a possibility that staphylococcal samples do not carry this gene or carry a small number of staphylococci that have not been detected. Another possibility is that the staphylococci participating in the experiment are not in the stage of *agr* activation, high expression, and role. It has been reported that *agr* has low expression at the initial and mature stage of biofilm formation. However, *agr* is activated only in the late stage of biofilm maturation.[22,23] *agr* regulation of staphylococcal growth is considered to be mainly in the exponential phase and stable phase. However, the gene still has some value as a reference gene.

Discussion on PCR testing results of the mec A gene

The *mec A* gene is 1 of the major factors of resistance in MRSA. The resistance mechanism of MRSA is that the *mec A* gene would express penicillin-binding protein PBP2a, which had a low affinity with β-lactam antibiotics. As a result, penicillin-binding protein PBP2a can catalyze the synthesis of cell walls with higher concentrations of β-lactam antibiotics.[20,24] Li *et al*[25] have proved that the resistance mechanism of *S aureus* is mainly caused by the expression of β-lactamase and the *mec A* gene. Cao *et al*[19] proved that the test of the *mec A* gene in blood culture, nonblood culture, and environmental skin–derived *S epidermidis* had a very high positive rate, Liu *et al*[20] found that about 94.6% of *S epidermidis* PCR samples detected show the positive result of the *mec A* gene, whereas more than 50% of *S epidermidis* in blood culture detected both the *mec A* gene and the *ica* gene at the same time.[21] The high detection rate of *mec A* in these findings is partly consistent with our experimental results, especially with the high detection rate of *mec A* and with the results in the *S epidermidis* group in our experiment. At the same time, the detection results of the high positive rate of *mec A* also indicated that the antibiotic resistance of *S epidermidis* become more serious.

In our actual experiments, it was found that *mec A* was highly sensitive, and the MRSA group samples were all positive in the *mec A* gene PCR test, which means all were detected. This high sensitivity shows that the *mec A* gene is a good choice for a PCR test used in the early diagnosis of PJI. However, according to our experiment results, it was found that there were many false-positive results in the *mec A* PCR gene test. This phenomenon may be caused by many reasons. First, researchers have reported that many *S aureus* had potential antibiotic resistance. This means some non–methicillin-resistant *S aureus* may also carry *mec A*, which may be the reason for the 1 positive case in the *S aureus* group. The strains may develop resistance during multiplication.[20,21] Increasing antibiotic resistance could be caused by antibiotic use in the perioperative period, and much research reported that MRSA infection may occur in many actual PJI cases.[13] Therefore, we put the *mec A* gene PCR test after 2 staphylococcus-specific genes, *ica D* and *agr*, were tested. The *mec A* gene test is performed in the samples that have been already tested for the 2 staphylococcus-specific genes. Therefore, the interference of *mec A* false positives in the actual results is not very serious. The test results of *mec A* still have high value in actual clinical work.

Discussion on the results of PCR testing of the mre B gene

The *mre B* gene expresses actin-like protein that would be exist in all non-cocci, and these proteins with a spiral net structure could guide protein movement in cell wall biosynthesis. Therefore, the main function of the mre B protein is to maintain cell

shape. All non-cocci have genes that express actin-like proteins. For example, there is the *mre B* gene in *E coli*.[21] According to some research about *E coli*, when the actin-like protein encoded by *mre B* is deficient or the expression of the *mre B* gene is impaired, the bacteria change from rod shaped to spherical.[21] In our experiment, we hoped we could detect *E coli* infection specimens through *mre B*. If the *mre B* gene test is positive, PJI can also be confirmed, and the pathogenic bacteria are non-cocci strains (in this experiment, it mainly refers to 2 strains of *E coli*).

However, the actual experimental results of the *mre B* gene PCR test showed that the *mre B* positive rate is low and unstable. In the second group of experiments, a total of 6 *E coli* samples belong to 2 different strains in 2 different groups, and 4 positive cases were detected, but no positive expression of *mre B* was found in other groups of *E coli* samples. The positive control expression was normal, and the control group *mre B* did not have false-positive results, which means that the results of our experiment is reliable.

There are several possible reasons for the negative test results of some *E coli* samples: on the one hand, false negatives would be possible; the other possible reason would be the primers design of *mre B*. Another aspect is the possibility of true negative; that is, the negative result is because of the low positive detection of the *mre B* gene by PCR, which means that the gene is not suitable as a detection index for diagnosing *E coli* infection.

In the second and third groups of experiments, false-positive results appeared in the test of *mre B* in the *S aureus* group and MRSA group, but the bands of each sample were uneven. This may be caused by *E coli* or other non-cocci contamination. According to the PCR test results of *mre B* in the experiment, we put *mre B* test at the end of all other genes. In clinical practice, only samples screened by the aforementioned genetic testing of *Staphylococcus* and MRSA will be considered for *E coli* infection, because the result of the impact of *mre B* false positive is also limited. Therefore, the results of *mre B* gene detection showed that there is high potential for improvement and development for non-cocci PJI detection including *E coli*.

Reasonable design and significance of sequential test of specific genes in PJI early diagnosis

According to the results of *ica D*, *agr*, *mec A*, and *mre B* in the PCR gene test, we rank these 4 genes and try to give full play to the advantages of each gene through the method of gene sequential test.

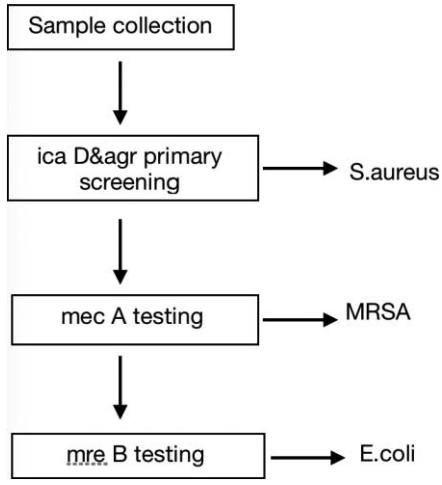

Fig. 18 Reasonable sequencing design and results of detection genes based on experiments.

First, we made full use of the test results of *ica D* and *agr* to conduct preliminary test of the samples, screening out the samples that may have staphylococcal infection and then used the MRSA main antibiotics resistance gene *mec A* for further screening. Finally, we used *mre B* as a reference and control. Then, the detection and screening of several common PJI pathogens such as *S aureus*, *S epidermidis*, MRSA, and non-cocci (mainly *E coli*) was achieved by a single sequential PCR test of these samples (Fig. 18). Our research established an in vitro PJI model successfully and then carried out real-time PCR detection with in-depth analysis test results. We designed a reasonable sequential detection of PJI common pathogenic bacteria-specific genes based on the experimental results, giving full play to the detection advantages of each gene. These provided new ideas for early PJI diagnosis, which is of great significance for us to further study and expand the test of other PJI pathogens and will have broad prospect in clinical application of early diagnosis of PJI.

Limitations

There are some limitations to this study. First, this study is a single center study, and the source of the strain is limited to the Ninth People's Hospital of Shanghai. There may be differences in the specific strains and virulence of PJI infection in different regions and hospitals, as well as the gene expression detected in this experiment. As a consequence, we

will broaden the source of strains in a follow-up study. Second, the sample size of this study is limited, so it is necessary to further increase the sample size. Finally, this study is limited to staphylococci, the most common pathogen of PJI, and the specific genes of rare bacteria and rare pathogens that often exist in clinical PJI multiple infection need to be further studied.

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