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# Evaluation of ERIC-PCR for fingerprinting Methicillin-resistant *Staphylococcus aureus* strains

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Staphylococcus aureus (S. aureus) has been long recognized as a common food-born pathogen in food safety. To study their individuality and drug resistance, a total of 179 Staphylococci strains were subject to the investigation. S. aureus was further confirmed with the detection of specific 16S rRNA and femA genes by multiplex-PCR. The molecular method was also used for amplification of mecA and orfX elements to identify Methicillin-resistant S. aureus (MRSA). Then fingerprinting for all strains was performed using ERIC-PCR. In this experiment, 179 staphylococci were isolated, and all of these strains were MRSA. In addition, 179 MRSA strains can be typed into 10 genotypes which have significant diversities, based on the results of ERIC-PCR. Through the above studies, a new protocol was brought out for rapid identification of MRSA using multiplex-PCR and MRSA typing using fingerprinting, which have a broad application prospect in food safety and epidemiology.

**Key words:** MRSA, Multiplex-PCR, ERIC-PCR, Fingerprinting

## INTRODUCTION

Staphylococci are a group of gram-positive, facultative aerobic and usually unencapsulated organisms, which are responsible for various tissues infection and a multitude of diseases. *Staphylococcus aureus* are typical foodborne microorganisms, which can cause food poisoning and toxic shock, bringing serious public health burden. About 14 million people are infected due to foodborne microorganisms per year [1]. Among these public health events, food poisoning caused by *Staphylococcus aureus* accounts for a large proportion, which has become an advanced project in food safety [2-4]. Since the first methicillin-resistance *Staphylococcus aureus* (MRSA) was found in Britain in 1961, it has been spreading among the world at an alerting rate. With the wide identification of MRSA in animal husbandry and food industry, a concept was put forward that MRSA strains are a potential risk factor, not only for food itself, but for those who engage in food production and processing [5~8]. Instead of restricting the resistance, invasion and propagation of MRSA into clinical fields, these events should be expanded into food safety [9]. It has been recognized that the infection caused by MRSA has high morbidity and mortality. According to the statistics published by CDC in 2003, hundreds of thousands of patients were hospitalized due to infection of MRSA, and the separation rate of MRSA might reach high up to 80%. The multi-drug resistance of MRSA increases the complicacy and difficulty in therapy as well as in the rapid growth of antibiotic and healthcare budget. Reducing the infection rate of MRSA has been a major challenge in clinical medicine, which decides that rapid identification and efficient typing method of MRSA are particularly crucial [10].

Increasing awareness of the risk of MRSA strains and demands for tests capable of early, cost-effective, timely, and sensitive detection of staphylococci with associated antibiotic resistance determinants has made these tests an urgent demand. This study used multiplex-PCR assay for identification of MRSA by detecting Staphylococcus-specific regions of the 16S rRNA gene, *S. aureus* specific femA gene and MRSA key drug-resistance gene mecA. Furthermore, in order to genotype MRSA strains, ERIC-PCR assay is used for fingerprinting. MRSA can be rapidly identified and genotyped using methods above.

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## MATERIALS AND METHODS

### *Bacterial strains*

Four reference strains, including MRSA 12513 and 10864 (with mecA, femA, 16S rRNA and orfX positive), MSSA 10501 (with femA, 16S rRNA and orfX positive, and mecA negative), MRCNS A110146 (with mecA, 16S rRNA and orfX positive, and femA negative), MSCNS 110830 (with 16S rRNA positive, and mecA, femA, orfX negative) were subjected to evaluation and optimization of multiplex-PCR assay. Application of the multiplex-PCR and RAPD assays were further performed on a total of 179 Staphylococcus isolates, which were isolated from The First Affiliated Hospital of Jinan University (FAHJU).

### *Culturing condition and template DNA preparation*

Genomic DNA from *S. aureus* strains used as template for PCR amplification was prepared from overnight Tryptic soy broth (TSB) cultures at 37 °C with shaking. Culture was performed according to the instruction of kits (Dongsheng Biotech, Guangzhou). Harvest 0.5-2.0ml culture in a sterile 1.5ml micro centrifuge tube by centrifuging for 1min at 12,000rpm, discard supernatant. Resuspend pellet in 200μl DS Buffer. Mix the mixture thoroughly with tip. Add 20μl Proteinase K and 220μl MS lysis buffer, mix thoroughly. Incubate at 65℃for 10min. Add 220μl EtOH, mix the mixture thoroughly with tip. Transfer the supernatant and floc to a new purify tube, centrifuging for 1 min at 12,000rpm, discard filtrate. Add 500μl PS Buffer, centrifuging for 1min at 12,000rpm, discard filtrate. Add 500μl PE Buffer, centrifuging for 1min at 12,000rpm, discard filtrate. Add 500μl PE Buffer, centrifuging for 1min at 12,000rpm, discard filtrate. Centrifuge the tube for 3 min at 12,000rpm, and transfer the spin column to a sterile 1.5ml centrifuge tube. Add 100μl TE Buffer, incubate at room temperature for 2min. At last, Centrifuge the tube for 2 min at 12,000rpm. Remove spin column, the buffer in the micro centrifuge tube contains the highly purified DNA. Stored at -20℃.

### *Primer design*

Three targets were selected to differentiate MRSA、MSSA、MRCNS、MSCNS and non-Staphylococci strains. The protocol was designed to (i) detect any staphylococcal species to the exclusion of other bacterial pathogens using as an internal control, with primers corresponding to Staphylococcus-specific regions of the 16S rRNA genes (C1: 5’-GATGAGTGCTAAGTGTTAGG -3’ and C2: 5’-TCTACGATTACTAGCGATTC-3’ , with an expected 542 bp amplicon); (ii) distinguish between S. aureus and CNS strains based on amplification of the S. aureus specific femA gene (F1: 5’-AAAGCTTGCTGAAGGTTATG-3’ and F2: 5’-TTCTTCTTGTAGACGTTTAC-3’, with an expected 823 bp amplicon) and (iii) provide an indication of the likelihood that the staphylococci present in the specimen are resistant to methicillin based on the amplification of the mecA gene (M1: 5’-GGCATCGTTCCAAAGAATGT-3’ and M2: 5’-CCATCTTCATGTTGGAGCTTT-3’, with an expected 374 bp amplicon). (iv) detect an open reading frame existing on both sides of SCCmec in order to identify MRSA strain (with mecA+) based on the amplification of the orfX gene (O1: 5’-ACCACAATCMACAGTCAT-3’ and O2:5’-CCCGCATCATTTGATGTG-3’). Besides, primer ERIC2 (5’-AAGTAAGTGACTGGGGTGAGCG-3’) is used for ERIC-PCR assay.All primers mentioned above are listed in Tab.1.

### *Establishment of multiplex-PCR assay*

Four reference strains were used to evaluate the multiplex-PCR assay, which was carried out in a total of 25 μL reaction mixture. Components for multiplex-PCR are listed in Tab.2. Primers are stored at -20℃ The stock solution of primers is 100pmol/μL. Work solution of primers is 10pmol/μL. Multiplex-PCR amplification was carried out using the thermal profile as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, annealing temperature for 30 sec, and 72°C for 1.5 min and a final extension cycle at 72°C for 7 min. Annealing temperature is presented in **Tab.1**. The amplified products (7μL/well) were analyzed by gel electrophoresis in 1.5% agarose gels and stained with ethidium bromide for 10 min. A negative control was performed using sterile water instead of DNA template.

### *Application of the multiplex-PCR assay on clinical strains*

179 clinical staphylococci isolates, were subjected to detection by the established multiplex-PCR assay with primers pairs F1 with F2, C1 with C2, O1 with O2, and M1 with M2, and PCR amplicons were evaluated by electrophoresis as aforementioned. These experiments were replicated to ensure reproducibility.

### *Fingerprinting analysis by ERIC-PCR on clinical strains*

179 isolated strains were genotyped using the ERIC-PCR assay, which was carried out in a total of 50 μL reaction mixture. Components for ERIC-PCR are listed in Tab.3. Primer ERIC-2 is stored at -20℃ The stock solution of primers is 100pmol/μL, while work solution is 10pmol/μL.

**Table 1.** Description of the primers used for amplification

| Primer | Sequence（5'-3'） | Target | Tm（°C） | Amplification（bp） |
|---|---|---|---|---|
| C1 | GGACTGTTATATGGCCTTTT | 16S rRNA | 50 | 542 |
| C2 | GAGCCGTTCTTATGGACCT | | | |
| M1 | GGCATCGTTCCAAAGAATGT | mecA | | 374 |
| M2 | CCATCTTCATGTTGGAGCTTT | | | |
| F1 | AAAGCTTGCTGAAGGTTATG | femA | | 823 |
| F2 | TTCTTCTTGTAGACGTTTAC | | | |
| O1 | ACCACAATCMACAGTCAT | orfX | | 212 |
| O2 | CCCGCATCATTTGATGTG | | | |
| ERIC2 | AAGTAAGTGACTGGGGTGAGCG | RAPD | 25 | — |

**Table 2.** Reaction system of multiplex PCR amplification

| Component | Volume (μL) |
|---|---|
| 2×UTaq PCR Master Mix | 12.5 |
| Primer M1, M2; C1, C2 (each) | 1 |
| Primer F1, F2; O1, O2(each) | 1.5 |
| DNA template | 1.5 |
| $ddH_2O$ | 1 |

**Table 3.** ERIC-PCR amplification of insert DNA

| Component | Volume (μL) |
|---|---|
| 2×Uaq PCR Master Mix | 25 |
| Primer ERIC-2 | 3 |
| DNA template | 1 |
| $ddH_2O$ | 21 |

ERIC-PCR amplification was carried out using the thermal profile as follows: 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, annealing temperature for 2 min, and 72°C for 2 min and a final extension cycle at 72°C for 7 min [11]. Annealing temperature is presented in Tab.1. The amplified products (7μL/well) were analyzed by gel electrophoresis in 1.5% agarose gels and stained with ethidium bromide for 10 min. A negative control was performed using sterile water instead of DNA template

## RESULTS AND DISCUSSION

### *Evaluation of the multiplex-PCR*

The specific amplification generated 4 bands on agarose gel (**Fig.1**), with sizes 823 bp for *femA*, 542 bp for *16S rRNA*, 374 bp for *mecA* and 212bp for *orfX* (**Tab.1**), respectively. Marker DS2000 (track 1) was used for control. MRSA strain 12513 (track 1) and strain 10864 (track 2) showed specific amplification for mecA, femA, 16S rRNA and orfX, MSSA strain 10501 (track 3) showed positive result for femA, 16S rRNA and orfX, MRCNS 110146 (track 4) was detected to carry mecA ,16S rRNA and orfX, MSCNS 110830 (track 5) had been found to be 16S rRNA positive, while $ddH_2O$ (track 6) cannot amplified any band.

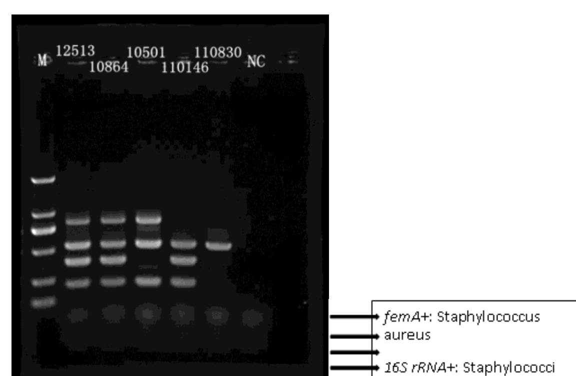



**Fig. 1.** Amplification results of multiplex-PCR

According to the amplification results mentioned above, the multiplex-PCR assay has fine resolution, not only to MRSA, but other Staphylococci such as MRCNS, MSCNS, and

MSSA etc. Besides, characters showed by multiplex-PCR, rapid identification and high specificity, for instance, may significantly increase efficiency both in clinical medicine and microbial safety in food industry.

### *Application of multiplex-PCR on 179 clinical strains*

One hundred and seventy-nine clinical Staphylococci strains had been subjected to the application of the multiplex-PCR detection using primers F1 with F2, C1 with C2, O1 with O2, and M1 with M2 together. Multiplex-PCR and subsequent detection by electrophoresis were performed as described previously. All 179 MRSA strains yielded three bands as 374 bp, 572 bp and 823 bp amplicon, corresponding to *mecA*, 16S rRNA and *femA*. No false positive amplification was observed, indicating the high specificity of the established multiplex-PCR assay (Fig.2).

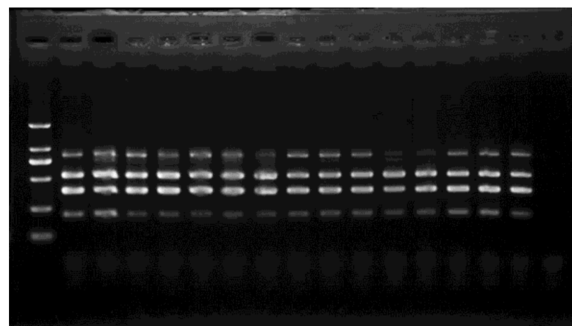
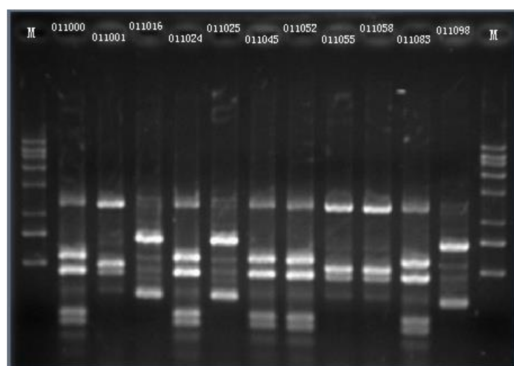
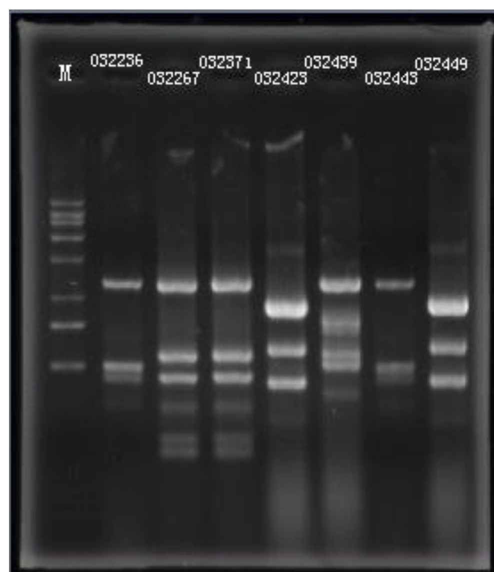
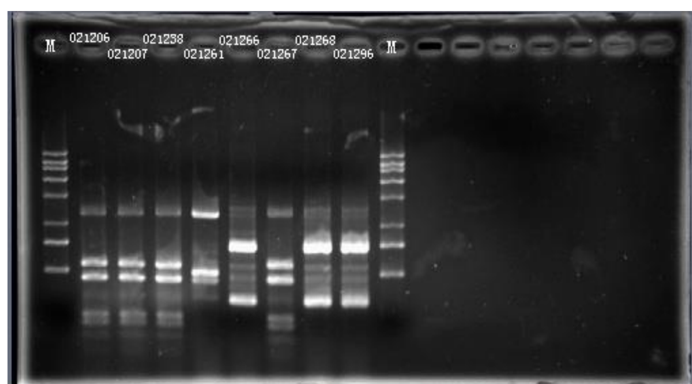


**Fig.2.** Part of amplification results of 179 clinical strains



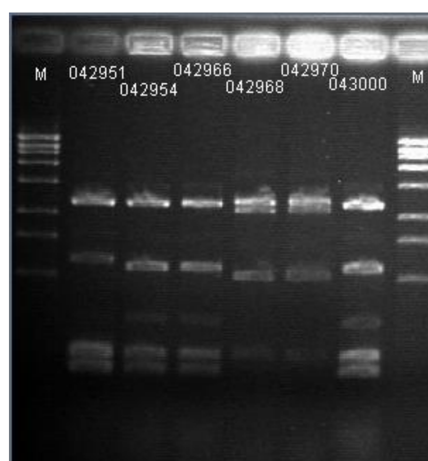




**Fig. 3.** The PCR amplification of part Staphylococcus aureus in primer AP1in primer ERIC2 (continues on the next page)

**Fig. 3.- *continuation*.**

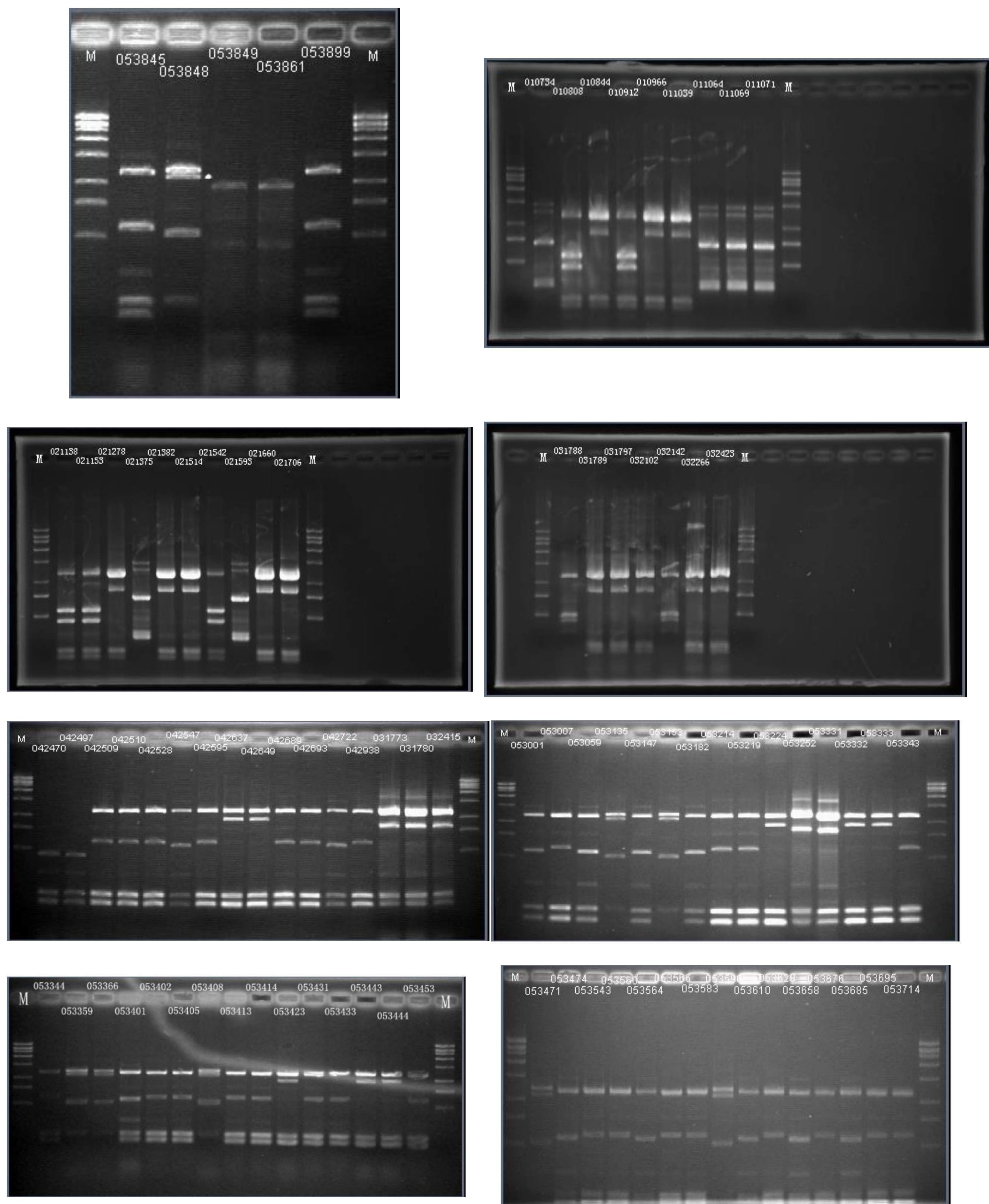



**Fig. 3.** The PCR amplification of part Staphylococcus aureus in primer AP1in primer ERIC2 (continues on the next page).

**Fig. 3.- *continuation.***

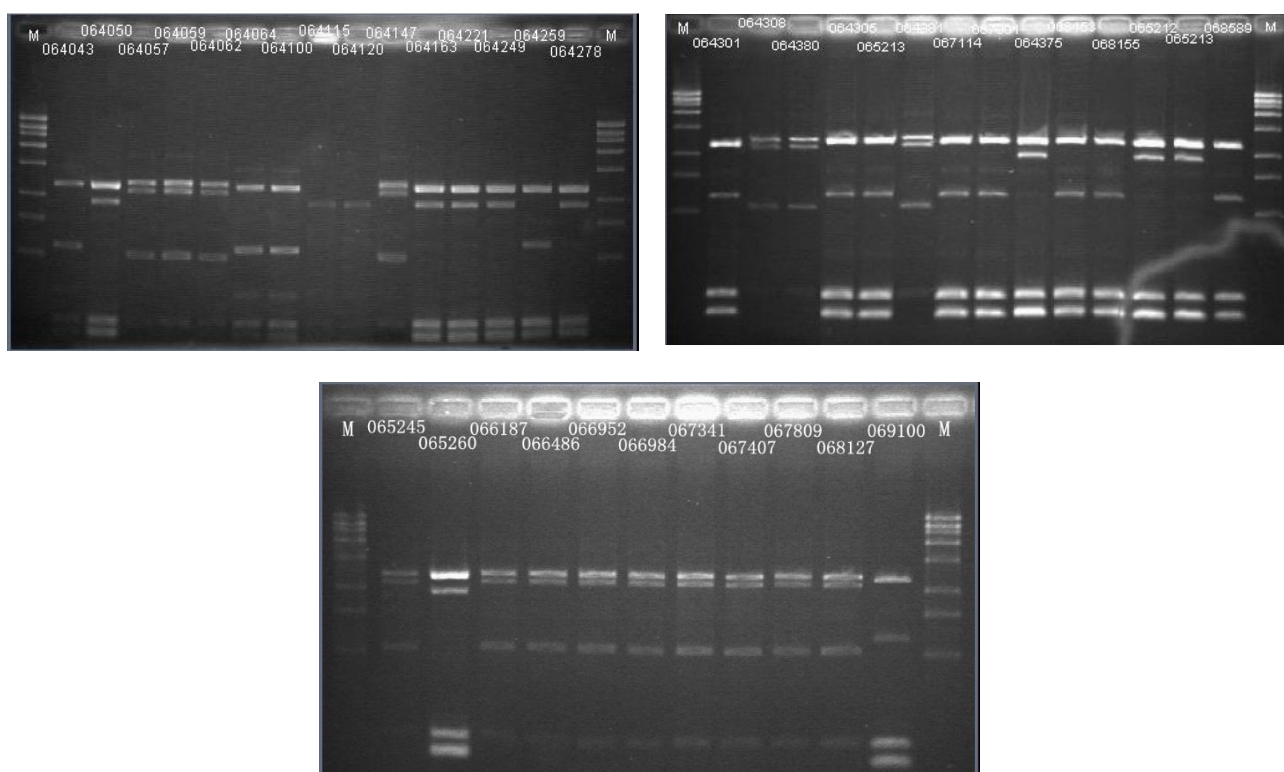



**Fig. 3.** The PCR amplification of part Staphylococcus aureus in primer AP1in primer ERIC2

*Application of Fingerprinting on 179 clinical strains*

One hundred and seventy-nine clinical MRSA strains which were rapidly identified by multiplex-PCR previously, had been subjected to the application of the ERIC-PCR detection with primer ERIC2 in order to genotyping in fingerprinting. ERIC-PCR and subsequent detection by electrophoresis were performed as described previously.

Based on the results of gel electrophoresis of ERIC-PCR, complex banding patterns were amplified in 2-5 bands, ranging from 400bp-5000bp. All 179 clinical MRSA strains were successfully genotyped with ERIC-PCR assay into 10 genotypes A-J (**Fig.4**). These clinical MRSA strains were isolated in separate ward and different organs of patients. All 179 clinical MRSA strains were listed in **Tab.4**. Fingerprinting results were recorded, then with the usage of Quantity One, the similarity between 179 MRSA strains was evaluated. The DNA relatedness between 179 MRSA strains was analyzed based on the unweighted pair group method using arithmetic averages (UPGMA).

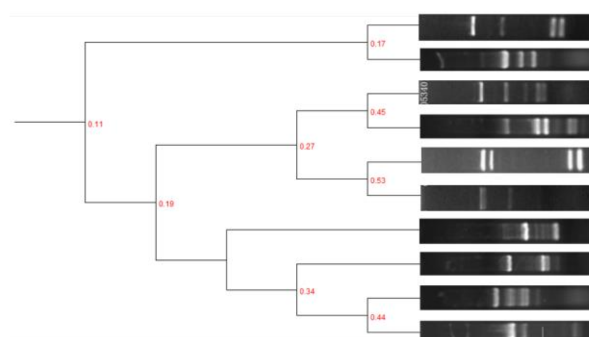


**Fig. 4.** Dendrogram analysis by ERIC-PCR of DNA fingerprinting results.

## CONCLUSION

MRSA are currently widespread pathogens throughout the world and have prompted a heightened interest and concern for the rapid detection [12] and accurate categorization of these pathogens. Thus, rapid and accurate detection and genotyping approaches are needed to reduce risk in nosocomial infection and food industry caused by MRSA strains.

This study was aimed to use rapid identification methods based on multiplex-PCR assays for differentiation of MRSA, MSSA, MRCNS, MSCNS even non-staphylococci strains.

**Table 4.** Summary of typing result of 179 MRSA strains using ERIC-PCR.

| Year | Source | Ward | Amount | Genotype |
|---|---|---|---|---|
| 2001 | Blood | Orthopedics | 1 | C |
| | | General | 1 | B |
| | Skin and parenchyma | Internal medicine | 4 | A |
| | | Surgery | 1 | C |
| | Respiratory | Infection | 1 | A |
| | | Orthopedics | 1 | B |
| | | Neurology | 1 | A |
| | | | 1 | B |
| | | | 1 | C |
| | Other | Orthopedics | 1 | A |
| | | | 1 | C |
| | | Neurology | 1 | C |
| | | General | 1 | D |
| | | Surgery | 1 | A |
| | Urethra | Internal medicine | 2 | D |
| | | Surgery | 1 | C |
| 2002 | Skin and parenchyma | Internal medicine | 5 | A |
| | | | 5 | D |
| | | Surgery | 1 | C |
| | | | 1 | D |
| | Blood | Orthopedics | 1 | C |
| | Urethra | Neurology | 1 | B |
| | | Surgery | 1 | C |
| | | Internal medicine | 1 | D |
| | Respiratory | Surgery | 1 | A |
| | | | 1 | C |
| | Other | Surgery | 1 | A |
| | | | 1 | C |
| 2003 | Skin and parenchyma | Internal medicine | 1 | A |
| | | | 2 | B |
| | | | 5 | D |
| | | | 1 | E |
| | | | 1 | F |

**Table 4.** Summary of typing result of 179 MRSA strains using ERIC-PCR. *(Continuation).*

| | | | | |
|---|---|---|---|---|
| | | Surgery | 1 | D |
| | | | 1 | E |
| | Blood | Internal medicine | 2 | B |
| | Other | Surgery | 1 | A |
| | Skin and parenchyma | Internal medicine | 1 | A |
| | | | 5 | D |
| | | | 4 | G |
| | | | 1 | H |
| | | | 3 | I |
| | | | 2 | J |
| | | Gynecology and obstetrics | 1 | D |
| | | | 1 | G |
| | | Infection | 1 | A |
| | | General | 1 | G |
| 2004 | Urethra | Internal medicine | 1 | A |
| | | General | 1 | A |
| | Blood | Pediatric | 1 | G |
| | | Internal medicine | 1 | G |
| | Other | Orthopedics | 1 | H |
| | | Internal medicine | 1 | A |
| | | | 2 | D |
| | | | 1 | G |
| | | | 1 | H |
| | | | 2 | I |
| | Respiratory | Gynecology and obstetrics | 1 | I |
| | | Pediatric | 1 | I |
| 2005 | Skin and parenchyma | Internal medicine | 2 | G |
| | | | 3 | H |
| | | | 3 | I |
| | | | 4 | J |
| | | General | 1 | I |
| | Blood | Internal medicine | 1 | I |
| | | | 1 | J |
| | | | 1 | H |

**Table 4.** Summary of typing result of 179 MRSA strains using ERIC-PCR. (*Continuation*).

| | | | | |
|---|---|---|---|---|
| | | Infection | 2 | G |
| | | Pediatric | 1 | D |
| | | | 1 | H |
| | | | 1 | I |
| | | | 1 | J |
| | | Neurology | 2 | G |
| | | | 2 | H |
| | | | 3 | I |
| | Respiratory | General | 2 | G |
| | | Surgery | 1 | D |
| | | | 1 | G |
| | | | 2 | I |
| | | Orthopedics | 3 | G |
| | | | 1 | H |
| | | Internal medicine | 1 | H |
| | | | 1 | G |
| | | Gynecology and obstetrics | 1 | I |
| | Urethra | Internal medicine | 6 | G |
| | | | 1 | H |
| | | | 1 | I |
| | Skin and parenchyma | Internal medicine | 5 | G |
| | | | 5 | H |
| | | | 1 | J |
| | | Gynecology and obstetrics | 1 | G |
| | | | 2 | H |
| | | | 1 | J |
| 2006 | | Infection | 1 | I |
| | | Orthopedics | 1 | J |
| | | | 1 | G |
| | | Pediatric | 1 | H |
| | Blood | Internal medicine | 1 | I |
| | | | 1 | J |
| | Respiratory | Pediatric | 1 | G |
| | | | 1 | I |

**Table 4.** Summary of typing result of 179 MRSA strains using ERIC-PCR. (Continuation).

| | | | |
|---|---|---|---|
| | | 1 | J |
| | Infection | 2 | H |
| | Surgery | 1 | G |
| | | 2 | H |
| | | 1 | G |
| | Neurology | 2 | H |
| | | 1 | J |
| | Gynecology and obstetrics | 1 | J |
| | | 1 | G |
| | Internal medicine | 3 | H |
| Urethra | | 1 | J |
| | Orthopedics | 1 | J |

Then apply fingerprinting to genotyping 179 clinical MRSA strains with ERIC-PCR assay. The experiment results indicated that both multiplex-PCR and ERIC-PCR showed strength in rapid identification and superior sensitivity.

In conclusion, the multiplex-PCR assay proved to be an efficient and accurate tool to identify and separate staphylococci, such as MRSA, MSSA, MRCNS, MSCNS, even non-staphylococci (Fig.1). All of the 179 clinical strains were identified as MRSA (Fig.2). On the other side, fingerprinting using ERIC-PCR also showed excellent resolution to 179 clinical MRSA strains (Fig.3). Each DNA of the 179 clinical strains was amplified into 2-5 bands, ranging from 400bp- 5000bp. Based on this result, the 179 clinical MRSA strains were successfully genotyped into 10 genotypes A-J (Fig.4).

Dendrogram analysis shows the similarity between 10 types MRSA strains. Among 10 types, type I and A, J and H, E and D had a relatively high affinity while type G and F was rather low. The results showed that the 179 clinical MRSA strains resolute by ERIC-2 system did not have close affinity, which could be considered to be evolved isolated. Resulted from the resolution, the ERIC-PCR assay in this study provided a sensitive method in the classification of 179 clinical MRSA strains while traditional genotyping means may not possess such high sensitivity and accuracy.

In the long term, the fingerprinting approach can be applied on rapid genotyping, genetic similarity analysis [9], genetic tracking [13] and epidemiology research [14]. Based on the character of simple, rapid and accurate, DNA fingerprinting are supposed to be listed in the common tools for genotyping.

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# ОЦЕНЯВАНЕ НА МЕТОДА ERIC-PCR ЗА ОПРЕДЕЛЯНЕ НА ПРЪСТОВИТЕ ОТПЕЧАТЪЦИ НА МЕТИЦИЛИН-РЕЗИСТЕНТЕН ЩАМОВЕ ОТ ВИДА *Staphylococcus aureus*

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(Резюме)

Бактериите от вида *Staphylococcus aureus* (*S. aureus*) дълго са били признавани като обикновени патогени в храните. За да се изследва тяхната индивидуалност и резистентност спрямо лекарства в настоящата работа 179 стафилококови щама са подложени на изследване. *S. aureus* са потвърдени с откриването на специфични гени 16S rRNA и femA чрез мултиплексен PCR. Използван е също така молекулен метод за усилването на елементите mecA и orfX за идентифицирането на метицилин-резистентен щам *S. aureus* (MRSA). След това са представени пръстовите отпечатъци за всички щамове при използването на техниката ERIC-PCR. В този експеримент са изолирани 179 стафилококови щамове, като всички те са MRSA. Допълнително, на основание ERIC-PCR-техниката тези 179 MRSA-щама могат да се разпределят в 10 генотипа с значимо разнообразие. Чрез тези резултати е съставен нов протокол за бърза идентификация на MRSA чрез пръстови отпечатъци. Тези щамове имат широко приложение за безопасността на храните и епидемиологията.