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Full Length Research Paper

Development and application of a novel multiplex polymerase chain reaction (PCR) assay for rapid detection of various types of staphylococci strains

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In this study, a novel multiplex- polymerase chain reaction (PCR) for rapid detection of various staphylococci strains, including methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus*, (MSSA) methicillin-resistant coagulase-negative staphylococci (MRCNS), methicillin-sensitive coagulase-negative staphylococci (MSCNS) and non-staphylococci strains, had been developed and applied. Six primers were specially designed on three target genes, which were *mecA*, *16S* Ribosomal ribonucleic acid *(rRNA)* and *femA*. The specific amplification generated 3 bands on agarose gel, with sizes 374 bp for *mecA*, 542 bp for *16S rRNA* and 823 bp for *femA*, respectively. The PCR product showed highest levels of resolution of DNA when 250 µM of dNTP, primer concentration of *mecA*, *16S rRNA* and *femA* reaching 1, 1 and 3 µM respectively. No false positive amplification was observed, indicating the high specificity of the established multiplex PCR assay. Application of this multiplex-PCR had been further performed on detection for 262 MRSA and MRCNS strains with primers pairs M1 with M2 and F1 and F2. According to the results, multiplex-PCR results showed expected products for either MRSA or MRCNS strains, demonstrating the multiplex-PCR assays established in this study to be useful and powerful methods for differentiation of MRSA, MSSA, MRCNS, MSCNS and non-staphylococci strains.

Key words: *Staphylococcus,* multiplex-PCR, rapid detection.

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. These bacterium, are carried, mostly transiently, by approximately 20 and 30% of healthy adults on the skin and anterior nares, respectively. Over 30 different types of staphylococci are infectious for humans, and its related illness can range from mild to severe, from no treatment required to even potentially fatal. Most of these infections are caused by *Staphylococcus aureus*, which has been regarded as leading issues both in medicine and food safety, and can typically causes a wide variety of infections, including skin infections and sometimes pneumonia, endocarditis, osteomyelitis, gastroenteritis, scalded skin syndrome and toxic shock syndrome (Xu et al., 2008b; 2011b, c). Coagulase-negative staphylococci (CoNS) are regarded as a frequent cause of nosocomial infection and bacteremia, especially in patients with indwelling medical devices (Ben-Ami et al., 2003). CoNS have also become the most frequently isolated pathogens in intravascular catheter related infections (CRI),

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accounting for an estimated 28% of all nosocomial bloodstream infections (Nouwen et al., 1998). Indiscriminate use of existing antibiotics contributes to proliferation of antibiotic resistance and poses a dilemma for the treatment of several bacterial infections, including therapy for individuals with food poisoning. Antibiotic resistance in microbes still remains one of the global major concerns in public health, with methicillin-resistant staphylococci (MRS) strains representing one important group, commonly referred to as "Super Bugs" (Xu et al., 2011a).

Since the first discovery in 1961, methicillin-resistant *Staphylococcus aureus* (MRSA) has become one of the most prevalent pathogens that cause nosocomial infections throughout the world. As this pathogen can spread easily by either direct or indirect contact between patients and environment, or via patients and medical personnel, it is considered to be an important risk factor for nosocomial infection, which continues to be a challenge for clinicians, hospital epidemiologists and administrators (Chang et al., 1997). Methicillin resistance in staphylococci is caused by PBP2a protein encoded by the *mecA* gene. The *mecA* gene is located on a mobile genetic element, designated as staphylococcal cassette chromosome *mec* (SCC*mec*), which contains the *mec* gene complex (the *mecA* gene and its regulators) and the *ccr* gene complex encoding site-specific recombinases responsible for the mobility of SCC*mec* (Katayama et al., 2000). MRCNS, which are more frequent carriers of SCC*mec* than MRSA, have been postulated to be the reservoir for the transfer of methicillin resistance to *S. aureus*. One assumes that the *ccr* and *mec* genes were bought together in CoNS from an unknown source, where deletion in the *mec* regulatory genes occurred, before the genes were transferred into *S. aureus* (Hanssen et al., 2004). As one example of the leading "Super Bugs", MRS strains show resistance to practically all β-lactam antibiotics and usually other multiple drugs due to the *mecA* and associated resistance genes carried by SCC*mec*, respectively (Schito 2006). China remains one of the worst areas for antibiotics abuse, with an estimate annual consumption of 140 gram per person, which is 10 times higher than that in the United Kingdom and the United States. General concerns for the threaten of unleashing waves of "Super Bugs" in China raised necessity for surveillance and investigation on antibiotic resistance mechanisms involved in clinical MRSA and MRCNS strains. Routine culture-based diagnostic detection and identification procedure for potentially pathogenic *Staphylococcus* strains includes: enrichment and enumeration in liquid media, subsequent recovery and isolation of colonies on selective culture broth such as Baird-Parker agar for 24 to 48 h at 37°C, followed by DNase or coagulase assays for suspicious colonies and further confirmation by biochemical tests. However, the lengthy recovery time to identify microbes at the species level (6 days), false negative results due to bacterial starvation and physical stress, as well as insufficient sensitivity have raised concerns for these conventional methodologies (Alarcon et al., 2006).

During the past decades, a number of PCR and real-time quantitative PCR (RQ-PCR) based assays have been employed and proposed for rapid detection of *Staphylococcus*. However, disadvantages for PCR (time required by post determination for each detection assay, high risk of cross contamination and low detection limit levels) and real-time PCR (requirement for trained personnel, operating space, expensive equipment and reagents) posed significant obstacles for their broad application. Increased awareness for the risk and hazard of MRSA and MRCNS strains and demands for tests capable of early, cost-effective, timely, and sensitive detection of staphylococci and associated antibiotic resistance determinants has made these tests an urgent necessity. This study aimed at developing and evaluating a rapid and flexible testing method based on multiplex-PCR assays for differentiation of MRSA, MSSA, MRCNS, MSCNS and non-staphylococci strains, and applying these assays to detection of a large scale of MRSA and MRCNS strains from various clinical samples.

MATERIALS AND METHODS

Bacterial strains

Five reference strains, including MRSA ATCC29212 and 85/2082 (with *mecA*, *femA* and *16S rRNA* positive), MSSA ATCC25923 (with *femA* and *16S rRNA* positive, and *mecA* negative), MRCNS ATCC700586 (with *mecA* and *16S rRNA* positive, and *femA* negative), MSCNS ATCC12228 (with *16S rRNA* positive, and *mecA* and *femA* negative) and *Escherichia coli* ATCC25922 (with *mecA*, *femA* and *16S rRNA* negative), were subjected to evaluation and optimization of multiplex-PCR assay. The optimized multiplex-PCR assay was further performed on a total of 262 various types of *Staphylococcus* isolates, including 209 MRSA and 53 MRCNS strains. These strains were isolated from various clinical samples, which had been preliminarily described (Xu et al., 2007; 2008a; 2009; 2010) or identified in the Laboratory of Clinical Microbiology, Zhongshan Supervision Testing Institute of Quality and Metrology.

Culturing condition and Template deoxyribonucleic acid (DNA) preparation

Cultural conditions and DNA extraction of Gram-positive and negative strains were performed as described previously (Xu et al., 2007; 2008a; 2009; 2010). In brief, Template DNA from *Staphylococcus* strains used for PCR were prepared from overnight Luria-Bertani (LB) broth cultures at 37°C with shaking. The culture was diluted 10-fold in 10 mM Tris-HCl (pH 8.0) containing 1 mM Ethylenediaminetetraacetic acid (EDTA) and the suspension was boiled for 10 min and kept on ice. After centrifugation at 12,000 g for 3 min, the resulting supernatant was used as templates for PCR amplification.

Primer design

Three targets were selected to differentiate MRSA, MSSA, MRCNS, MSCNS and non-Staphylococci strains. The protocol was designed

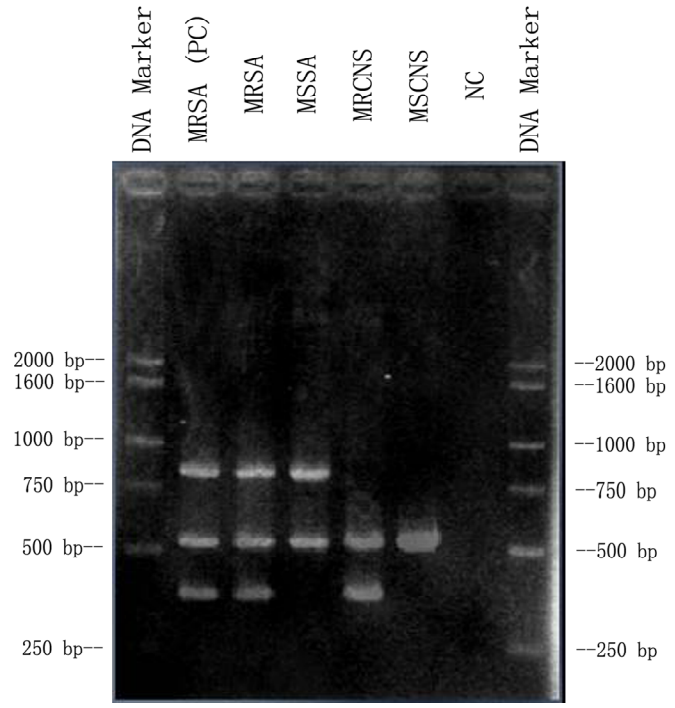

Figure 1. Multiplex-PCR assays for differentiation of MRSA, MSSA, MRCNS, MSCNS and non-staphylococci strains.

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 amplification of the *mecA* gene (M1: 5'-GGCATCGTTCCAAAGAATGT-3' and M2: 5'-CCATCTTCATGTTGGAGCTTT-3', with an expected 374 bp amplicon).

Establishment of the multiplex-PCR assay

Six reference strains were used to develop and evaluate the multiplex-PCR assay, which was carried out in a total of 25 µl reaction mixture. Primer concentration for had been selected as 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 µM (each) of the primers, and dNTP concentration included 100, 150, 200, 250, 300 and 350 µM. 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 min and a final extension cycle at 72°C for 7 min. Eight annealing temperature, including 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C and 55°C were subjected to optimize the PCR assay. The amplified products (5 µl/well) were analyzed by gel electrophoresis in 2% agarose gels and stained with ethidium bromide for 10 min. A negative control was performed using sterile water instead of culture or DNA template.

Cloning and DNA sequencing of *16S rRNA*, *femA* and *mecA*

The PCR products of *16S rRNA*, *femA* and *mecA* were cut out from the agarose gel, purified by the QIAquick Gel Extraction kit (Qiagen, Hilden and Germany) and ligated with the pGEM-T easy vector (Promega, Madison, WI and USA). The ligation mixture was transformed into *E. coli* DH5α strain and the recombinants were selected on LB agar containing ampicillin (100 µg/ml). Recombinant plasmid DNA was purified by standard method and subjected for DNA sequencing for further analyses. The nucleotide sequences of gene cassette were determined by Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit on ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Japan Applied Biosystems, Tokyo and Japan). Nucleotide sequence homology searches were performed against all sequences in the GenBank database by using the BLAST algorithm, which is available through the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

Application of the multiplex-PCR assay on clinical strains

Three hundred and ten clinical staphylococci isolates, including 209 MRSA and 53 MRCNS strains were subjected to detection by the established multiplex-PCR assay with primers pairs F1 with F2 and M1 with M2, and PCR amplicons were evaluated by electrophoresis as aforementioned. These experiments were replicated to ensure reproducibility.

RESULTS AND DISCUSSION

Optimization of the multiplex-PCR

In order to determine the optimal condition, DNA from MRSA 85-2082 was used as target template. The specific amplification generated 3 bands on agarose gel, with sizes 823 bp for *femA*, 542 bp for *16S rRNA* and 374 bp for *mecA*, respectively. The PCR product showed highest levels of resolution of DNA when 250 µM of dNTP, primer concentration of *mecA*, *16S rRNA* and *mecA* reaching 1, 1 and 3 µM (1:1:3) and amplification at an annealing temperature of 50°C when compared to other conditions (data not shown). MRSA ATCC29212 and 85/2082 yielded specific amplification for *mecA*, *femA* and *16S rRNA*, MSSA ATCC25923 showed positive result for *femA* and *16S rRNA*, MRCNS ATCC700586 was detected to carry *mecA* and *16S rRNA*, MSCNS ATCC12228 had been found to be *16S rRNA* positive, while *E. coli* ATCC25922 was observed to be negative for all the three targets (Figure 1).

Application of multiplex-PCR on a large scale of clinical strains

Two hundred and sixty-two clinical MRSA and MRCNS strains had been subjected to the application of the multiplex-PCR detection using primers F1 with F2 and M1 with M2. Multiplex-PCR and subsequent detection by electrophoresis were performed as described previously. All 209 MRSA strains yielded 2 bands as 374 bp and 823

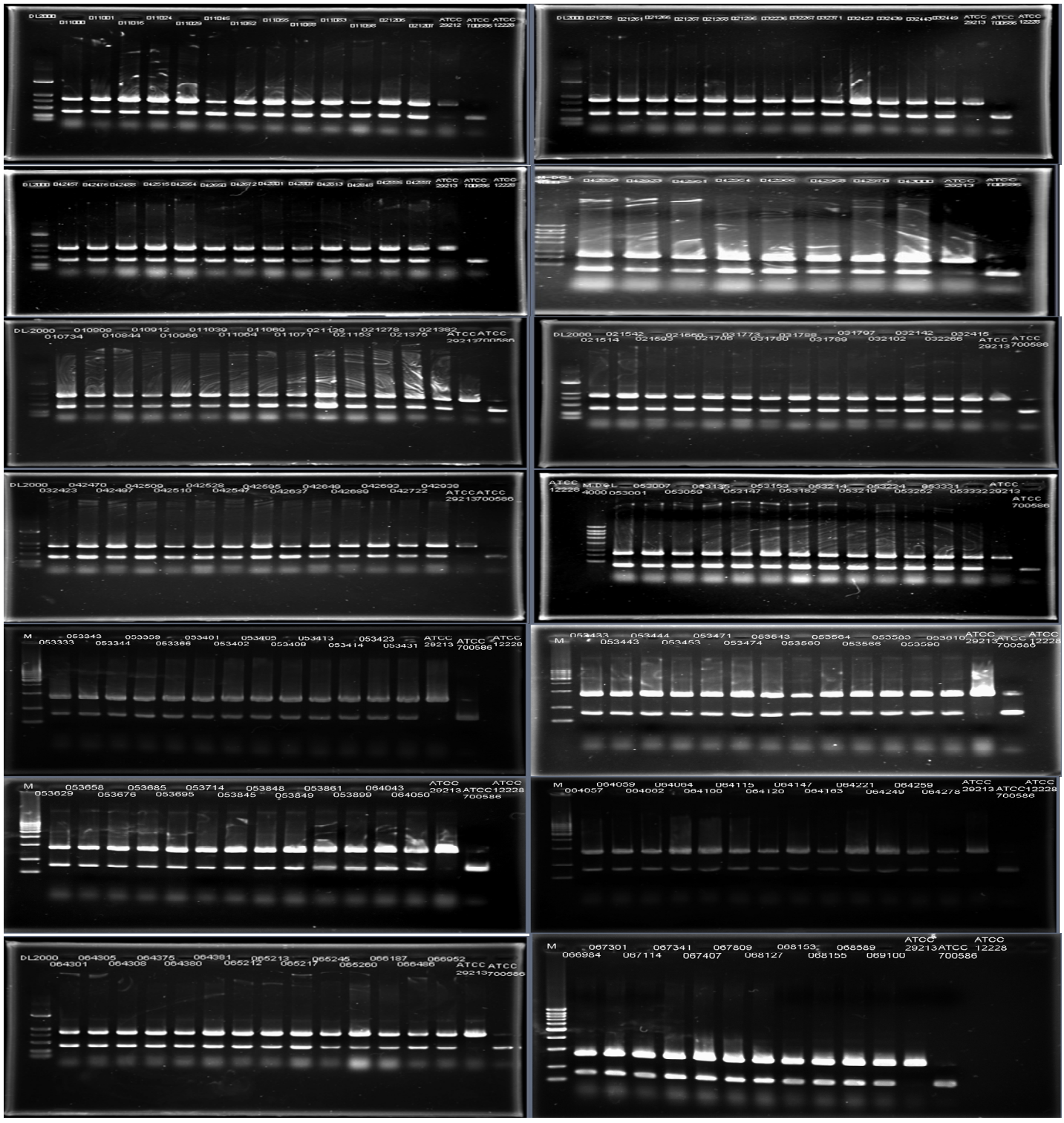

Figure 2. Application of multiplex-PCR assays on a large scale of MRSA strains.

bp amplicon, corresponding to *mecA* and *femA*; for 53 MRCNS strains, all isolates had been detected positive for *mecA* and negative for *femA*. No false positive amplification was observed, indicating the high specificity of the established multiplex PCR assay (Figure 2).

Conclusion

Staphylococcus is currently widespread pathogens throughout the world and has prompted a heightened interest and concern for the rapid detection of these

pathogens as well as its related antibiotic resistance determinant. Thus, rapid and accurate detection approaches are needed to reduce risk of nosocomial infection caused by *Staphylococcus*. This study aimed to establish simple and rapid testing methods based on multiplex-PCR assays for differentiation of MRSA, MSSA, MRCNS, MSCNS and non-staphylococci strains. The optimal condition for multiplex-PCR was detected to be 250 µM of dNTP, primer concentration of *mecA*, *16S rRNA* and *femA* reaching 1, 1 and 3 µM (1:1:3) and amplification at an annealing temperature of 50°C when compared to other conditions. No false positive amplification was observed, indicating the high specificity of the established multiplex PCR assay.

In conclusion, this established multiplex-PCR assay was demonstrated to be useful and powerful tools for the rapid detection of various *Staphylococcus* strains, with advantages on the extension and flexibility in application to either separate detection of staphylococci, *S. aureus* and methicillin-resistance or combined use for differentiation of MRSA, MSSA, MRCNS, MSCNS and non-staphylococci. Nevertheless, implementation of multiplex-PCR assay to routine clinical laboratory diagnoses requires accumulation of data on clinical samples and the validation and connection with current procedures. Further investigation should focus on the application to a large scale of clinical samples and comparative sensitivity and specificity with current culture- or PCR-based approaches.

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Abbreviations: MRSA, Methicillin-resistant *staphylococcus aureus;* **MRCNS,** methicillin-resistant coagulase-negative staphylococci; **MSCNS,** methicillin-sensitive coagulase-negative staphylococci; **MSSA,** methicillin-sensitive *staphylococcus aureus;* **PCR,** polymerase chain reaction; ***rRNA,*** ribosomal ribonucleic acid; **CoNS,** coagulase-negative staphylococci; **CRI,** catheter related infections; **MRS,** methicillin-resistant staphylococci; **SCC*mec*,** staphylococcal cassette chromosome *mec*; **RQ-PCR,** real-time quantitative polymerase chain reaction; **DNA,** deoxyribonucleic acid; **LB,** luria-bertani; **EDTA,** ethylenediaminetetraacetic acid; **NCBI,** national center for biotechnology information.

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