DEVELOPMENT OF MULTIPLEX PCR FOR DETECTION OF METHICILLIN RESISTANCE AND MLS_B RESISTANCE GENES IN *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Nosocomial infection caused by multidrug resistant staphylococci such as methicillin or macrolide-lincosamide-streptogramins B (MLS_B) resistance is a growing problem for many health care institutions. Of all species of staphylococci, *Staphylococcus aureus* (*S. aureus*) has the greatest pathogenic potential. Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are associated with increased morbidity and mortality. Rapid identification of the organism as well as drug resistance is crucial for infected patients. Therefore, in this study, a new multiplex PCR for identification of *S. aureus* and detection of certain antimicrobial resistant genes was developed, in order to improve the speed of detection from suspected bacterial colonies. New specific primers of five target genes, *fem*A (for *S. aureus* identification), *mec*A (for oxacillin or methicillin resistance), *erm*A, B and C (for MLS_B resistance) were designed and tested against 250 clinical isolates of *Staphylococcus* spp. comparing with the conventional identification and susceptibility test in routine process. All five primers revealed 100% specificity to their target genes and the results showed 100% concordance with the phenotypic testings. All methicillin resistant isolates contained the *mec*A gene. The prevalence of the *erm*A, *erm*B and *erm*C genes in erythromycin resistant *S aureus* were 53.4%, 1.1% and 12.6% respectively while prevalence of the *erm*A, *erm*B, and *erm*C in coagulase negative staphylococci (CoNS) were 6.6%, 1.3%, and 46.1% respectively . In contrast to *S. aureus*, the *erm*C was found to be predominated among erythromycin resistant CoNS isolates. With this specific multiplex PCR, the identification of *S. aureus* and CoNS as well as two groups of most important resistant genes could be performed rapidly and reduced approximately 24 hr of turnaround time comparing to the routine process.

KEY WORDS: METHICILLIN RESISTANCE AND MLS_B RESISTANCE / MULTIPLEX PCR

67 pages

การพัฒนาชุดการตรวจวิเคราะห์เชื้อ *Staphylococcus aureus* ที่ดื้อต่อยา Methicillin และ ยีนดื้อยา MLS_B โดยวิธี Multiplex PCR

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บทคัดย่อ

การติดเชื้อในโรงพยาบาลโดยเชื้อกลุ่ม Staphylococci ที่ดื้อยาหลายขนานเช่น Methicillin หรือ macrolide-lincosamide-streptograminsB (MLS_B) เป็นปัญหาที่เพิ่มขึ้นในสถานพยาบาลจำนวนมาก โดยในบรรดาสายพันธุ์ของ Staphylococci ทั้งหมด *Staphylococcus aureus* (*S. aureus*) มีความสามารถในการก่อโรคได้มากที่สุด การติดเชื้อกลุ่ม Methicillin-resistant *Staphylococcus aureus* (MRSA) ก่อให้เกิดพยาธิสภาพและอัตราการเสียชีวิตที่สูง การตรวจวินิจฉัยเชื้อและการทราบการดื้อยาที่รวดเร็วจึงมีความสำคัญต่อผู้ป่วยเป็นอย่างมาก ดังนั้นการศึกษานี้จึงได้พัฒนาการตรวจเพื่อหาเชื้อ *S. aureus* และ ยีนดื้อยาของเชื้อดังกล่าวโดยวิธี Multiplex PCR เพื่อให้เกิดความรวดเร็วยิ่งขึ้นในการตรวจวิเคราะห์จากโคโลนีของเชื้อที่เพาะเลี้ยงได้ การทดสอบนี้ใช้ Primer ที่จำเพาะต่อ ยีนเป้าหมายจำนวน 5 ยีน ได้แก่ *fem*A (สำหรับการวิเคราะห์ *S. aureus*), *mec*A (สำหรับวิเคราะห์ยีนดื้อยา oxacillin หรือ methicillin), *erm*A, B และ C (สำหรับวิเคราะห์ยีนดื้อยา MLS_B) การทดสอบได้ใช้เชื้อ Staphylococcus Spp. จากตัวอย่างผู้ป่วยจำนวน 250 ตัวอย่าง เปรียบเทียบผลกับการตรวจวิเคราะห์โดยวิธีเพาะเชื้อและการทดสอบความไวต่อยาในงานประจำของห้องปฏิบัติการ ซึ่งจากการทดลองพบว่า ทั้ง 5 primers ที่สร้างขึ้นมีความจำเพาะต่อยีนเป้าหมาย และแสดงผลไปในทิศทางเดียวกับการวิเคราะห์ทางห้องปฏิบัติการ 100% เชื้อที่ที่มีการดื้อยา methicillin ทั้งหมด สามารถตรวจพบยีน *mec*A นอกจากนี้เชื้อ S. aureus ที่ดื้อยา erythromycin ตรวจพบยีนดื้อยาได้แก่ *erm*A, *erm*B *erm*C จำนวน 53.4%, 1.1% และ 12.6% ตามลำดับ ในขณะที่เชื้อ กลุ่ม coagulase negative staphylococci (CoNS) ตรวจพบเฉพาะยีน *erm*A 6.6%, *erm*B 1.3%, and *erm*C 46.1% โดยตรวจพบยีน ermC มากที่สุด และด้วยวิธี Multiplex PCR ที่พัฒนาขึ้นใหม่นี้ ทำให้สามารถลดเวลาของการตรวจวิเคราะห์ *S. aureus* และ CoNS ลงได้ประมาณ 24 ชั่วโมง เมื่อเทียบกับการตรวจวิเคราะห์โดยการเพาะเชื้อและทดสอบความไวต่อยาทางห้องปฏิบัติการในปัจจุบัน

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LIST OF ABBREVATIONS

%	Percent
bp	Base pair (s)
CFU/ml	Colony forming unit/ milliliter
CLSI	Clinical Laboratory Standards Institute
°C	Degree Celsius
DNA	Deoxyribonucleic acid
h	Hour (s)
ICU	Intensive Care Unit
mm	Millimeter
n	Number
ng	Nanogram
pmol	Picomol
µg	Microgram
µl	Microliter
µm	Micrometer or micron

CHAPTER I
INTRODUCTION

There are several types of *Staphylococci* that can infect humans, but most infections are caused by *Staphylococcus aureus*. The organism can be found in nose as well as on skin. Therefore, many cases of *S. aureus* infection occur either at or via the skin. (1, 2). *Staphylococcus aureus* is considered as an important cause of serious infections in community-acquired and hospital-acquired. Treatment of these infections has become more difficult because of the emergence of multidrug-resistant strains. Only limited antibiotics are available for treatment. (3).

Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates have been associated with nosocomial infections and rapidly developed resistance to multiple drug classes. The major transmission of MRSA is impermanent colonization of the hands of health care workers (4). The MRSA infections are associated with prolonged hospitalizations. Risk factors associated with MRSA infection include patient in long-term care facilities, prior antibiotic exposure, insulin dependent diabetes, urinary catheterization, nasogastric tube placement, prior surgery, patients undergoing hemodialysis, having an underlying disease, and HIV-infected patients. Moreover, mortality rates for patients that develop MRSA bacteremia range between 20–40% (3, 5-8). The methicillin-resistance determinant, *mec*A, encodes PBP-2a. PBP-2a has low affinity for binding β-lactam antibiotics (9-11). Over the years, MRSA has gained multiple mechanisms of resistance to several classes of antimicrobials, including macrolides, aminoglycosides, fluoroquinolones, tetracyclines, and lincosamide antibiotics such as clindamycin (6, 12). Among those, the macrolide resistance in combination with wide clindamycin and/or streptogramin B has been more recognized worldwide in clinical isolates of gram-positive bacteria (mainly *staphylococc*i and *streptococci*). The main causes of resistant based on two mechanisms, the modification of the ribosomal target site encoded by a variety of *erm (erythromycin ribosome methylase)* genes (*erm*A, *erm*B, and *erm*C). These *erm* genes result in

resistance to macrolides, lincosamides and streptogramins B (MLS_B phenotype). The resistance of *msr*A gene is encoded by ATP-dependent efflux pump mechanism and mediated resistance mechanism is responsible for resistance to macrolides and streptogramin B only (MS phenotype). Another resistance mechanism, inactivation appears to be rare (13-15). Macrolide resistance in *Staphylococcus aureus* may be inducible or constitutive and the former can be detected by D-test (3).

Routine antimicrobial susceptibility testing according to The Clinical and Laboratory Standards Institute (CLSI), disk diffusion or broth microdilution were recommended for detection of methicillin resistance and MLS_B resistance. However, the accuracy of the results by these two methods rely on proper techniques as well as the expression level of resistant genes (14). It is also time consuming which requires at least 24 hours for the result. The delay of the test result directly effects to the treatment of infected patients (10).

Molecular detection of specific target genes has become widely used in microbiology field. This technique provides the result rapidly and accurately. There are several molecular methods such as PCR, or another nucleic acid amplification method. Variants of the PCR technique have been proposed, in order to amplify and detect more than one gene in the same reaction. The use of multiplex PCR can provide the solution for detection of various target genes in the same reaction. Therefore, in this study the multiplex PCR was selected as a tool for detection of *S. aureus* and its resistant genes.

CHAPTER II
OBJECTIVES

To develop multiplex PCR for detection of methicillin resistance and MLS_B resistance *S. aureus* using newly design primers.

CHAPTER III
LITERATURE REVIEW

3.1 *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive coccus. Microscopically, *S. aureus* has the size of 0.5- to 1.5-μm and arranges as single, pair, tetrad or cluster, non-motile, non-spore forming, and facultative anaerobe. The organism produces coagulase enzyme which is used as a signature of this species. Apart from coagulase, *S. aureus* also produces various enzymes including staphylokinase (sak), nuclease, hyarulonidase, etc. It can be found in human, animals and environment. *S. aureus* carriers are at higher risk of infection, and they are presumed to be an important source of the *S. aureus* strains that spread among individuals. The pathogen can cause a wide variety of infections and able to survive inside host cells (16). This organism is known to be a major cause of skin and soft tissue infection. Certain strains of *S. aureus* may produce toxins such as enterotoxin causing food poisoning, exofoliative (epidermolytic) toxin causing scald skin syndrome (SSS), or septic shock syndrome toxin-1 (TSST-1) which is a life threatening toxin causing septic shock in patients. This makes *S. aureus* being the most significant pathogen among staphylococci (17, 18). Invasive *S. aureus* related conditions most commonly reported include septic shock (56%), pneumonia (32%), endocarditis (19%), bacteremia (10%), and cellulitis (6%) (19). In addition to various virulent factors of *Staphylococcus aureus*, the organism is a potent pathogen developing resistance to antibiotics which lead to difficulty and treatment failure (19).

3.2 Identification of *Staphylococcus aureus*

The infection of *S. aureus* is the most common problem in the hospital. This has warranted the establishment of rapid and reliable methods for screening and

confirmation. The detection methods can be divided into groups including phenotype and molecular methods.

3.2.1 Gram strain

Staphylococcus aureus is gram-positive cocci, approximately 0.5 – 1.0 μm in diameter and grow in clusters (20).

3.2.2 Isolation and Identification

The organism is isolated by streaking material from the clinical specimen onto solid media such as blood agar, tryptic soy agar or heart infusion agar. The organism grows well on plain media. Colonies of *S. aureus* are round, opaque, white to golden yellow. On blood agar, *S. aureus* produces beta-hemolysis. Specimens likely to be contaminated with other microorganisms can be plated on mannitol salt agar containing 7.5% sodium chloride, which allows the halo-tolerant staphylococci to grow (20).

3.2.3 Catalase Test

The catalase test is important in staphylococci which are catalase positive. The test is performed by drops of 3% hydrogen peroxide onto suspected colonies. This test can be performed either flooding red blood cell-free media or broth culture with several. Catalase-positive cultures bubble at once. The catalase should not be tested on blood agar due to false positive result from catalase in red blood cells (20). The enzyme catalase, differentiates catalase positive staphylococci from catalase negative streptococci and enterococci (21).

3.2.4 Tube coagulase test

The tube coagulase test with rabbit plasma and examination of tubes after incubation for 4 h and 24 h is the standard test for routine identify of *S. aureus*. Tests negative at 4 h should be re-incubation at 24 h because a small proportion of strains require longer than 4 h for clot formation. Some other species of staphylococci, including *Staphylococcus schleiferi* and *Staphylococcus intermedius*, may also give positive results in tube coagulase tests but are not common isolates from human

infections. In addition, rare strains of *S. aureus* are negative in coagulase tests. For routine testing more rapid tests are now widely used, particularly latex agglutination tests. *S. aureus* can be confirmed by testing colonies for agglutination with latex particles coated with immunoglobulin G and fibrinogen which bind protein A and the clumping factor, respectively, on the bacterial cell surface. In addition, any test including clumping factor may give false-positive results with *S. lugdunensis* and *S. schleiferi.*(20, 22).

3.2.5 DN*ase* and heat-stable nuclease tests

Deoxyribonuclease (DNase) plates can be used to screen isolates but, as various amounts of DNase are produced by CoNS, positives should be confirmed with an additional test. Heat-stable nuclease tests can be used to identify *S. aureus*, although some rare coagulase-negative species can be positive. However, they can be identified to a species specific by their characteristic pattern of biochemical test (Figure 3.1).

3.2.6 Latex agglutination tests

Latex agglutination tests for *S. aureus* detected protein A and/or clumping factor. These tests had problems with some MRSA which produce little or no clumping factor and protein A. In addition, any test including clumping factor may give false-positive results with *S. lugdunensis* and *S. schleiferi* (23).

3.2.7 Commercial biochemical tests

There are many commercial kits and automated instruments which include identification of *S. aureus.* While performance of these tests may be good, they are slower, technically more time consuming or more expensive than tests (22).

3.3 Antimicrobial susceptibility test of *Staphylococcus aureus*

Antimicrobial susceptibility test of *S. aureus* for detect the antibiotic resistance was determined by standard disk diffusion and broth microdilution tests

were performed according to the Clinical and laboratory Standard Institute (CLSI). Disk diffusion tests were set up from a bacterial suspension equivalent to 0.5 McFarland in normal saline was prepared and used for inoculation of Mueller-Hinton agar plates and zone of inhibition was determined after 16–18 h incubation at 35 °C. The results were interpreted according to CLSI guidelines (Table 3.1) (24).

Table 3.1 Zone diameter interpretive standards for *Staphylococcus aureus* (24).

Antimicrobial agent	**Zone diameter interpretive criteria (nearest whole mm.)**		
	R	**I**	**S**
Pennicilin 10 unit	≤ 28	-	≥ 29
Cefoxitin 30 µg	≤ 21	-	≥ 22
Teicoplanin 30 µg	≤ 10	11-13	≥ 14
Erythromycin 15 µg	≤ 13	14-22	≥ 23
Clindamycin 2 µg	≤ 14	15-20	≥ 21
Levofloxacin 5 µg	≤ 15	16-18	≥ 19
Ciprofoxacin 5 µg	≤ 15	16-20	≥ 21
Chloramphenical 30 µg	≤ 12	13-17	≥ 18
Trimethoprim sulfamethoxazone 1.25/23.75 µg	≤ 10	11-15	≥ 16
Tetracyclin 30 µg	≤14	15-18	≥ 19
Linezolid 30 µg	≤ 20	-	≥ 21
gentamicin 10 µg	≤ 12	13-14	≥ 15

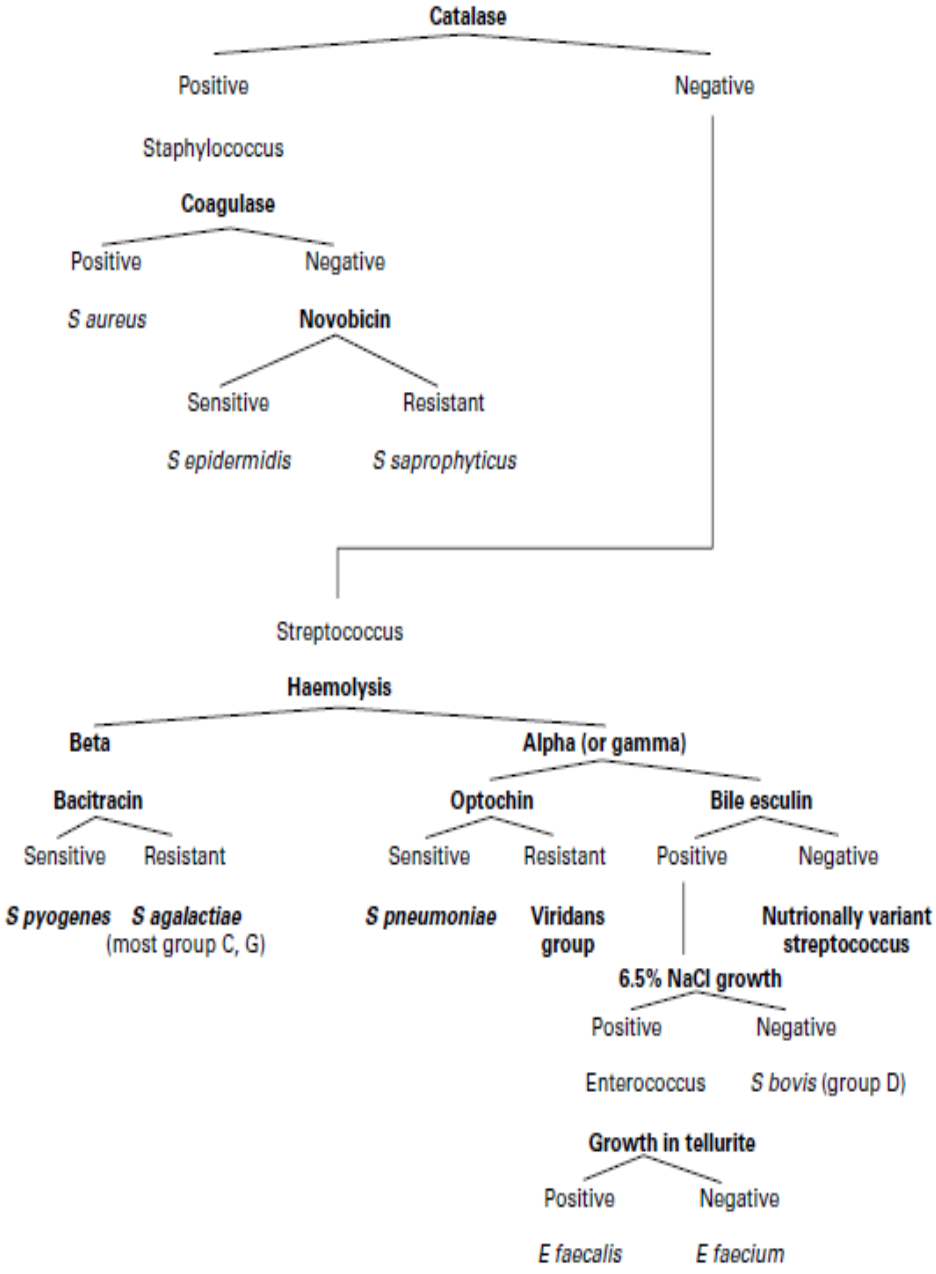

Figure 3.1 Biochemical tests for identified *Staphylococcus aureus* (21).

3.4 Methicillin-resistant *Staphylococcus aureus* (MRSA) and mechanism of resistance

Methicillin was introduced in 1959 to treat infections caused by penicillin resistant *Staphylococcus aureus*. The therapeutic outcome of infections that result from MRSA is worse than the outcome of those that result from methicillin-sensitive strains. In 1961, *S. aureus* isolates that had acquired resistance to methicillin (methicillin-resistant *S. aureus*, MRSA) was reported from the United Kingdom (25). This multidrug resistant pathogen has played an important role amongst hospital-acquired infection or patients who have stayed in long-term health care facilities (12). Prevalence of MRSA infection varies greatly according to geographic regions ranging from less than 5% in some European countries to over 50% in the United States and Latin American countries as well as some Asian countries (Figure 3.2) (12, 19).

3.4.1 Mechanism of methicillin-resistant *Staphylococcus aureus* (MRSA)

Methicillin resistance in *S. aureus* is primarily mediated by the *mec*A gene. By alteration of penicillin binding proteins 2 (PBP2), the new PBP2, so called PBP2a, differs from regular PBPs in its active site allowing low affinity binding of all β-lactams antibiotics, including cephalosporins (13-16).

The *mec*A gene is part of a 21 to 60 kb and carried on a specific integrative genetic element known as the staphylococcal cassette chromosome (SCC), a mobile genetic element that may also contain genetic structures (7, 15, 21, 26). Five SCC*mec* types have been identified for *S. aureus*. The gene elements differ in size, composition, and associated antimicrobial resistance expression.

In particular, the *mec*A gene encodes for PBP2a when the methicillin arrives, *mec*R1 gene for membrane-bound signal transduction protein (*mec*R1 protein) and *mec*I gene for a transcriptional regulator. Therefore, PBP2a confers resistance by contributing to the function of native PBPs during cell wall synthesis (8). There is no *mec*A homologue in susceptible strains. The MRSA can be classified according to where the infection was acquired: hospital-acquired MRSA (HA-MRSA) or community associated MRSA (CA-MRSA) (27).

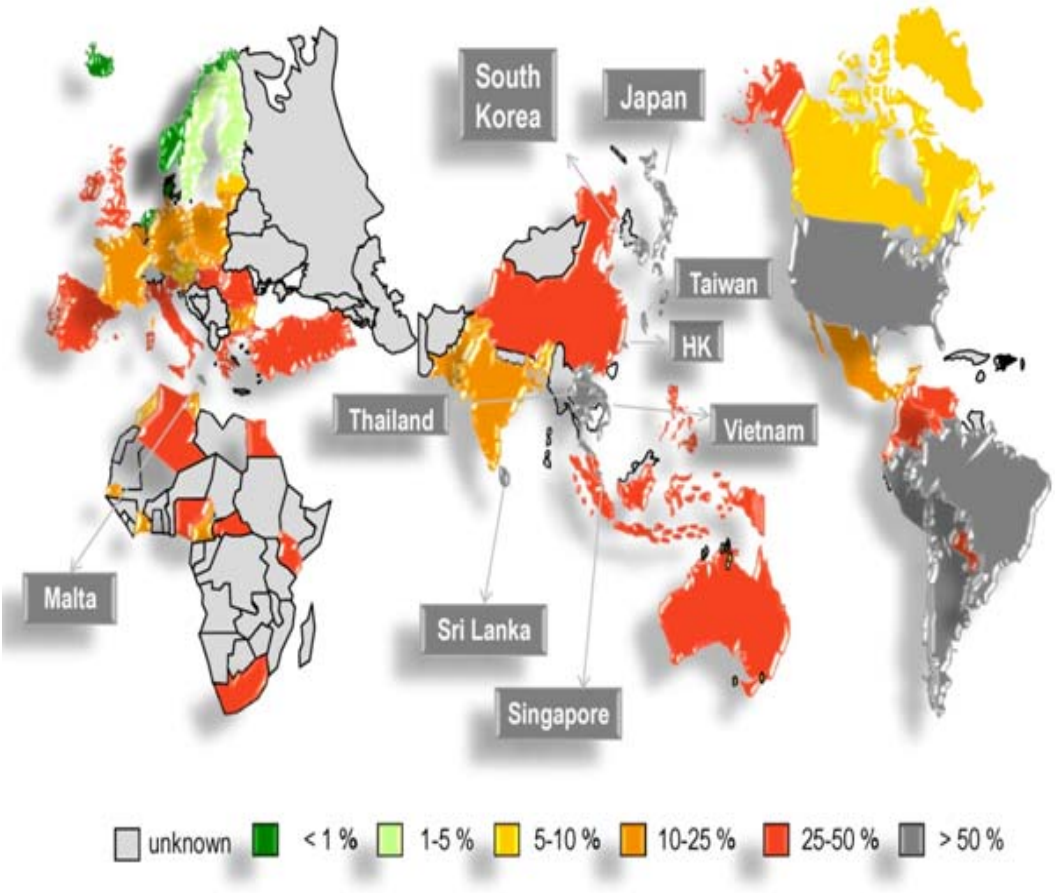

Figure 3.2 Worldwide prevalence of hospital-acquired meticillin-resistant *Staphylococcus aureus* (2012) (28). Prevalence of MRSA infection varies greatly according to geographic regions. The highest rates (>50%) are reported in North and South America, Asia and Malta. Intermediate rates (25–50%) are reported in China, Australia, Africa and some European countries (e.g. Portugal (49%), Greece (40%), Italy (37%) and Romanian (34%). Other European countries have generally low prevalence rates.

3.4.2 Community-acquired (CA) and Hospital-acquired (HA) MRSA

Community-acquired MRSA (CA-MRSA) infections have been reported worldwide, including several outbreaks in the United States. Transmission has occurred by close physical contact in situations involving children in day-care centers, athletes, military personnel, correctional facilities. Of concern, these patients are otherwise healthy individuals and persons who have not been hospitalized recently or had a medical procedure. Conversely, Hospital-acquired (HA-MRSA) infection was patient in

long-term health care facilities, have comorbidities (such as diabetes), and are on dialysis, have prolonged hospitalization, and are ICU patients. Community isolates tend to be susceptible to a variety of non beta-lactam antibiotics, and frequently carry Panton-Valentine leucocidin (PVL) genes. Whereas, HA-MRSA are typically resistant to multiple antibiotics, and seldom carry the genes for the PVL gene (19).

The molecular analysis, SCC*mec* type IV (resistant to beta-lactam antibiotics) is typically found in CA-MRSA strains. This SCC*mec* type is smaller in size and lacks other multidrug-resistance genes. HA-MRSA strains carry a relatively large staphylococcal chromosomal cassette *mec*. The SCC*mec* types I, II, and III are found predominatingly in HA-MRSA isolates. The SCC*mec* types II and III are responsible for the multiple non beta-lactam antimicrobial resistance often expressed in these HA-MRSA related strains. (6). However, Both HA-MRSA and CA-MRSA are usually susceptible to trimethoprim/sulfamethoxazole, but are resistant to erythromycin. While HA-MRSA is usually resistant to fluoroquinolones, susceptibility of this drug class among CA-MRSA strains is various in different geographic regions (12). The difference between CA-MRSA with HA-MRSA is shown in table 3.1.

Table 3.2 Differences between CA-MRSA and HA-MRSA (29).

HA-MRSA	DIFFERENCES	CA-MRSA
Panton Valentine gene (rare 5%), Staphylococcal cassette chromosome Types I, II,III (most common USA100, USA200)	**Genetic traits**	Panton Valentine gene (almost 100%), Staphylococcal Cassette chromosome IV(most common USA300, USA400)

Table 3.2 Differences between CA-MRSA and HA-MRSA (29). (cont.)

HA-MRSA	DIFFERENCES	CA-MRSA
Blood stream, Surgical site, Site of implant	**Area affected**	Skin (boil, and/or red, swollen, painful area), Lungs
Immunocompromised Residency in long term care facilities Recent hospitalizations Dialysis patients	**Who is affected**	Young, otherwise healthy patients (most common) No recent hospitalizations
Recent surgery		Anyone
Many agents	**Antibiotic resistance**	Some agents

3.4.3 Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* (LA-MRSA)

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) of the clonal complex (CC) 398 became primarily known as colonizers of livestock animals. The CC398 was first identified in pigs and swine workers in a number of countries in Europe, Asia, and North and South America, as well as Australia. The LA-MRSA CC398 has no clearly host specificity and can colonize or infect other animals (including cattle, poultry, and dogs as well as humans), and has led to a reexamination of the idea of host specificity in *S. aureus*.

Staphylococcus aureus may be transmitted to humans from meat products by handling of contaminated products or by the cross contamination of household surfaces. While antibiotic use on farms may drive selection of antibiotic-resistant strains of *S. aureus* that eventually end up in meat products, eliminating consumer exposure to such bacteria is not as straightforward as simply purchasing products raised in an antibiotic-free environment. Additionally, antibiotic resistance genes, including *mec*A and *mec*C, have been suggested to have an animal origin.

Nonetheless, the majority of reported infections with CC398 strains appear to be similar in scope to community-associated *S. aureus* strains, causing skin and soft tissue infections and, more rarely, serious invasive infections and death (30-32).

3.4.4 *mec*A gene

The specific genetic mechanism of its resistance has been identified as a mobile genetic element (staphylococcal cassette chromosome *mec,* SCC*mec*) integrated into the *S. aureus* chromosome, within which the *mec*A gene encodes a specific methicillin-resistant transpeptidase (penicillin-binding protein 2a or PBP-2a). This protein has a low affinity for β-lactam antimicrobial drugs. Thus, bacteria expressing this protein are resistant to all types of these drugs (33, 34).

The *mec* element SCC*mec* has been identified in several different MSSA genetic backgrounds. The distributions of the *mec* regulator genes *mec*I and *mec*R1, which were identified on the chromosome of *mec*A-carrying *Staphylococcus aureus* N315, in methicillin-resistant staphylococci isolated in Japan and various countries were studied. The expression of *mec*A gene results in production of PBP2a and is controlled by proteins encoded by the pennicillinase-associated *bla*R1-*bla*RI inducer-repressor system and the corresponding genomic *mec*R1-*mec*I elements. The *mec*I gene code for repressor protein and the *mec*R1 gene for a beta-lactam sensing transmembrane signaling protein (35). The presence of the *mec*I-*mec*Rl regulator genes in some staphylococcal strains has been shown to be correlated with a strong repression of the *mec*A gene (18).

3.4.5 *fem*A gene (factors essential for methicillin-resistance)

Staphylococcus aureus-specific targets used defined molecular assays for detection of MRSA: *nuc* encodes heat-stable DNA nuclease gene, *spa* encodes *S. aureus*-specific protein A, and factors essential for methicillin-resistance A (*fem*A), *fem*B encode enzymes important in cross-linking peptidoglycan.

fem (factors essential for methicillin-resistance) or the auxiliary genes like *fem* A/B/X in addition to *mec*A are also important in the expression of methicillin resistance. The *fem*ABX operon encodes factors which are responsible for the formation of pentaglycine bridges in the cell wall of *Staphylococci.* Constitutively

expressed genes such as *fem*A, *fem*B and *nuc* are being used as molecular targets for the identification of *Staphylococcus aureus* (36, 37). *fem*A is a chromosomally encoded factor, occurring naturally in *Staphylococcus aureus*. The product of *fem*A is a 48-kDa protein with encoded two proteins required for the formation of the pentaglycine interpeptide bridge that serves as the crosslink of peptidoglycan. The *fem*A gene was shown to be related to high-level methicillin resistance in MRSA because the level of resistance was decreased when *fem*A was inactive (38, 39).

3.5 Phenotypic detection of methicillin resistance

Phenotypic expression of resistance can vary depending on the growth conditions making susceptibility testing by standard microbiological methods potentially difficult.

3.5.1 Cefoxitin disk diffusion test

Cefoxitin is a cephamycin type antibiotic and has been described as an inducer of the PBP2a encoding *mec*A gene. CLSI has recommended cefoxitin disk diffusion method for the detection of MRSA. A direct colony suspension of each *S. aureus* isolate is prepared to a 0.5 McFarland standard. A 30 μg cefoxitin disk is placed on Mueller-Hinton plate and zone of inhibition was determined after 16–18 h incubation at 35 °C. Zone size was interpreted according to CLSI criteria. An inhibition zone diameter of ≤ 21 mm is reported as methicillin resistant and ≥ 22 mm is considered as methicillin susceptible. Cefoxitin can be detect only MRSA with a *mec*A-mediated resistance mechanism (25, 40).

3.5.2 Oxacillin disk diffusion test

A direct colony suspension of each *S. aureus* isolate is prepared to a 0.5 McFarland standard. An oxacillin (1 μg) disk is placed on the surface Mueller-Hinton plate and zone of inhibition was determined after 24 h incubation at 35 ºC. Zone size was interpreted according to CLSI criteria: susceptible, ≥13 mm; intermediate, 11–12 mm; and resistant ≤ 10 mm (25).

3.5.3 Agar dilution test

The colonies isolated from an overnight growth are transferred to sterile saline. The suspension is adjusted to a 0.5 McFarland standard (10^8 cfu/ml) and spot inoculated on Mueller–Hinton agar plates supplemented with 2% NaCl and containing 256 – 0.125 μg oxacillin/ml in serial doubling dilutions. The oxacillin Mueller–Hinton plates are incubated at 35°C for 24 hours. MIC of ≥ 4 μg/ml is considered resistant and MIC of ≤ 2 is considered susceptible (40).

3.5.4 Broth microdilution

This involves the use of Mueller-Hinton broth with 2% NaCl, an inoculum density of 5 x 10^5 cfu/mL and incubation at 33–35°C for 24 hours. Interpretation any growth equates to resistant (40).

3.5.5 E-test

The inoculum is standardized to 0.5 McFarland turbidity and plated on Mueller-Hinton agar supplemented with 2% NaCl. E-test strips are placed and incubation at 35°C for a full 24 hours. The E-test has an advantage over other MIC methods in that it is as easy to set up as a disk diffusion test (40).

3.5.6 Oxacillin screening agar

Mueller-Hinton agar plates containing 4% NaCl and 6 μg/ml of oxacillin are inoculated with 10 μL of 0.5 McFarland suspension of the isolate by streaking in one quadrant and plates were incubated at 35 °C for 24 h. Any strains showing growth on the plate containing oxacillin were considered to be resistant to meticillin (25, 40)

3.5.7 Chromogenic media for MRSA

Selective chromogenic media FDA approved for MRSA screening such as MRSA*Select*™ (Bio-Rad), Spectra MRSA (Remel), ChromID MRSA (bioMérieux), and BBL CHROMagarMRSA (Becton Dickinson). The currently available chromogenic media for MRSA detection show almost uniformly high specificities after 24 h of incubation, although sensitivities tend to vary widely both between media and between studies. Prolonging incubation time to 48 h can improve sensitivities;

however, specificities are adversely affected, necessitating confirmatory tests before reporting MRSA (40, 41).

3.5.8 PBP2a latex agglutination

The method involves extraction of PBP2a from suspensions of colonies and detection by latex agglutination. The kit contains latex particles sensitized with a monoclonal antibody against PBP2a. Visible agglutination indicates a positive result and the presence of PBP2a, the *mec*A gene product. The test is rapid (10 minutes for a single test) and very sensitive and specific with S. aureus, but may not be reliable for colonies grown on media containing NaCl (40).

3.6 Genotypic detection of MRSA

Detection of *mec*A gene by PCR is considered as the gold standard for MRSA. DNA extraction is performed on the isolate and *mec*A gene is amplified using specific primers. Most molecular methods for identification of *S. aureus* such as polymerase chain reaction (PCR), multiplex-PCR, but a range of primers designed to amplify species-specific targets have now been developed. Rapid detection methods for use either directly in clinical samples and/or in the clarification of enrichment broths have been long desired (13, 22)

3.6.1 hyplex StaphyloResist™

The hyplex StaphyloResist™ are qualitative multiplex PCR assays for the direct detection of clinically relevant staphylococci. The assay consists of PCR modules that contain labeled oligonucleotide primers, enabling simultaneous and specific amplification of different staphylococcal DNA regions in a single PCR reaction. The PCR is followed by recerse hybridization procedures using single-stranded specific probes immobilized on microtitre plates. Hybridization of PCR products with specific probes is detected using the ELISA principle.

3.6.2 GenoType MRSA Direct, BD GeneOhm™MRSA, and GeneXpert MRSA assay

The MRSA detection is based on the detection of a single amplicon, which includes the right junction of the SCC*mec* downstream of the *mec*A gene and a part of the adjacent *S. aureus*-specific *orf*X gene.

The Genotype MRSA Direct targets SCC*mec* types I to V in a multiplex PCR using biotinylated primers followed by a reverse hybridization step. An updated version of the assay, the GenoQuick™ MRSA assay is a direct test for MRSA. Following DNA extraction and conventional PCR, the single stranded amplicon hybridizes with a fluorescein labeled probe included in the master mix. The amplicon-probe complex is selectively labeled with gold and generated a band on the dipstick that is inserted in a tube that contains the amplicon. The assay aslo has an amplification control dipstick.

The BD GeneOhm™ MRSA is a multiplex assay comprised of six primers that amplify target sequences near the insertion site of SCC*mec*. Amplified targets within *SCCmec* and *orf*X genes are detected with four molecular beacons. This assay has an internal control not found in MRSA. The fluorescence emitted by each beacon is measured and interpreted. Results are displayed as postive, negative or unresolved. It can differentiate MRSA from MSSA and *mec*A positive CoNS in clinical sample.

3.7 Macrolides, lincosamides and type B streptogramins (MLS_B) and mechanism of resistance

Macrolide, lincosamide, and streptogramins B (MLS_B) antibiotics are chemically distinct but have a similar mode of action. They have a narrow spectrum of activity that includes gram-positive bacteria (in particular, staphylococci, streptococci, and enterococci) and bacilli and gram negative cocci. Erythromycin and clindamycin are widely used in treatment of *Staphylococcus aureus* infections, and clindamycin represents an attractive option for several reasons. It is an attractive option for clinicians because it is available for parenteral and oral use, distributes well in tissues. Clindamycin is an alternative choice for patients allergic to penicillin (42, 43).

Commercially available macrolides have a 14-membered (clarithromycin, dirithromycin, erythromycin, and roxithromycin) or 15-membered (azithromycin) lactone ring. 16-membered ring macrolides (josamycin, midecamycin, miocamycin, rokitamyin, and spiramycin) are available in certain countries or in veterinary practice (tylosin). Lincosamides (clindamycin and lincomycin) are devoid of a lactone ring.Macrolides, lincosamides and streptogramins are distinct antibiotic families, with different chemical structure, but with share a similar mode of mechanisms (21). Their mechanisms consist in inhibiting protein synthesis (44-46).

The target site of MLS_B antibiotic being 50S subunit of the bacterial ribosome, the binding sites being different for the different MLS_B classes (21). Bacteria resist MLS_B is mainly based on three mechanisms. Modification of the ribosomal target has resulted in cross-resistance in the macrolides, lincosamides and streptogramins B (MLS_B), so called MLS_B phenotype. In addition, this resistance is attributed to the presence of erythromycin-resistant methylase (*erm* genes) and major classes of *erm* genes are detected in pathogenic microorganisms: *erm*A, *erm*B, and *erm*C, whereas the resistance of *msr*A gene is encoded by ATP-dependent efflux pump and mediated resistance mechanism is responsible for resistance to macrolides and streptogramins B only (MS phenotype), while the inactivation mechanism is very rare (6, 7)**.** The e*rm* gene encoding for enzymes that confer inducible or constitutive resistance to MLS_B antibiotics. In constitutive resistance, r-RNA methylase is always produced ($cMLS_B$); where as in inducible, methylase is produced only in the presence of an inducing agent ($iMLS_B$).

3.7.1 The *erm*A gene (Erythromycin resistance methylase)

The *erm*A is found on the transposon Tn*554*, which is derived from *Staphylococcus aureus* E1206. Tn*554* is 6691 bp in length and contains six major open reading frames. The nucleotide sequence of *erm*A contains an open reading frame of 243 amino acids, the transcriptional start site of *erm*A at position 5493 bp (AUG codon) and stop site at position 4553 bp (UAA codon) of Tn*554,* which potentially encodes a protein with a calculated molecular mass of 28,380 daltons (47-49).

3.7.2 The *erm*B gene (Erythromycin resistance methylase)

*Erm*B was discovered in Japan it typically occurs on a transposon, Tn*551*, as part of a 28-kilobase (kb) and associated with the penicillinase plasmid, pI258. Although borne by a self-transferable plasmid, Tn*551* is rarely found in staphylococci (15, 50).

3.7.3 The *erm*C gene (Erythromycin resistance methylase)

The classic *erm*C is plasmid containing the 3.7 kb, pE194, which plasmid regulation of expression the *erm*C gene. Two types of *ermC* gene expression are distinguished: inducible and constitutive expression. The inducible *erm*C genes is referred to as the translational attenuator and comprises two pairs of inverted repeated sequences as well as a small open reading frame for a regulatory peptide. The constitutive type of *erm*C gene expression involves structural alterations in the translational attenuator. So, two types of structural changes have been reported: sequence deletions of varying extent sequence duplications at different locations in the translational attenuator (51).

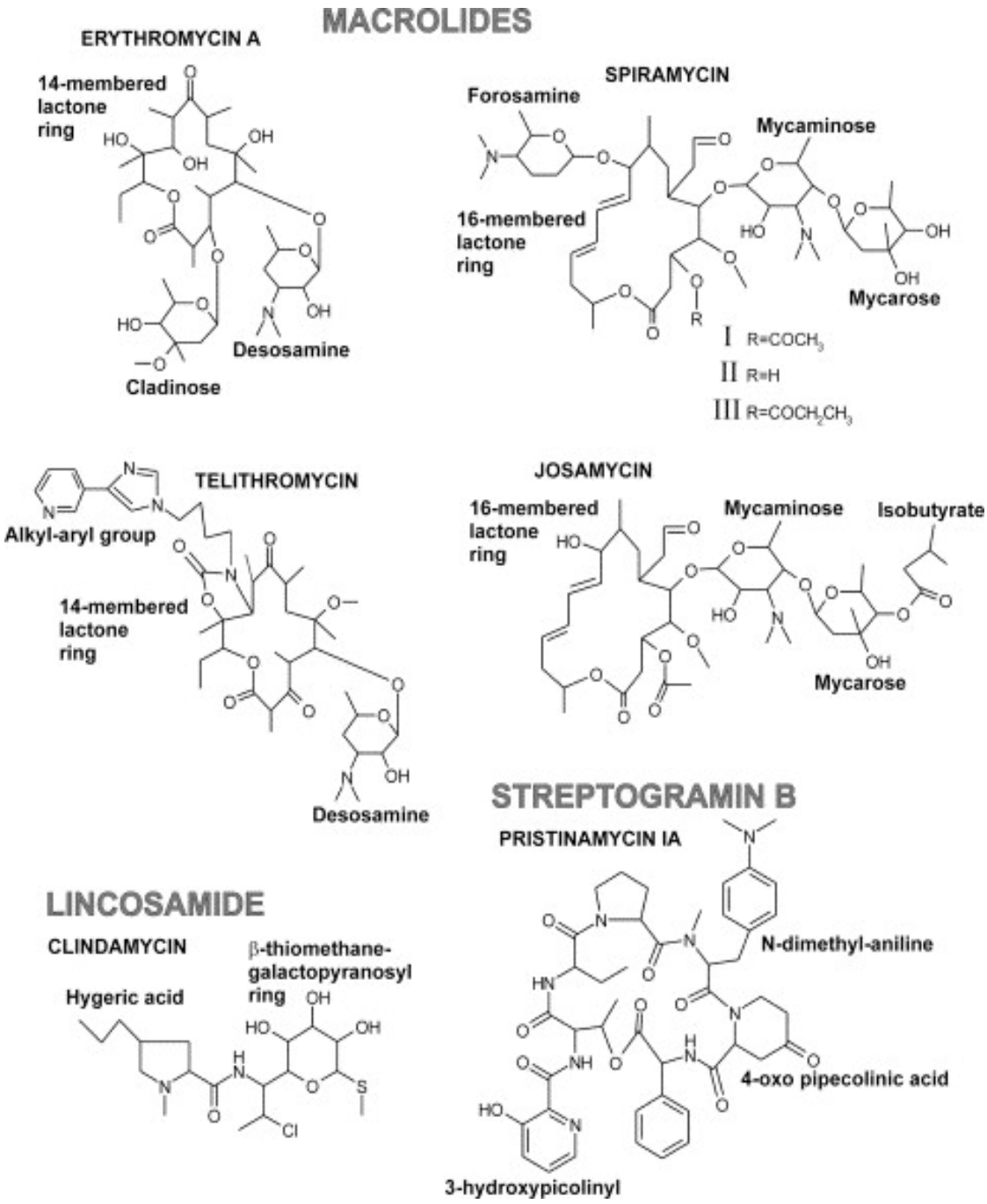

Figure 3.3 Chemical structures of Macrolides, Lincosamide and Streptogramin B antibiotics (52).

3.8 Phenotypic detection for inducible clindamycin resistance

Detection of resistance phenotypes is crucial to guide antimicrobial therapy. Clinical and Laboratory Standards Institute (CLSI) recommends disk diffusion test (D-test) to detect the presence of inducible clindamycin resistance. D-testing was performed for erythromycin-resistant *S. aureus* strains according to the guidelines of the CLSI. Suspension equivalent to 0.5 McFarland of each freshly cultured isolate in normal saline was prepared and used for inoculation of Mueller-Hinton agar plates. Erythromycin 15 μg and clindamycin 2 μg disks were placed on inoculated plates 15 mm apart (edge-to-edge). Plates were read after 18 h of incubation at 35°C and the shape of the clindamycin zone was verified. A flattening of the zone of inhibition around the clindamycin disk proximal to the erythromycin disk (producing a zone of inhibition shaped like the letter D) is considered a positive result and indicates that the erythromycin has induced clindamycin resistance (a positive "D-zone test"). For Erythromycin-resistant isolates, induction tests can help laboratories determine whether results for clindamycin should be reported as susceptible (when the induction test is negative) or as resistant (when the induction test is positive) (13, 43, 52). MLS_B phenotype can be constitutive (rRNA methylase is always produced) or inducible (methylase is produced only in the presence of an inducing agent). While strains with constitutive MLS_B resistance ($cMLS_B$) phenotypes can be detected by routine disk diffusion testing, strains with inducible MLS_B resistance ($iMLS_B$) phenotypes show resistance to erythromycin and sensitivity to clindamycin, similar to strains containing the MS phenotype, which had resistance to only macrolide and streptogramins B, not to clindamycin. The expression of phenotype can be further classified following the table 3.2

Table 3.3 Characteristics of clindamycin induction test phenotypes as tested by disk diffusion (13).

phenotype	Resistance phenotype	CLI result	ERY result	Induction test description
D	Inducible MLS_B	S	R	Blunted, D-shaped clear zone around CLI disk proximal to the ERY disk (A).
D^+	Inducible MLS_B	S	R	Blunted, D-shaped zone around CLI disk proximal to the ERY disk and small colonies growing to CLI disk in otherwise clear zone (B).
Negative	MS_B	S	R	Clear zone around CLI disk (C).
HD	Constitutive MLS_B	R	R	Two zones of growth appear around the CLI disk. One zone is a light, hazy growth extending from the CLI disk to the second zone where the growth is much heavier. The inner, hazy zone is blunted proximal to the ERY disk as in phenotype D (D).
R	Constitutive MLS_B	R	R	No hazy zone. Growth up to CLI and ERY disks (E).
S	No resistance	S	S	Clear, susceptible zone diameters(F).

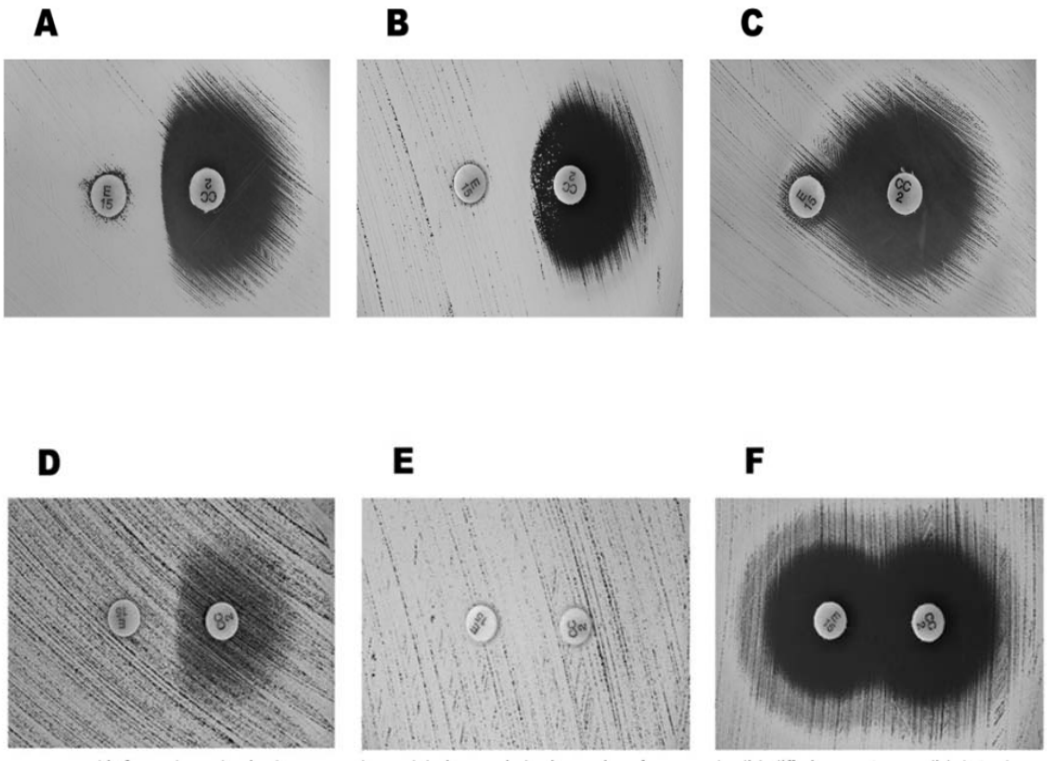

Figure 3.4 Six phenotypes of clindamycin resistant induction test in *S. aureus* by disk diffusion. 15-µg Erythromycin disk (E15); 2-µg Clindamycin disk (CC2): A = D phenotype (D-shaped clear zone around CC disk proximal to the E disk) , B = D^+ phenotype (D-shaped zone around CC disk proximal to the E disk and small colonies growing to CC disk in otherwise clear zone), C = Negative phenotype (Clear zone around CC disk), D = HD phenotype (Two zones of growth appear around the CC disk. One zone is a light, hazy growth extending from the CC disk to the second zone where the growth is much heavier and the inner, hazy zone is blunted proximal to the E disk as in phenotype D), E = R phenotype (No hazy zone. Growth up to CC and E disks), F = S phenotype (Clear, susceptible zone diameters) (13).

CHAPTER IV
MATERIALS AND METHODS

4.1 Bacterial isolates

A total of 250 clinical isolates of *Staphylococcus* spp. (174 isolates of *S. aureus* and 76 isolates of coagulase negative *Staphylococcus*) was obtained from Microbiology Laboratory, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand during July 2013 to November 2014. These isolates were selected from different sites of individual patient. The organisms were kept in skimmed milk at -80^{o}C until used. The details of methicillin and erythromycin resistance are shown in Table 4.1.

4.2 Quality control strains

Five *S. aureus* strains, ATCC 25923, RAMA 5-3046, ATCC BAA-977, UCN07 and HM1054/R were used as control in the experiments. Although all 5 isolates of *S. aureus* contained the *fem*A gene, the ATCC 25923 was used as positive control for this gene in all experiments unless stated and *S. epidermidis* (RAMA 0-1112) was used as negative control of this gene. The positive control strains of *mec*A and *erm* genes are shown in Table 4.2. The ATCC 25923 strain was also used as a negative control of *mec*A and *erm* genes.

Table 4.1 Distribution of *Staphylococcus* clinical isolates and their resistant patterns

***S. aureus* (n = 174)**		**CoNS (n = 76)**	
Methicillin resistance (n=101)	**No. of isolates**	**Methicillin resistance** (n=54)	**No. of isolates**
Ery-R, Clinda-S (D-shape +)	6	Ery-R, Clinda-S (D-shape +)	1
Ery-R, Clinda-S (D-shape -)	0	Ery-R, Clinda-S (D-shape -)	10
Ery-R, Clinda-R	92	Ery-R, Clinda-R	31
Ery-S, Clinda-S	2	Ery-S, Clinda-S	12
MSSA (Total 73)	**No. of isolates**	**MSCoNS** (Total24)	**No. of isolates**
Ery-R, Clinda-S (D-shape +)	11	Ery-R, Clinda-S (D-shape +)	0
Ery-R, Clinda-S (D-shape -)	0	Ery-R, Clinda-S (D-shape -)	3
Ery-R, Clinda-R	8	Ery-R, Clinda-R	8
Ery-S, Clinda-S	54	Ery-S, Clinda-S	12

Ery = Erythromycin, Clinda = Clindaycin, S = Susceptible, R = Resistant

D-shape = inducible clindamycin phenotype

Table 4.2 The positive and negative control strains of *S. aureus* and their antibiotic resistant genes.

Organism	Gene	Source
S. aureus ATCC 25923	*fem*A	ATCC[a]
S. aureus 5-3046	*fem*A , *mec*A	Bangkok, Thailand
S. aureus ATCC BAA-977	*fem*A, *erm*A	Caen, France[b]
S. aureus UCN07	*fem*A, *erm*B	Caen, France[b]
S. aureus HM1054/R	*fem*A, *erm*C	Caen, France[b]

S. aureus ATCC BAA-977: positive control for the inducible clindamycin resistant "D-test".

S. aureus UCN07: methicillin resistance and D-test positive.

S. aureus HM1054/R: methicillin susceptible but erythromycin and clindamycin resistance.

a: ATCC: American Type culture collection, USA.

b: kindly provided by Professor Vincent Cattoir, CHU de Caen, Service de Microbiologie, Caen, France.

4.3 Phenotypic detection of methicillin resistance

Detection of *mec*A mediated methicillin resistance of *S. aureus* and CoNS was determined by standard disk diffusion procedures as described in the Clinical and laboratory Standards Institute (CLSI) guideline (53). Briefly, bacterial colonies were suspension in sterile normal saline until the turbidity was equivalent to 0.5 McFarland standard solution. The adjusted suspension was spread onto Mueller-Hinton agar using

sterile cotton swab. The cefoxitin (30 μg) disk was placed onto the surface of the agar. The plate was incubated at 35°C in ambient air for 18 hours. The inhibition zone diameters were measured and interpreted according to the zone diameter interpretive standard for *Staphylococcus* recommended by CLSI M100-S24 (Table 4.3) (53).

Table 4.3 Zone diameter interpretive standards for detection of *mec*A mediated oxacillin resistance *Staphylococcus* spp. by cefoxitin disk (30 mg) (53).

Organisms	***mec*A positive**	***mec*A negative**
S. aureus and *S. lugdunensis*	≤ 21	≥ 22
CoNS (except *S. lugdunensis*)	≤ 24	≥ 25

S.aureus : Susceptible (S) = ≤ 21 mm, Resistant (R) = ≥ 22 mm

CoNS (except *S. lugdunensis*): Susceptible (S) = ≤ 24 mm, Resistant (R) = ≥ 25 mm

4.4 Phenotypic detection for inducible clindamycin resistance

Detection of inducible clindamycin resistance of *S. aureus* and CoNS was determined by standard disk diffusion procedures as described in the Clinical and laboratory Standards Institute (CLSI) guideline (53). Briefly, bacterial colonies were suspended in sterile normal saline until the turbidity was equivalent to 0.5McFarland standard solution. The adjusted suspension was spread onto Mueller-Hinton agar using sterile cotton swab. The erythromycin (15 □g) and clindamycin (2 □g) disks were placed onto the surface of the agar at the distance of 15 mm (edge to edge) apart. The plate was incubated at 35°C in ambient air for 16 to 18 hours. The inhibition zone diameters were measured and interpreted according to the zone diameter breakpoints for *Staphylococcus* spp. recommended by CLSI M100-S24 (Table 4.4) (53). The interpretative patterns of MLS_B resistance in *Staphylococcus* spp. was determined according to the information given in Table 4.4 and figure 3.4. (54).

Table 4.4 Zone diameter interpretive breakpoints of erythromycin and clindamycin for *Staphylococcus* spp. (53).

Antibiotics	Resistant	Intermediate	Susceptible
Erythromycin (15 μg)	≤ 13	14-22	≥ 23
Clindamycin (2 μg)	≤ 14	15-20	≥ 21

4.5 Genotypic characterization of MRSA and MLS_B by Multiplex PCR amplification

Novel oligonucleotide primers designed for *fem*A, *mec*A, *erm*A, *erm*B and *erm*C genes were used in this study. The details of these primers are shown in Table 4.5.

4.5.1 Primer design

The chosen primers were tested for sequence-alignment with each other as obtained by nucleotide-nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search and analyzed for hair-pin loop and primer dimer formation using program primer designer version 2.01. Target annealing positions were checked by using alignment tool (Bioedit)

Table 4.5 The nucleotide primers for detection *S. aureus* and antibiotic resistant genes.

Primer	Nucleotide Sequence	Melting Temperature (°C)	Amplicon size (bp)	Reference (Genbank accession No.)
femA-F	5'-TAC GCA GCA TAT ACC GCA CT-3'	57.3	300	X17688.1[a]
femA-R	5'-CCA TTA CTG GAC CAC GAT TC-3'	57.3		
mecA-F	5'-CAG GTT ACG GAC AAG GTG AA-3'	57.3	493	X52593.1[b]
mecA-R	5'-GAA GCA ACC ATC GTT ACG GA-3'	57.3		
ermA-F	5'-GAA GCG GTA AAC CCC TCT GA-3'	59.3	224	BA000017.4[c]
ermA-R	5'-AAC CCA AAG CTC GTT GCA G-3	56.7		
ermB-F	5'-AAG CCA TGC GTC TGA CAT CT-3	57.3	192	U35228.1[d]
ermB-R	5'-GTG GTA TGG CGG GTA AGT TT -3'	57.3		
ermC-F	5'-AAT CGG CTC AGG AAA AGG-3'	53.7	715	AB982226.1[e]
ermC-R	5'-GAA GCG AAT GCG CAA AAG-3'	53.7		

a: Genbank accession No. X17688.1 = Sequence of *fem*A gene

b: Genbank accession No. X52593.1 = Sequence of *mec*A gene

c: Genbank accession No. BA000017.4 = Sequence of *erm*A gene

d: Genbank accession No. U35228.1 = Sequence of *erm*B gene

e: Genbank accession No. AB982226.1 = Sequence of *erm*C gene

4.5.2 DNA extraction

The total DNA was extracted by a rapid boiling procedure. Briefly, the overnight growth bacterial colonies on blood agar were suspended in 200 µl of lysis buffer and heated at 100°C for 20 minutes. The supernatant was obtained after centrifugation at 13,000 rpm for 5 minutes, was used as template DNA immediately.

4.5.3 Multiplex PCP amplification

Each sample was amplified in a total volume of 25 µl. mixture. The PCR mixture contained a DNA template and 2X Phusion Blood Direct PCR Master Mix (Thermo Scientific), each of forward and reverse primers femA-F, femA-R, mecA-F, mecA-R, ermA-F, ermA-R, ermB-F, ermB-R, ermC-F and ermC-R (Table 4.5).

Amplification was performed in the thermal cycler (Labcycler, SensoQuest GmbH., Germany) according to the Table 4.6.

Table 4.6 Multiplex PCR condition

Step	Temperature(°C)	Time	Cycle	
Initial activation step	94 °C	5 min.	1	
Denaturation	95 °C	30 sec.	1	35 cycles
Annealing	54 °C	30 sec.	1	
Extension	72 °C	30 sec.	1	
Final elongation	72 °C	7 min.	1	

4.5.4 Multiplex PCR amplicon detection

Multiplex PCR products were analysed in 2% (w/v) agarose gel (Promega, Madison, USA) electrophoresis in 1X Tris-borate-EDTA buffer (TBE) at pH 8.3, which were stained with GelRed™ Nucleic Acid Gel Stain. The gel pattern with respect to amplicon was visualized on an UV light transilluminator and photographed on Gel document (Biovision+ 1000/20M, Vilber Laumat). The reference DNA ladders were needed to evaluate amplicon size and concentration. The designed

primers were tested for specificity of PCR products using a DNA template prepared from control strains of target genes.

4.6 Sequencing analysis

4.6.1 DNA sequencing

For sequencing, 30 µl of each PCR product were further purified by the polyethylene glycol precipitation method. The corresponding amplification primers or inner primers were used as the sequencing primers (1st BASE DNA Sequencing Services, Selangor DarulEhsan, Malaysia).

4.6.2 Nucleotide analysis

The nucleotide sequencing was analyzed and compared with other available sequences in public databases as Genbank and EMBL, using the BLAST program software via the NCBI web site (http://www.ncbi.nlm.nih.gov/).

4.7 Sensitivity and specificity calculations

The sensitivity and specificity of the applied PCR assays were calculated for each target. Hereby, true-positive samples were defined as those positive for resistance genes and resistant to the corresponding antibiotics. False-negative samples were defined as those negative for resistance genes but resistant to the corresponding antibiotics. True-negative samples were defined as those negative for resistance genes and susceptible to the corresponding antibiotics. False-positive samples were defined as those positive for resistance genes but susceptible to the corresponding antibiotics. The following formulas were used, as previously described (36, 55).

4.7.1 Sensitivity

The sensitivity of a clinical test refers to the ability of the test to correctly identify those patients with the disease.

$$\text{Sensitivity (\%)} = \frac{\text{no. of true positive samples}}{\text{(no. of true positive samples + no. of false negative samples)}} \times 100$$

4.7.2 Specificity

The specificity of a clinical test refers to the ability of the test to correctly identify those patients without the disease.

$$\text{Specificity (\%)} = \frac{\text{no. of true negative samples}}{\text{(no. of true negative samples + no. of false positive samples)}} \times 100$$

The following terms are fundamental to understanding the utility of clinical tests:

1) True positive: the patient has the disease and the test is positive.
2) False positive: the patient does not have the disease but the test is positive.
3) True negative: the patient does not have the disease and the test is negative
4) False negative: the patient has the disease but the test is negative.

CHAPTER V
RESULTS

5.1 Designing of oligonucleotide primers using bioinformatics tool

Newly designed oligonucleotide primers for *fem*A, *mec*A, *erm*A, *erm*B and *erm*C genes were used in this study, all primers were checked for self-complementary potential hairpin conformation, 3'complementary and all potential self-anneal sites by using program Primer designer version 2.01. This resulted in no self-complementary aspects. The primers design for *fem*A, *mec*A, *er*mA, *erm*B, and *erm*C genes show in figure 5.1-5.5, respectively.

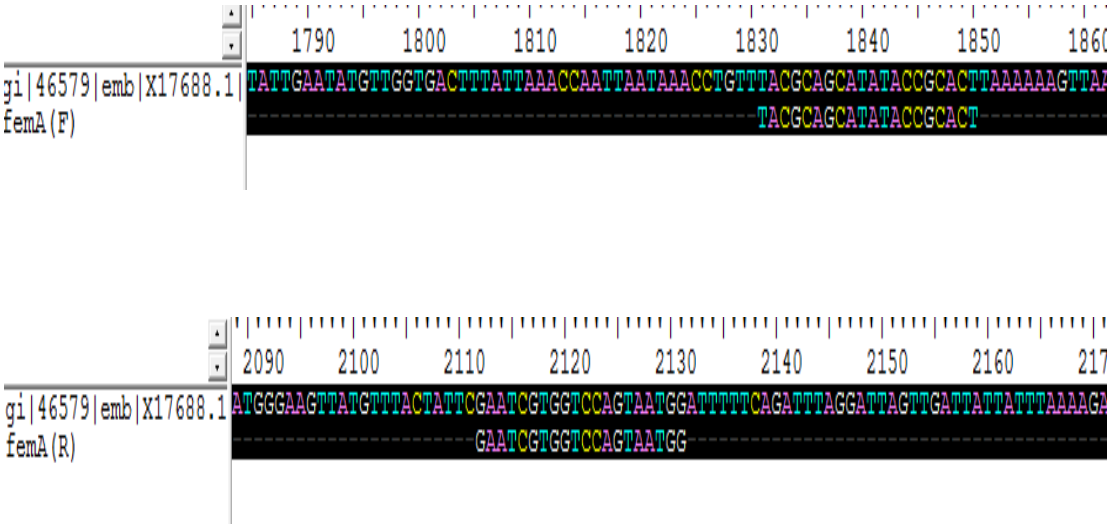

Figure 5.1 The nucleotide sequence of *fem*A gene and femA primer.

X17688.1 = accession number of *fem*A gene sequence

femA(F) = femA forward primer

femA(R) = femA reverse primer

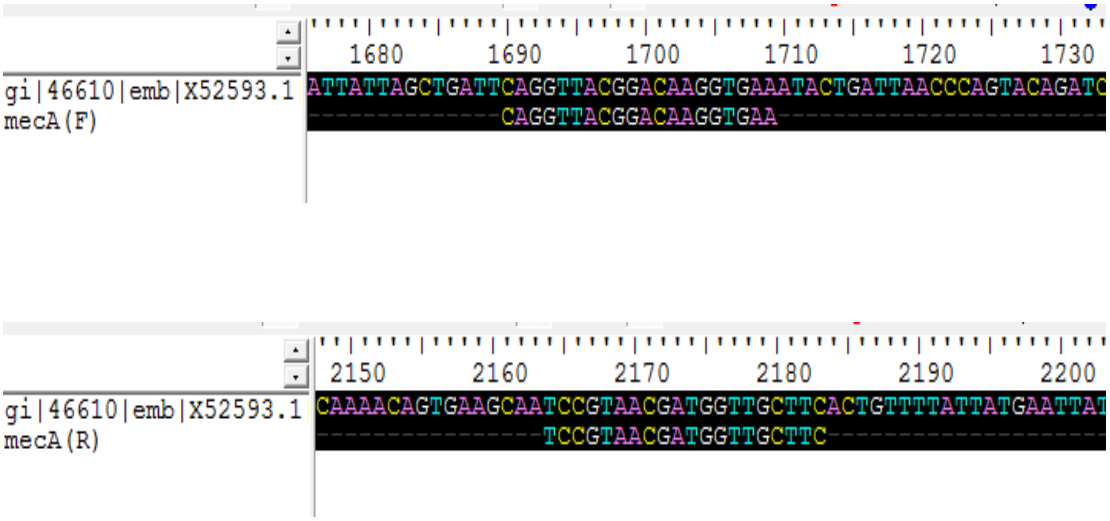

Figure 5.2 The nucleotide sequence of *mec*A gene and mecA primer.

X52593.1 = accession number of *mec*A gene sequence

*mec*A(F) = *mec*A forward primer

*mec*A(R) = *mec*A reverse primer

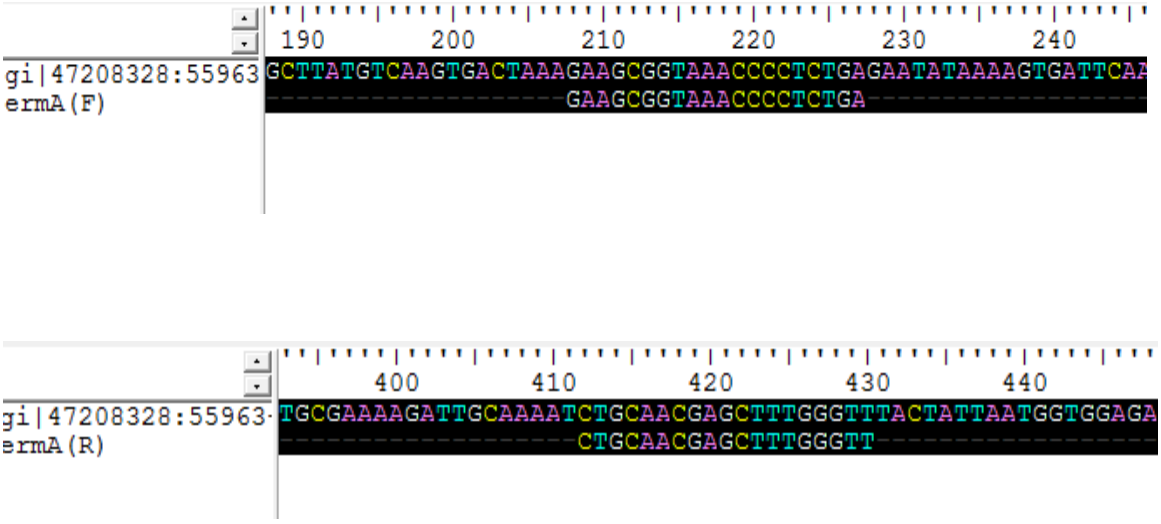

Figure 5.3 The nucleotide sequence of *erm*A gene and ermA primer.

BA000017.4 = accession number of *erm*A gene sequence

*erm*A(F) = *erm*A forward primer

*erm*A(R) = *erm*A reverse primer

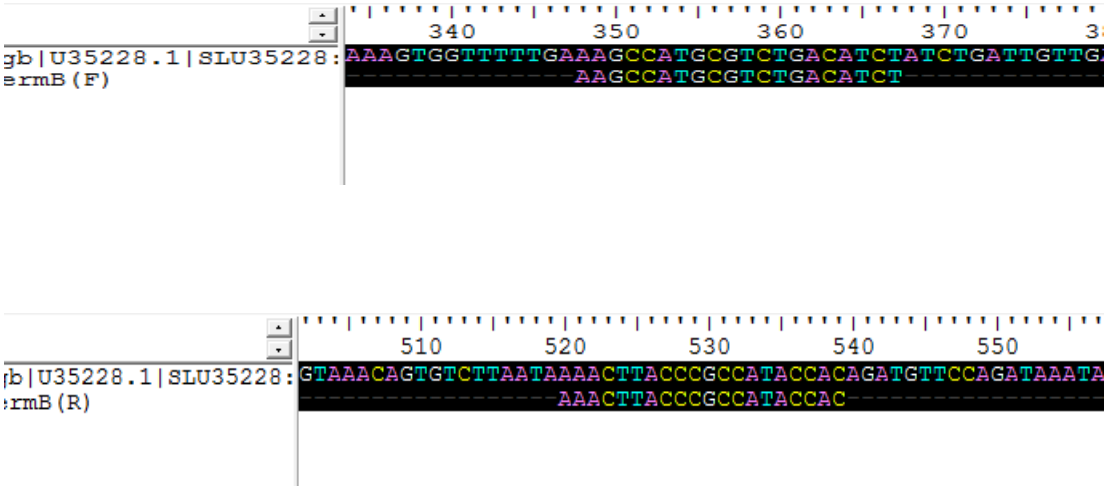

Figure 5.4 The nucleotide sequence of *erm*B gene and ermB primer.

U35228.1 = accession number of *erm*B gene sequence

*erm*B(F) = *erm*B forward primer

*erm*B(R) = *erm*B reverse primer

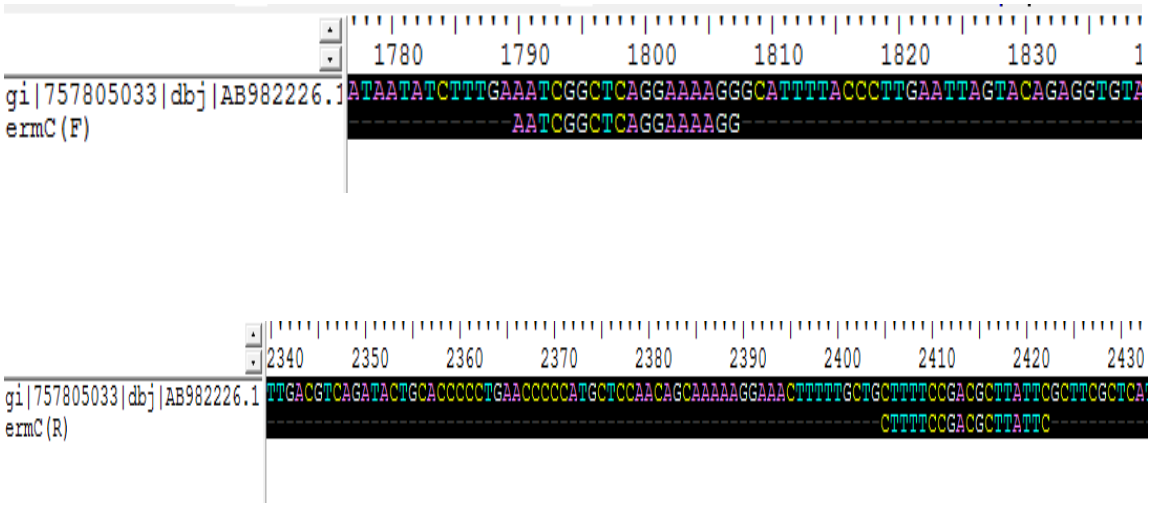

Figure 5.5 The nucleotide sequence of *erm*C gene and ermC primer.

AB982226.1 = accession number of *erm*C gene sequence

*erm*C(F) = *erm*C forward primer

*erm*C(R) = *erm*C reverse primer

5.2 The developing of multiplex PCR

A total of 5 candidate genes from *S. aureus* including *fem*A, *m*ecA, *erm*A, *erm*B, and *erm*C genes were tested for development of multiplex PCR method for detection *S. aureus* and antibiotic resistant genes.

5.2.1 Study A

The designed primers were tested for specificity in separate PCR using a DNA template prepared from control strains (Table 4.2) known to produce specific of target genes. The PCR amplification of DNA template prepared from *S. aureus* ATCC 25923 (methicillin, macrolide, and clindamycin susceptible and negative for *mec*A, *erm*A, *erm*B and *erm*C genes) resulted in a single amplified product of 300 bp when *fem*A primers were used. Other *S. aureus* control strains used in the experiments revealed similar band of *fem*A gene and no band was observed when tested with *S. epidermidis* (0-1112) strain (data not shown). The *mec*A primers amplified a single 493 bp fragment when DNA template prepared from *S. aureus* No.5-3046 (positive control for *mec*A gene, confirmed by DNA sequencing). The *erm*A primers amplified a 224 bp product from DNA template from *S. aureus* ATCC BAA-977 contained *erm*A gene. For *erm*B, *S. aureus* UCN07 was used and the amplified product of 192 bp was observed. Meanwhile, *erm*C primers amplified a 715 bp product from DNA template which prepared from *S. aureus* HM1054/R. The DNA template of control strain, *S. aureus* ATCC 25923 showed no amplified product of *mec*A, *erm*A, *erm*B and *erm*C genes. The representative gel indicating the specific of the primer pair is shown in figure 5.6.

5.2.2 Study B

In order to test for optimal annealing temperature of the PCR reaction in this study, singleplex PCR for the *fem*A, *mec*A, *erm*A, *erm*B and *erm*C genes using the above reference control strains was tested. Three different annealing temperatures (52, 54 and 56°C) were tested according to the initial Tm of the primers. Each of PCR mixture contained a DNA template, PCR Master Mix II (Thermo Scientific) and 10 pmols of each other primers. The singleplex PCR amplification with 5 genes control strains exhibited distinct bands corresponding to expected sizes. The optimum cycling

conditions for amplifying five genes were performed in an automated thermal cycler as follows: initial denaturation for 5 min at 94°C, 35 cycles of 30 sec at 95°C for denaturation, 30 sec of either 52, 54 or 56°C for annealing and extension for 2 min at 72°C, followed by final elongation for 7 min at 72°C.

The PCR products from 5 target genes (*fe*mA, *mec*A, *erm*A, *erm*B and *erm*C genes) were observed in all three annealing temperatures. However, the *erm*C gene amplified products were observed only at 52 and 54°C but not at 56°C (figure 5.7). Regarding to the annealing temperatures, although both 52 and 54^{o}C revealed positive result in all primers, the clearer band of *erm*C was observed at the 52^{o}C. Therefore, the annealing temperature at 52^{o}C was selected for further studies.

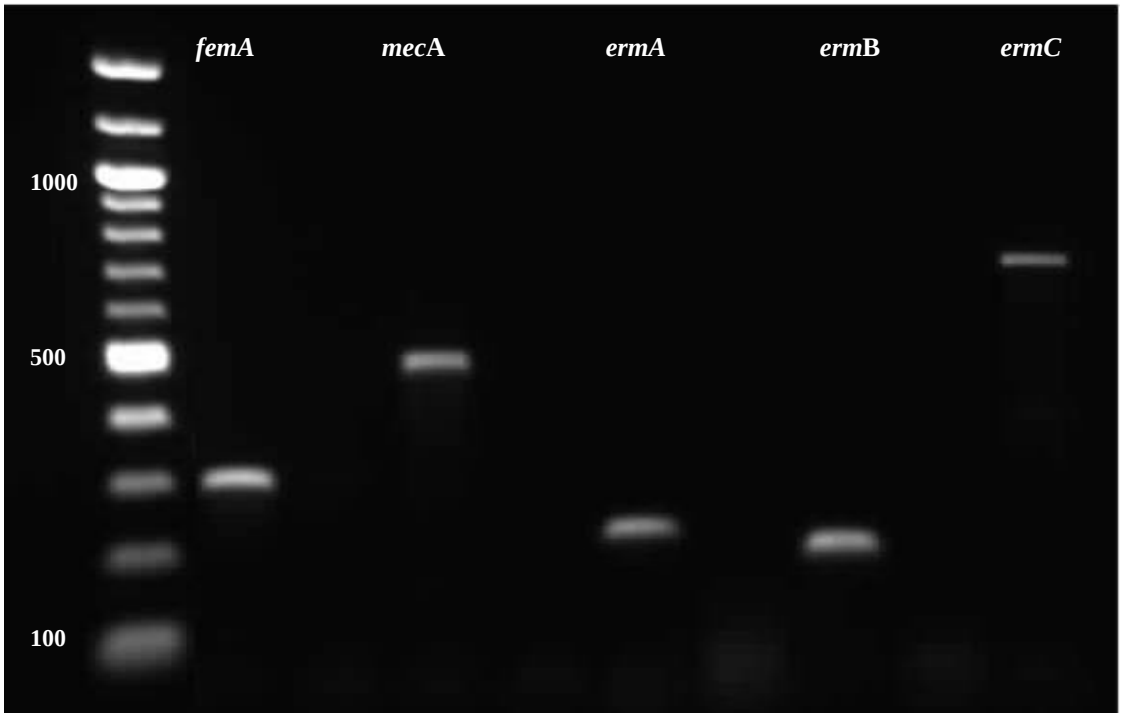

Figure 5.6 The result of primer specific testing using control strains of *S. aureus* (study A). The ATCC 25923 for *fem*A (300 bp), 5-3046 for *mec*A (493 bp), BAA-977 for *erm*A (224 bp), UCN07 for *erm*B (192 bp) and HM1054/R for *erm*C genes (715 bp).

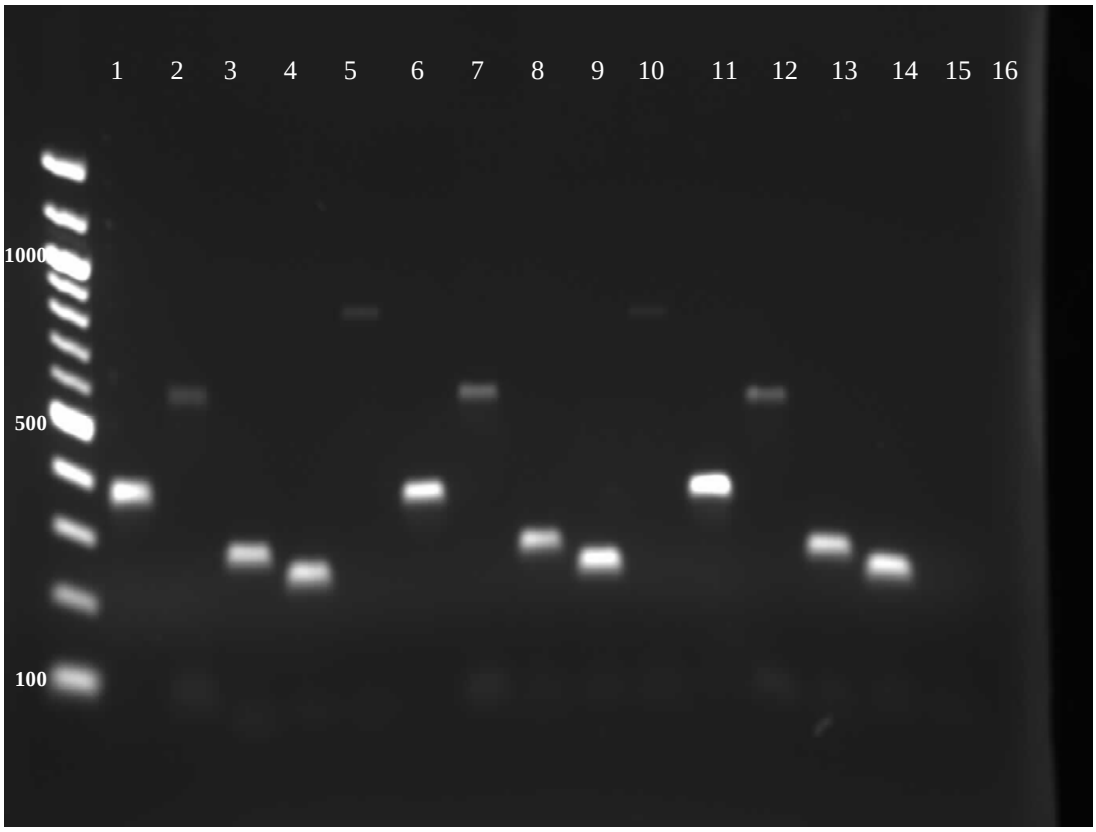

Figure 5.7 The result of optimization of annealing temperature for *fem*A, *mec*A, *erm*A, *erm*B and *erm*C genes by singleplex PCR (study B).

Lane 1, 6, 11 = *fem*A at temperature 52, 54 and 56^{o}C, respectively.

Lane 2, 7, 12 = *mec*A at temperature 52, 54 and 56^{o}C, respectively.

Lane 3, 8, 13 = *erm*A at temperature 52, 54 and 56^{o}C, respectively.

Lane 4, 9, 14 = *erm*B at temperature 52, 54 and 56^{o}C, respectively.

Lane 5, 10, 15 = *erm*C at temperature 52, 54 and 56^{o}C, respectively.

5.2.3 Study C

In order to step further in our experiments, the primers of five candidate targets were brought to test for suitable condition of multiplex PCR. The amounts of the component consisted of all 5 pairs of primers, master mix, distilled water, and DNA of five target genes (*fem*A, *mec*A, *erm*A, *erm*B and *erm*C genes). The details of multiplex PCR composition are shown in Table 5.1. The PCR running condition was similar to study B using the annealing temperature at 52^{o}C. The results showed that only 3 bands including *fem*A, *mec*A, and *erm*C were observed (figure 5.8).

Table 5.1 The components of multiplex PCR mixture for 5 target genes (*fem*A, *mec*A, *erm*A, *erm*B and *erm*C) used in study C.

DNA (µl)	PCR Primers (µl)										DW (µl)	Master mix (µl)
	femA (F)	femA (R)	mecA (F)	mecA (R)	ermA (F)	ermA (R)	ermB (F)	ermB (R)	ermC (F)	ermC (R)		
5	0.5	0.5	1.5	1.5	1	1	1	1	3	3	1	5

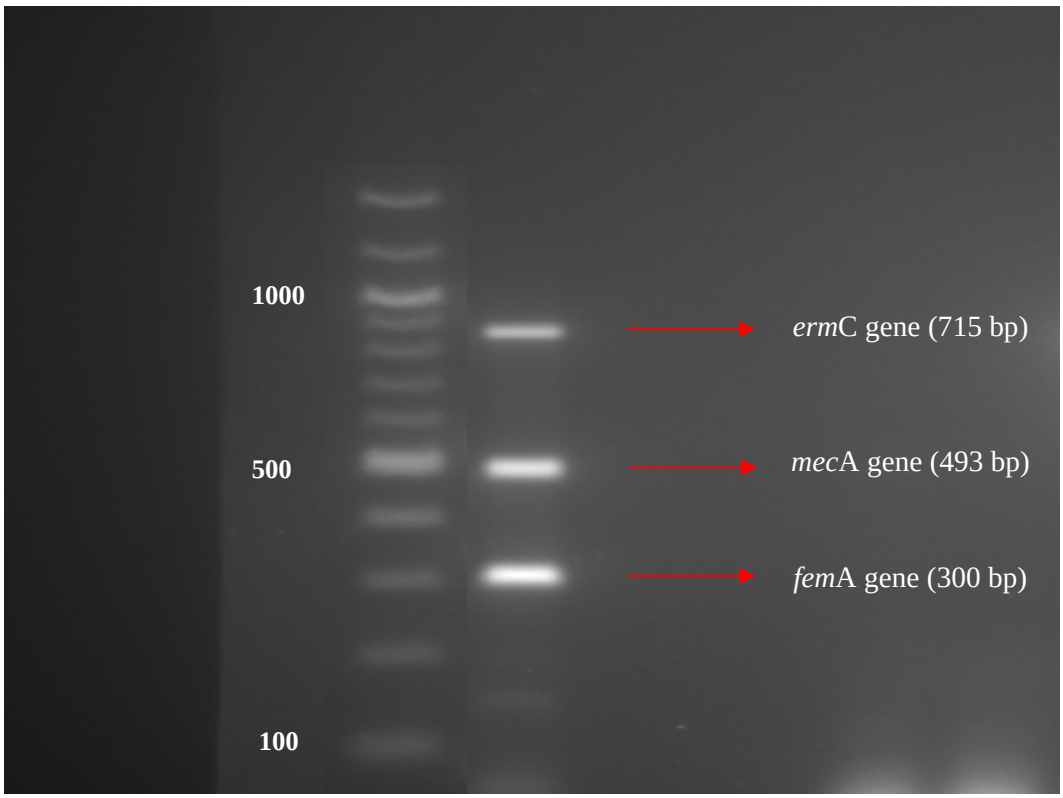

Figure 5.8 The multiplex PCR products from study C show only 3 bands including: *fem*A, *mec*A, and *erm*C. The products of *erm*A and *erm*B were not observed.

5.2.4 Study D

Referred to the problem in study C where only 3/5 bands were observed in the initial multiplex PCR, one possibility of the missing bands could be the effect of the proportions of each primer pair. In this experiment, the amount of *fem*A and *mec*A primers were reduced due to the thick bands observed in study C while the amount of *erm*A and *erm*B primers was increased. (Table 5.2) However, the result remained similar to the study C.

Table 5.2 The components of multiplex PCR mixture for 5 target genes (*fem*A, *mec*A, *erm*A, *erm*B and *erm*C) used in study D.

DNA (µl)	PCR Primers (µl)										DW (µl)	Master mix (µl)
	fem A (F)	fem A (R)	mec A (F)	mec A (R)	erm A (F)	erm A (R)	erm B (F)	erm B (R)	erm C (F)	erm C (R)		
3	0.25	0.25	0.5	0.5	2	2	1.5	1.5	3	3	2.8	10

5.2.5 Study E

From the results of study D, the reason of missing bands from the multiplex PCR may be caused by components in the master mix, such as dNTP, Mg^{2+}, or Taq polymerase. Therefore, the master mix for multiplex PCR was changed to 2X Phusion Blood Direct PCR Master Mix. The amount of each primer was initially similar to the study D. With this new master mix, all 5 target bands were observed. However, the thickness of each band was rather unequal. Therefore, the amount of each primer was re-adjusted according to the thickness appearance of product bands on the agarose gel. The optimal amount of primers is shown in Table 5.3 and the product bands are shown in figure 5.9.

Table 5.3 The components of multiplex PCR mixture for 5 target genes (*fem*A, *mec*A, *erm*A, *erm*B and *erm*C) used in study E. The 2X Phusion Blood Direct PCR Master Mix was used.

DNA (µl)	PCR Primers (µl)										DW (µl)	Master mix (µl)
	fem A (F)	fem A (R)	mec A (F)	mec A (R)	erm A (F)	erm A (R)	erm B (F)	erm B (R)	erm C (F)	erm C (R)		
3	0.25	0.25	1	1	0.5	0.5	0.35	0.35	2.5	2.5	2.8	10

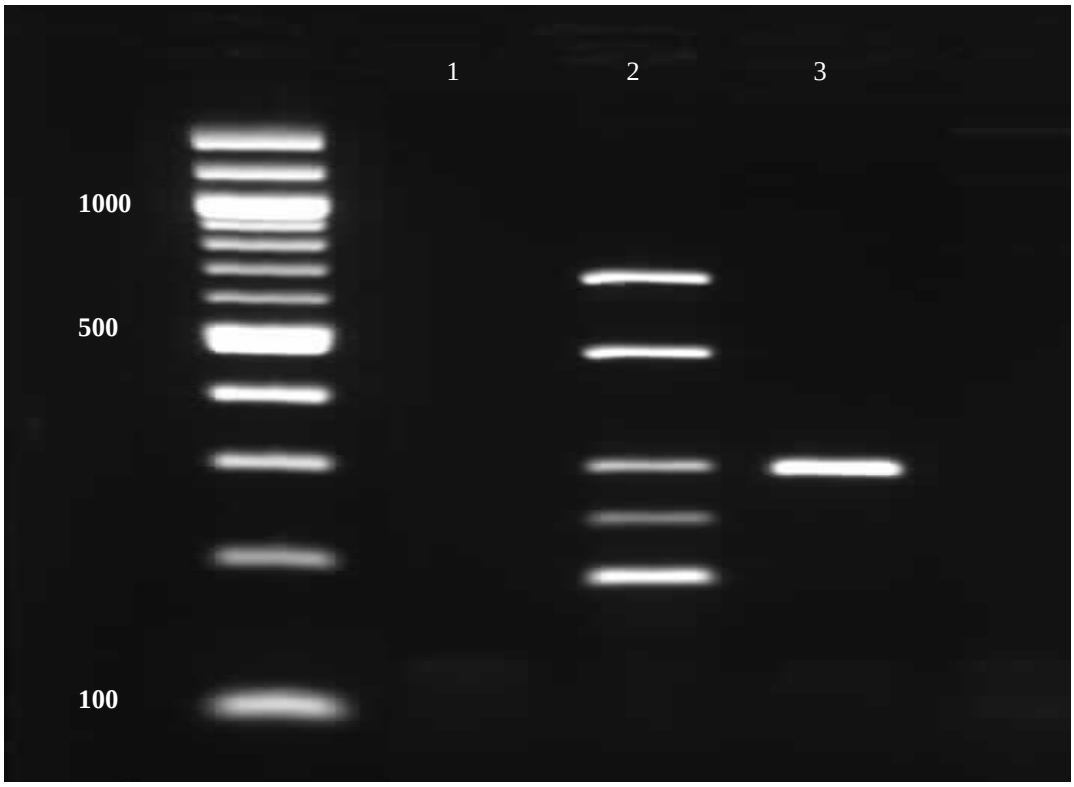

Figure 5.9 The result of study E shows complete all 5 bands of target genes: *fem*A (300 bp), *mec*A (493 bp), *erm*A (224 bp), *erm*B (192 bp) and *erm*C (715 bp).

Lane 1 = Negative control

Lane 2 = Positive control (5 bands of multiplex PCR in one reaction)

Lane 3 = The result of multiplex PCR show the band of *fem*A gene (S. *aureus* ATCC 25923)

5.2.6 Study F

After the multiplex PCR condition which allowed all targets bands to be observed in study E was set, the annealing temperatures were again taken into account in order to test for optimal condition of this multiplex. Three annealing temperatures of 52, 54 and 56^{o}C were tested. The result revealed that compete bands of five target genes were demonstrated in all tested annealing temperatures (Figure5.10). Therefore, the annealing temperature at 52^{o}C was selected for the next study with unknown isolates.

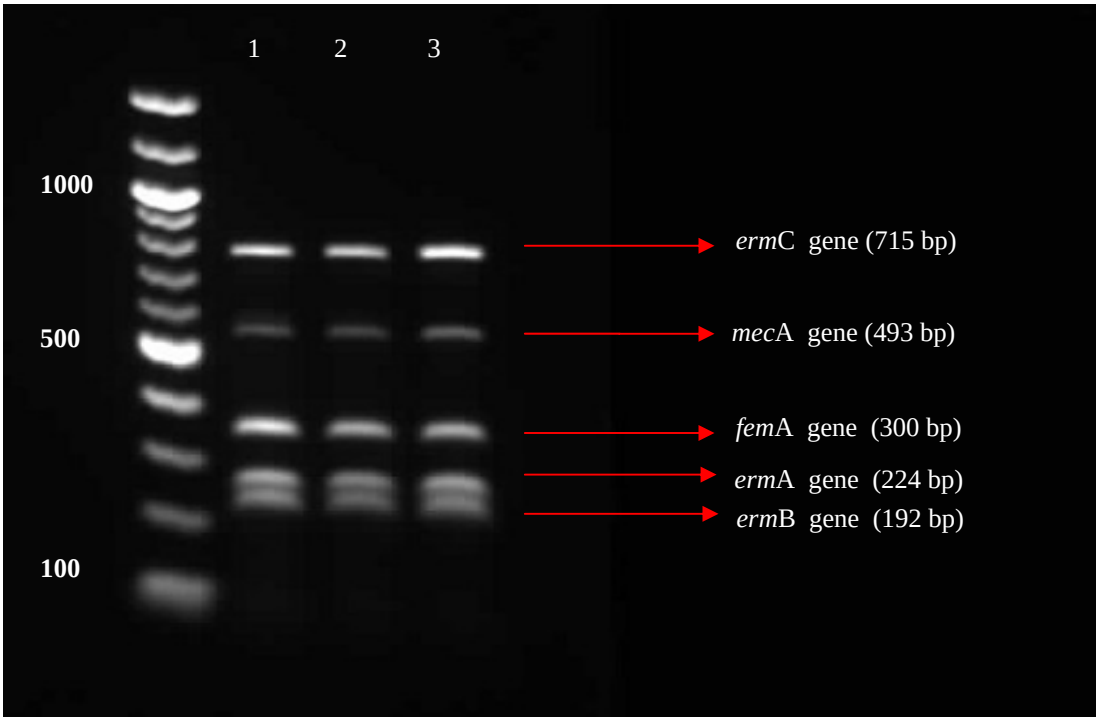

Figure 5.10 The result of optimization of annealing temperature for *fem*A, *mec*A, *erm*A, *erm*B and *erm*C genes in multiplex PCR (study F).

Lane 1 = Annealing temperature of multiplex PCR at 52 °C

Lane 2 = Annealing temperature of multiplex PCR at 54 °C

Lane 3 = Annealing temperature of multiplex PCR at 56 °C

5.2.7 Study G

After establishment of the optimal multiplex PCR condition in study F, 13 isolates of *Staphylococcus* spp. were performed using the above PCR protocol with the annealing temperature at 52^{o}C. These isolates were identified for their species as well as phenotypically tested against oxacillin, erythromycin and clindamycin. By comparing with phenotypic susceptibility test of the above antibiotics, the PCR results revealed non-specific bands in most isolates (data not shown). These non-specific bands had the size close to *mec*A gene and *erm*C gene bands. The non-specific bands were sequenced and none revealed as either *mec*A or *erm*C genes. The first attempt to eliminate the non-specific bands was performed by increasing the annealing temperature to 54^{o}C. However, the non-specific bands remained observable (Figure 5.11). Further attempt was established by diluting the extracted DNA in half and performed the PCR using 54^{o}C annealing temperature. With this procedure, the non-specific bands were removed (Figure 5.11, lane 14-17). Follow the optimal condition for multiplex PCR, larger scale of unknown isolates was established for the specificity of this multiplex PCR. The results of some unknown isolates are shown in Figure 5.12.

5.2.8 Study H

In order to determine the sensitivity of the assay, three isolates of MRSA containing *erm* genes were selected. The multiplex PCR was performed using three different amounts of template DNA including 1.5, 15 and 150 ng in 25 µl mixture. The result revealed the positive bands in all concentration with minimal different between 15 and 150 ng of template DNA. Nevertheless, at the DNA template of 1.5 ng, the bands were noticeable fader than 15 and 150 ng (Figure 5.13).

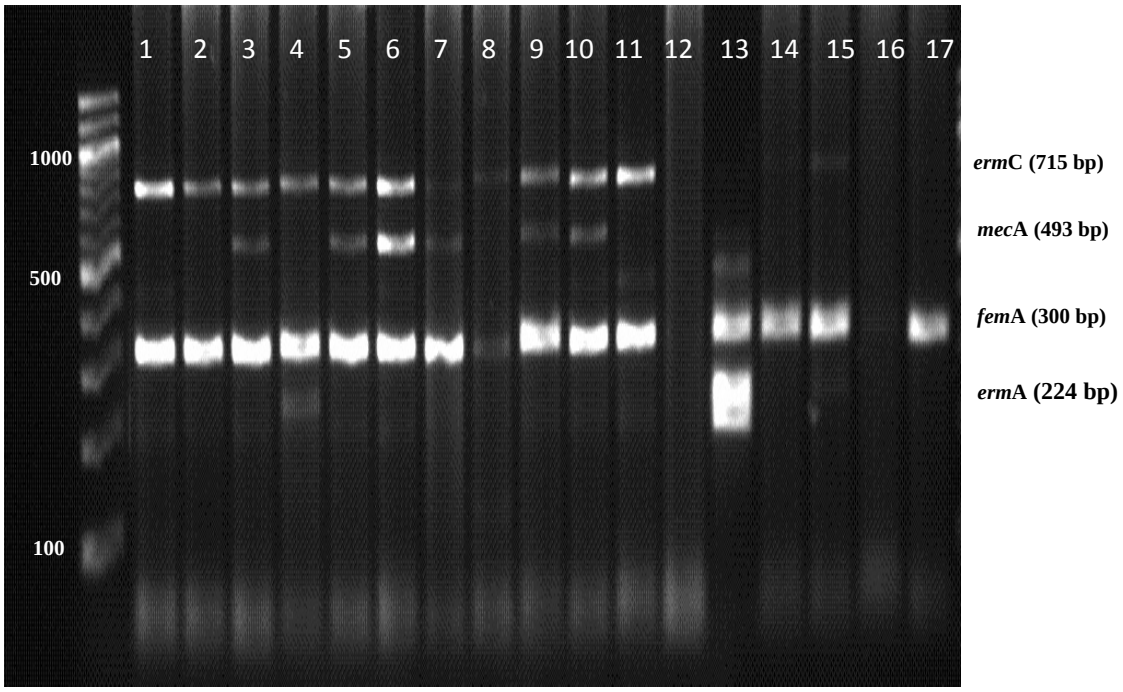

Figure 5.11 The results of unknown samples tested by multiplex PCR (study G).

Lane 1, 11: MSSA resisted to E & DA with *fem*A and *erm*C

Lane 2, 4: MSSA susceptible to E & DA with non-specific band size close to *erm*C

Lane 3, 5, 9, 10: MSSA susceptible to E & DA with non-specific band size close to *mec*A and *erm*C

Lane 6: MRSA resisted to E & DA with *fem*A , *mec*A and *erm*C

Lane 7: MSSA with non-specific band size close to *mec*A

Lane 8: MSSE susceptible to E & DA with non-specific band size close to *fem*A and *mec*C

Lane 12: MSSE susceptible to E & DA (negative control)

Lane 13: MSSA resisted to E & DA with *fem*A and *erm*A

Lane 14: MSSA from lane 3 after DNA was diluted to remove non-specific bands

Lane 15: MSSA from lane 4 after DNA was diluted to remove non-specific band

Lane 16: MSSE from lane 8 after DNA was diluted to remove non-specific band

Lane 17: MSSA from lane 3 after DNA was diluted to remove non-specific bands

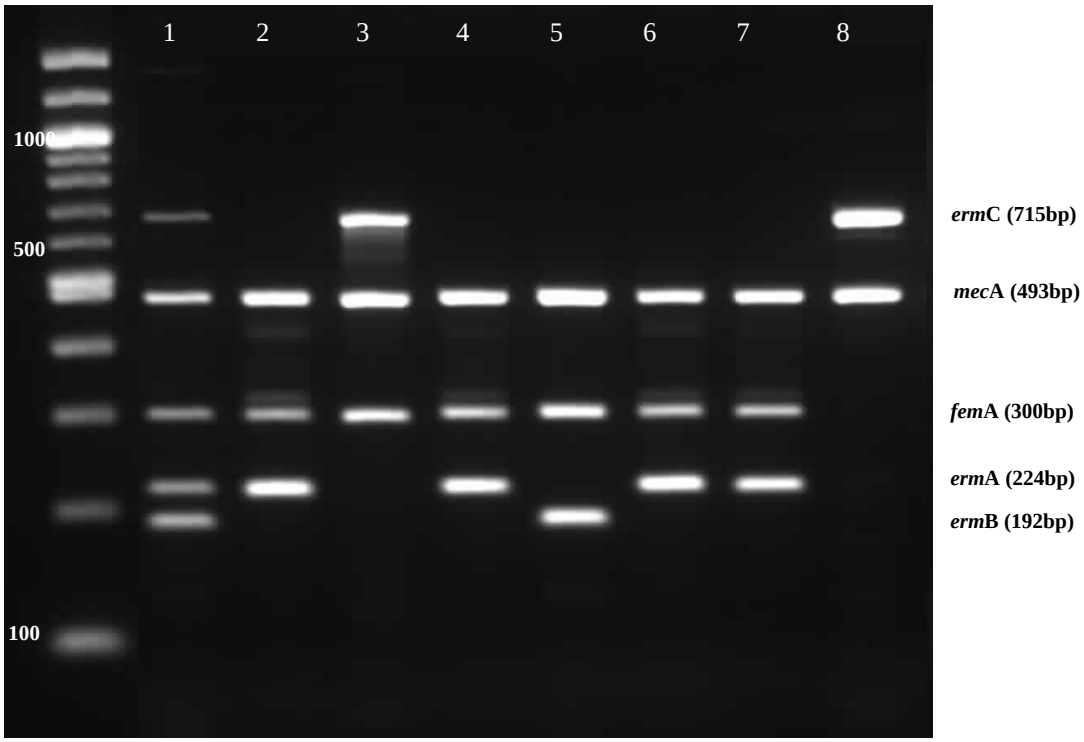

Figure 5.12 Multiplex PCR amplifications on template DNA of 7 different *Staphylococcus* isolates.

Lane 1 = Positive control (DNA mixture with all targets)

Lane 2 = No.391 (MRSA with *erm*A gene)

Lane 3 = No.366 (MRSA with *erm*C gene)

Lane 4 = No.6672 (MRSA with *erm*A gene)

Lane 5 = No.6010 (MRSA with *erm*B gene)

Lane 6 = No.6801 (MRSA with *erm*A gene)

Lane 7 = No.7023 (MRSA with *erm*A gene)

Lane 8 = No.6764 (Coagulase-negative *staphylococci* with *mec*A and *erm*C genes

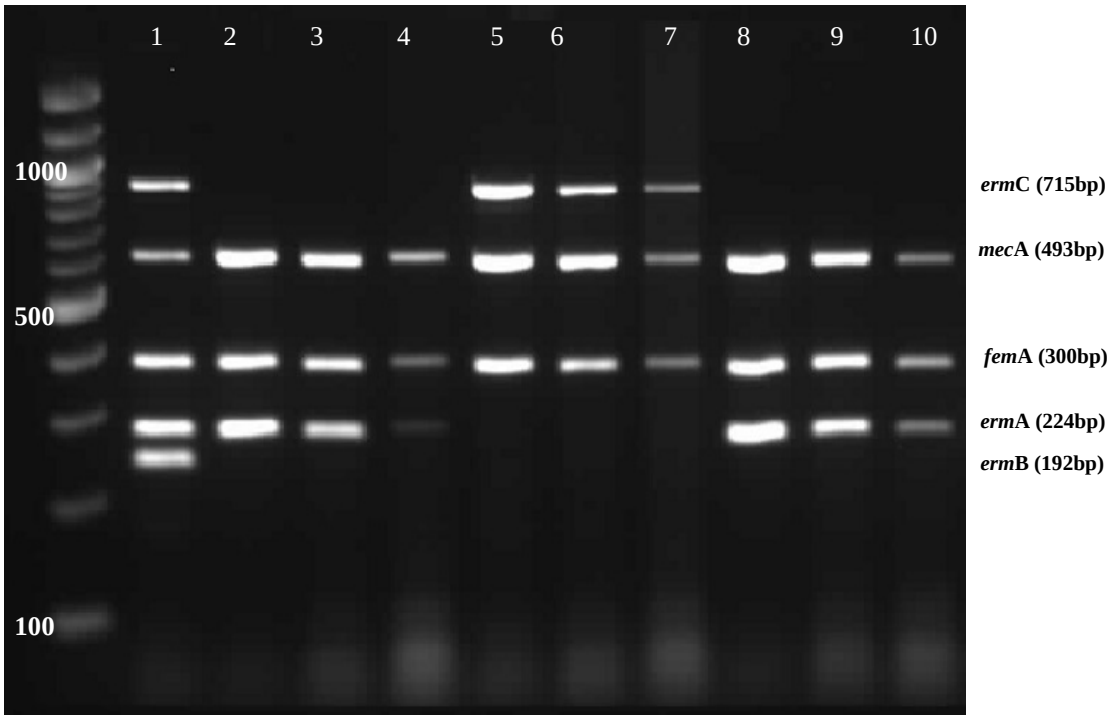

Figure 5.13 The result of Multiplex PCR amplifications in three different amounts of template DNA.

Lane 1: Positive control

Lane 2, 3, 4: DNA of *erm*A containing MRSA at the amount of 150, 15 and 1.5 ng, respectively

Lane 5, 6, 7: DNA of *erm*B containing MRSA at the amount of 150, 15 and 1.5 ng, respectively

Lane 8, 9, 10: DNA of *erm*A containing MRSA at the amount of 150, 15 and 1.5 ng, respectively

5.3 Correlation between biochemical test for bacterial identification and molecular method

A total number of 250 isolates of *Staphylococcus* spp. were identified by multiplex PCR based on the existence of *fem*A gene comparing with conventional biochemical test and MALDI-TOF technique. The results of PCR revealed that 174 isolates contained *fem*A which were identified as *S. aureus.* Other 76 isolates were negative for *fem*A gene which was referred as CoNS. These PCR results showed 100% concordance with both biochemical test and MALDI-TOF (table 5.3).

Table 5.4 Correlation between biochemical test for bacterial identification and multiplex PCR method

Organisms (Total 250 isolates)	No. of strains positive for:	
	Biochemical test	Multiplex PCR (*fem*A +)
S. aureus (n=174)	174	174
CoNS (n=76)	76	76

5.4 Correlation between phenotypic testing for MRSA and the multiplex PCR.

Of 174 *S. aureus* from the above experiment, 101 isolates carried *mec*A gene. All of these isolates showed resistant to cefoxitin according to the breakpoint recommended in the CLSI guideline while all 73 *mec*A-negative isolates revealed susceptible to cefoxitin. Similar results were observed among CoNS isolates. A total

of 54/76 isolates of CoNS contained *mec*A gene and showed total agreement with the phenotypic test against cefoxitin. All *mec*A-negative CoNS isolates revealed susceptible to cefoxitin (Table 5.5).

Table 5.5 Correlation between phenotypic of methicillin-resistance and multiplex PCR results

Organism and Phenotype[a]	No. of isolates with genotype	
	*mec*A (+)	*mec*A (-)
S. aureus (n=174)		
MRSA[b]	101	0
MSSA[c]	0	73
CoNS (n=76)		
MRS[d]	54	0
MSS[e]	0	22

[a] Determined by standard disk diffusion test

[b] MRSA = Methicillin-resistant *Staphylococcus aureus*

[c] MSSA = Methicillin-susceptible *Staphylococcus aureus*

[d] MRS = Methicillin-resistant *Staphylococcus*

[e] MSS = Methicillin-susceptible *Staphylococcus*

5.5 Correlation between phenotypic testing for erythromycin resistance and the multiplex PCR.

A total of 170/250 isolates of *Staphylococcus* spp. were resistant to erythromycin. Of these isolates, 117 were *S. aureus* and 53 isolates were CoNS. Among 117 isolates of erythromycin resistant *S. aureus*, 100 isolates (85.47%) were also resistant to clindamycin while only 17 isolates showed susceptible to clindamycin but all of these isolates revealed inducible clindamycin resistant phenotype (D-shape positive). In contrast, only 1/13 (7.69%) of erythromycin resistant with clindamycin susceptible was D-shape positive.

Although in general, the *erm*A gene showed to be predominating in erythromycin resistant *S. aureus* (94/117 isolates), the *erm*C gene was found more frequently (11/17 isolates) among D-shape positive isolates. In contrast to *S. aureus*, 34 of 40 isolates (85%) of erythromycin-clindamycin resistant CoNS carried the *erm*C gene. In addition, all of the D-shape negative CoNS which revealed the MSB phenotype were negative for all tested *erm* genes. The *erm*B gene was rarely found. Only 3 isolates of *Staphylococcus* spp. in this study carried the *erm*B gene. The details of *erm* genes distribution is shown in Table 5.6.

Table 5.6 Correlation between phenotypic of erythromycin-resistance and multiplex PCR results

Organism and Phenotype[a]	No. of isolates with genotype		
	*erm*A	*erm*B	*erm*C
S. aureus (n= 174)			
E^R, CC^S (D-shape +) (17)	5	1	11
E^R, CC^R (100)	88	1	11
E^S, CC^S (57)	0	0	0
CoNS (n= 76)			
E^R, CC^S (D-shape +) (1)	0	0	1
E^R, CC^S (D-shape -) (12)	0	0	0
E^R, CC^R (40)	5	1	34
E^S, CC^S (23)	0	0	0

[a] Determined by disk diffusion test

E = erythromycin, CC = clindamycin, S = susceptible, R = resistance

D-shape = Inducible clindamycin resistant phenotype

5.6 Correlation between phenotypic of disk diffusion test of erythromycin and clindamycin among methicillin resistant *S. aureus* (MRSA) and methicillin susceptible *S. aureus* (MSSA) and the presence of *erm* genes

Of 174 *S. aureus* isolates, 101 and 73 were MRSA and MSSA, respectively. Among 101 isolates of MRSA, only 3 isolates (2.97%) were susceptible to erythromycin while 54 isolates (73.97%) of MSSA were susceptible. All of these isolates also susceptible to clindamycin and contained none of the tested *erm* genes. The majority of erythromycin resistant MRSA at 92/98 isolates (93.88%) were also resistant to clindamycin and 88/92 isolates (95.65%) carried the *erm*A gene. In contrast, the *erm*C gene showed to be predominating among MSSA as 16/19 (84.21%) of erythromycin resistant MSSA carried this gene. Interestingly, all of the erythromycin-clindamycin resistant MSSA were controlled by the *erm*C gene. In addition, the D-shape positive phenotype was more frequently found among erythromycin resistant MSSA (57.89%) than the erythromycin resistant MRSA (5.94%). The details of *erm* genes distribution is shown in Table 5.7.

Table 5.7 Correlation between phenotypic of disk diffusion test of erythromycin (E) and clindamycin (CC) among methicillin resistant *S. aureus* (MRSA) and methicillin susceptible *S. aureus* (MSSA) and the presence of *erm* genes

Organism and Phenotype[a]	No. of isolates with genotype		
	*erm*A	*erm*B	*erm*C
MRSA (n=101)			
E^R, CC^S (D-shape +) (6)	2	1	3
E^R, CC^R (92)	88	1	3
E^S, CC^S (3)	0	0	0

Table 5.7 Correlation between phenotypic of disk diffusion test of erythromycin (E) and clindamycin (CC) among methicillin resistant *S. aureus* (MRSA) and methicillin susceptible *S. aureus* (MSSA) and the presence of *erm* genes (cont.)

Organism and Phenotype[a]	No. of isolates with genotype		
	*erm*A	*erm*B	*erm*C
MSSA (n=73)			
E^R, CC^S (D-shape +) (11)	3	0	8
E^R, CC^R (8)	0	0	8
E^S / CC^S (54)	0	0	0

[a] Determined by disk diffusion test

E = erythromycin, CC = clindamycin, S = susceptible, R = resistance

D-shape = Inducible clindamycin resistant phenotype

5.7 Correlation between phenotypic of disk diffusion test of erythromycin and clindamycin among methicillin resistant CoNS (MRCoNS) and methicillin susceptible CoNS (MSCoNS) and the presence of *erm* genes

Similar to *S. aureus*, isolates, only 3 isolates (5.56%) of MRCoNS were susceptible to erythromycin while 11 isolates (50%) of MSCoNS were susceptible. However, the *erm*C gene was the most common *erm* genes distributed in CoNS population both MR and MS isolates. The prevalence of ermC gene among the erythromycin-clindamycin resistant MRCoNS and MSCoNS was 83.87 and 88.87%, respectively. In addition, most of the erythromycin resistant – clindamycin susceptible isolates were D-shape negative and these isolates contained none of the tested erm genes. The details of *erm* genes distribution is shown in Table 5.8.

Table 5.8 Correlation between phenotypic of disk diffusion test of erythromycin (E) and clindamycin (CC) among methicillin resistant CoNS (MRCoNS) and methicillin susceptible CoNS (MSCoNS) and the presence of *erm* genes.

Organism and Phenotype[a]	No. of isolates with genotype		
	*erm*A	*erm*B	*erm*C
MRCoNS (n=54)			
E^R, CC^S (D-shape +) (1)	0	0	1
E^R, CC^R (31)	5	0	26
E^R, CC^S (D-shape -) (10)	0	0	0
E-S, CC^S (3)	0	0	0
MSCoNS (n=22)			
E^R, CC^S (D-shape-) (2)	0	0	0
E^R, CC^R (9)	0	1	8
E-S, CC^S (11)	0	0	0

[a] Determined by disk diffusion test

E = erythromycin, CC = clindamycin, S = susceptible, R = resistance

D-shape = Inducible clindamycin resistant phenotype

CHAPTER VI
DISCUSSION

Rapid identification of *S. aureus* and detection of antibiotic resistant genes by multiplex PCR has been described previously (11, 56-58). Five target genes including *fem*A gene for species specific (found only in *S. aureus*) and 4 different antibiotic resistance genes (*mec*A, *erm*A, *erm*B, and *erm*C genes) were selected for multiplex PCR. Selection of the antibiotic resistant genes was based on frequent resistance found in *S. aureus* to clinically relevant antibiotics (58-60).

Prior to the unknown testing, the multiplex PCR of these target genes was developed. Testing of specific primers was started by performing as singleplex PCR against control strains. Each individual primer revealed a proper size. The annealing temperature was further varied from 52 -56^{o}C in order to find the optimal condition of each primer. The result demonstrated that the annealing temperature at 52^{o}C was the optimal temperature for all primers. The band of *erm*C gene was unable to be observed after rising the annealing temperature to 56^{o}C. Therefore, the annealing temperature at 52^{o}C was selected for multiplex PCR development.

The first attempt of multiplex PCR development was partly success. Only 3 bands of *fem*A, *mec*A and *erm*C were demonstrated. In order to resolve the problem of missing bands (*erm*A and *erm*B), the amounts of each primer were adjusted according to the appearance of each band. However, the result remained the same. It suggested that the amount of primers might not be the major cause of missing bands. Therefore, the next attempt was focused on other factors such as component in the master mix. By changing the master mix to 2X Phusion Blood Direct PCR Master Mix, the multiplex PCR was completed. All five target bands were observed.

A pilot testing of the multiplex PCR was performed with a small group of unknown samples. The results revealed some of non-specific bands with the size close to either *mec*A or *erm*C bands. Therefore, the initial attempt to solve this problem was performed by rising the annealing temperature from 52 to 54^{o}C. However, the non-

specific bands remained. The next approach was to dilute the extracted DNA in order to increase the purity of DNA as well as maintained the annealing temperature at 54^{o}C. This protocol could remove the non-specific bands from the multiplex PCR. Therefore, it is recommended that DNA sample from boiling extraction procedure need to be diluted in order to avoid the non-specific bands and increase the accuracy of the result from multiplex PCR.

In this study, the *fem*A gene was selected as a species specific marker for *S. aureus*. A total number of 250 isolates of *Staphylococcus* spp. consisted of 176 *S. aureus* isolates and 74 CoNS. The *fem*A gene showed to be an excellent marker for S. aureus with 100% agreement with both biochemical tests and MALDI-TOF. These data confirmed the use of *fem*A for identificatio of *S. aureus* as it was mentioned in other study (63).

Among 250 isolates of *Staphylococcus* spp. used in this study, approximately 62% were methicillin resistance and carried the *mec*A gene. The data also demonstrated that resistance to erythromycin was associated with methicillin resistance. The *erm*A appeared to be the highest prevalence of *erm* genes among *S. aureus* in which over 90% of erythromycin resistance isolates carried this gene. In addition, the *erm*A gene was also associated with MRSA isolates whereas *erm*C genes are mostly spread in MSSA.

In contrast to *S. aureus*, the *erm*C gene was found to be common among CoNS isolates. The frequency of erythromycin resistance in CoNS which was controlled by the *erm*C gene was approximately 85%. These results were concordant with previous studies in other countries (61-63). The *erm*B gene was rare detected in *staphylococci* (2 strains of *S. aureus* and one strain of CoNS). Twelve isolates of CoNS showed the MS phenotype (erythromycin resistant – clindamycin susceptible without D-shape phenomenon). All of these isolates contained none of the tested *erm* genes. It was likely that the efflux pump mechanism controlled by *msr*A gene was responsible for resistance to macrolides and streptogramins B (64). The prevalence of macrolide resistance due to *msr*A gene found in coagulase-negative *staphylococci* more than in *S. aureus* (61, 65).

The method was evaluated by testing 250 isolates from clinical sample, with 100% sensitivity and specificity for *S. aureus* identification, methicillin resistant

mediated by *mec*A as well as erythromycin-clindamycin resistance or erythromycin resistant – clindamycin susceptible with D-shape positive mediated by *erm* genes. However, this multiplex PCR could not distinguish between erythromycin resistant – clindamycin susceptible with D-shape negative (MS phenotype) from erythromycin susceptible isolates due to lack of *erm* genes. Fortunately, from the data, the MS phenotype was rarely found in *Staphylococcus* spp. in particular *S. aureus*.

CHAPTER VII
CONCLUSION

Epidemiology of MRSA is a major problem in many countries around the world. Non β-lactam antibiotics such as macrolide, lincosamide, and streptogramins B (MLS_B) were taken into account for treatment of MRSA infection. In many countries, resistance to these antibiotics was also observed among *Staphylococcus* spp. Routine detection of methicillin as well as MLS_B resistance was time consuming and relied very much on the proper antimicrobial susceptibility testing techniques. Therefore, this study was designed in order to resolve these limitations by using of molecular approach. Five target genes including *fem*A for *S. aureus* identification, *mec*A for methicillin resistance and *erm*A, *erm*B and *erm*C for erythromycin-clindamycin resistance were selected as compositions in a novel multiplex PCR panel. By testing the newly developed PCR panel, the results revealed an excellent correlation with either biochemical testing or MALDI-TOF for identification of the organisms as well as the resistant genes comparing with phenotypic antimicrobial susceptibility results. With only small limitation, this newly developed multiplex PCR panel had shown to be a promising solution for detection of *S. aureus* with methicillin and/or erythromycin resistance.

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APPENDIX

Materials

1. Instruments

- Incubator 35-37º C (Labnet Internationational, Inc., USA)
- Freezer -80 ºC (Forma Scientific, USA)
- Refrigerator 4-8 ºC
- Vortex mixture (Scientific Industries, USA)
- Dry bath (Labnet Internationational, Inc., USA)
- Automated thermal cycler (Biometra, Geramany)
- Electrophoresis system (SCIE-PLUS, LTD, England)
- UV light transilluminator (Biovision+100/20M, Biogenomed-Thailand)
- Automatic pipette (Eppendorf, Germany)
- Microcentrifuge
- Erlenmeyer flasks 500 ml
- Filter tips

2. Supplies

- Sterile loops
- Gloves
- PCR tube (Corning Incorporated, Corning, NY)
- Microcentrifuge tube - 1.5 ml (Treff, Switzerland)

3. Reagent

3.1 Reagent for Multiplex PCR (Master-Mix)

1. Phusion Blood Direct PCR Master Mix (Thermo Scientific)
2. DNase-free and RNase-free Sterile Distilled water (Gibco, USA)
3. primer sets (BioDesign Co., Ltd.)
4. Total genomic DNA

3.2 Reagent for preparation of Gel electrophoresis

1. 100 bp Ladder marker (New England Biolabs, Inc)
2. GelRed (Biotium)
3. Distilled water, Sterile
4. 1X Tris-borate-EDTA buffer

4. Reagents preparation

Lysis buffer

- 1% Triton x-100
- 10 mM Tris-HCl (pH 8.0)

1 mM Disodium ethylene diamine tetra acetate (EDTA)

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