ISOLATION, IDENTIFICATION AND PCR DETECTION OF METHICILLIN RESISTANT *Staphylococcus aureus* (MRSA) IN COW MILK SAMPLES FROM PERAK

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

~	Approximately
λ	Lambda
µg	Microgram
µg/ml	Microgram per millilitre
µl	Micro Litter
API-STAPH	Analytical Profile Index for Staphylococci identification
bp	Base pair
DNA	Deoxyribonucleic Acid
g	Gram
Kb	Kilo base pair
l	Liter
MgCl2	Magnesium Chloride
ml	Milliliter
NaCl	Sodium Chloride
CONS	Coagulase negative *Staphylococcus*
COPS	Coagulase positive *Staphylococcus*
EDTA	Ethylene diamine tetra acetic acid
M	Molar
Min	Minute
MRSA	Methicillin resistant *S. aureus*
MSSA	Methicillin susceptible *S. aureus*

ng	Nano gram
ORF	Open reading frame
PBP2a	Penicillin-binding protein 2a
PCR	Polymerase chain reaction
pmol	picomole
pvl	Panton-Valentine leukocidin
rpm	Revolution per minute
SCC	Staphylococcal cassette chromosome
SCC*mec*	Staphylococcal chromosome cassette methicillin-resistance
TBE	Tris - Borate – EDTA
U	Unit
w/v	Weigh per volume
SEs	*Staphylococcal* enterotoxins
MDR	Multidrug resistant

PEMENCILAN, PENGENALPASTIAN DAN PENGESANAN PCR *Staphylococcus aureus* RINTANG TERHADAP METHICILLIN (MRSA) DALAM SAMPEL SUSU LEMBU DI PERAK

Abstrak

Staphylococci mengkoloni haiwan dan manusia dan keadaan ini biasanya tidak berbahaya. Walau bagaimanapun terdapat beberapa jenis *Staphylococcus* yang mempunyai keupayaan untuk menyebabkan penyakit. Kaedah konvensional yang diguna pakai sekarang iaitu berdasarkan ujian biokimia untuk mengenalpasti staphylococci mengambil masa yang lama. Tambahan pula, kit yang diguna pakai sekarang tidak dapat mengenalpasti perbezaan antara *Staphylococcus* koagulase-negatif (CoNS). Sebagai alternatif, kaedah PCR amat berguna untuk mengenal pasti strain patogen *Staphylococcus*. Dalam kajian ini sampel *Staphylococcus* telah diasingkan daripada susu lembu dan dicirikan berdasarkan kaedah fenotipik. Sebanyak dua ratus dua puluh lima pencilan dipencil dari empat pusat pengumpulan iaitu Taiping, Tapah, Parit dan Sungai Siput telah diuji. Strain *S. aureus* telah diuji berdasarkan hemolisis beta, pewarnaan Gram, ujian koagulase, ujian katalase, ujian gula mannitol dan ujian gula maltosa. Sampel yang positif diuji dengan ujian rintangan terhadap kesan antibiotik dengan menggunakan 1μg cakera oksasillin. Keputusan ujian rintangan terhadap kesan antibiotik mengesahkan bahawa 19 pencilan *S. aureus* (12.41%) telah didapati menunjukkan ciri-ciri rintangan terhadap ancaman antibiotik (MRSA), manakala 1 pencilan (0.65%) menunjukkan rintangan pertengahan dan 133 (86.93%) sensitif terhadap kesan ancaman antibiotik (MSSA). Di samping itu, pengesanan MRSA dalam susu telah dilakukan menggunakan kaedah PCR untuk mengesahkan kehadiran fragmen 310 bp gen *mecA* dan fragmen 686 bp

gen *femA*. Sepuluh pencilan memiliki gen *mecA* manakala dua daripada pencilan tersebut tidak memiliki gen *femA* yang juga diklasifikasikan sebagai *S. aureus* koagulase Negatif (CoNS). Satu dikategorikan sebagai bukan *Staphylococcus* kerana memberi keputusan negatif dalam ujian koagulase dan katalase. Satu daripada strain tersebut adalah sensitif terhadap ujian kesan antibiotik tetapi mempunyai gen *mecA* dan *femA*. Ini mungkin kerana pencilan tersebut menggunakan mekanisme yang berbeza untuk menunjukkan rintangan terhadap metisillin. Data daripada kajian ini menunjukkan bahawa kaedah fenotip sahaja tidak mencukupi untuk mengenal pasti MRSA. Kajian ini meningkatkan kebolehpercayaan kaedah PCR dalam pengesanan *S.aureus* dan MRSA serta akan menjadi pelengkap kepada ujian biokimia yang sedia ada.

ISOLATION, IDENTIFICATION AND PCR DETECTIONOF METHICILLIN RESISTANT *Staphylococcusaureus* (MRSA) IN COW MILK SAMPLES FROM PERAK

ABSTRACT

Staphylococci colonize a diverse range of animals and human and this association is normally harmless. Some strains of *Staphylococcus* have increased ability to cause disease. Biochemical tests used to identify staphylococci are lengthy. Furthermore, commercially available panels do not allow a reliable distinction between different coagulase-negative staphylococci (CNS). As an alternative, the development of a PCR based technique will be useful to identify pathogenic strains of *Staphylococcus*. In this study *Staphylococcus* samples were isolated from cow milk and characterized using phenotypical methods. Two hundred and twenty five isolates identified from four different collection centers in Taiping, Tapah, Parit and Sungai Siput were tested. *S. aureus* strains were characterized by beta hemolysis, Gram stain, coagulase test, catalase test, mannitol fermentation and maltose fermentation. The positive samples were further investigated by antibiotic susceptibility test by using 1µg oxacillin disc. Result of antibiotic susceptibility test confirmed that 19 *S.aureus* isolates (12.41%) were found to be methicillin resistant *S. aureus* (MRSA), while 1 isolate (0.65%) showed intermediate resistance and 133 (86.93%) as methicillin sensitive *S. aureus* (MSSA). In addition, detection of MRSA in milk was performed using PCR to confirm the presence of a 686 bp *femA* fragment and a 310 bp *mecA* fragment which are essential in MRSA identification. Ten samples were positive for the *mecA* gene whereas three samples did not possess the *femA* gene. Two of them were classified as Coagulase Negative *S. aureus*. One was categorised as non-*Staphylococcus* isolate since it was negative for coagulase and

catalase test. One strain was susceptible in oxacillin disk diffusion test but possesses *mecA* and *femA* genes. This may be because the isolate is heteroresistant. Data from this study indicated that phenotypic method alone was not adequate for identifying MRSA. This study will improve the reliability of detection for *Staphylococcus* using PCR to complement existing biochemical tests and will be very useful for rapid detection of pathogenic species of *S. aureus* and MRSA.

CHAPTER 1

INTRODUCTION

1.1 Study Background

Staphylococcus is a bacteria which does not form spore, it is non-motile and it is spherical in shape. The size of *Staphylococcus* is approximately 1 µm in diameter. This Gram positive bacteria look like bunches of grapes. The name *staphyle,* came from a Greek word which means *"bunches* of grapes*".* Pathogenic strains of *S. aureus* strains grow in a hemolytic pattern in which red blood cells ruptured by *Staphylococcus*. *Staphylococcus* can be easily noticeable when there is clearing of zones in blood agar (Vanderhaeghen *et al*., 2010). Coagulase production is the key factor to identify pathogenic *Staphylococcus* since it is not present in other genus. There are some strains which are atypical which do not produce coagulase and are known as "Coagulase Negative *Staphylococcus*" (CoNS) (Graveland *et al*., 2011). *S. aureus* has become a major concern in the genus of *Staphylococcus* because it causes serious problem in animals.

Antibiotic usage is a common method of treating infection caused by *Staphylococcus* in veterinary medicine (Van-Bambeke *et al*., 2008). Introduction of β-lactam antibiotics such as penicillin in the early 1940s in healthcare industries were continued with the emergence of penicillin resistance (Bhattacharya, 2014).

To overcome penicillin resistance semisynthetic penicillinase-stable penicillin related compounds such as methicillin and oxacillin were synthesized. Methicillin were started to be used in hospitals in 1961. In a few years after introduction *S. aureus* have lost their effectiveness against methicillin and this phenomenon known as methicillin-resistant *S. aureus* (MRSA) emerged (File, 2008). Altered penicillin-binding protein PBP2a is responsible for the rise in methicillin resistance (There & Wadhai, 2013).

The emergence of Multidrug resistant (MDR) MRSA cause increase in the cost and treatment time. Sometimes improper treatment also increases the rates of morbidities in livestock animals. Correct selection of antimicrobial agent for treatment is extremely important that methicillin resistant *S. aureus* must be quickly recognised. Fast and reliable method is important to identify resistant isolate so that proper treatment can be given (Liu *et al*., 2011).

One of the genes responsible for methicillin resistance has been designated as *mecA*. The *mecA* gene is found in mobile genetic element named as SCC*mec* is present in most of the MRSA strain (Popovich & Weinstein, 2009). The *mecA* gene that found in MRSA acts by synthesizing penicillin binding protein 2A (PBP2A). The *mecA* gene in MRSA encodes a new β-lactam insensitivity to penicillin (Aklilu *et al*., 2010).

The conventional method in identifying *S. aureus* and MRSA is insufficient because there are reports of some MRSA which has *mecA* but unable to be detected

by biochemical test. There could also be coagulase negative MRSA. This problem can be overcome with the use of PCR which is highly sensitive tool to detect strain of *S. aureus* which carries the *mecA* gene. Simultaneous application of conventional method with PCR method could be very useful in determining MRSA strains which harbour the *mecA* gene (Holmes & Zadoks, 2011).

The studies on MRSA that were performed in Malaysia mainly focused on humans and healthcare industries. However, livestock associated reports are still available but very limited. It mainly focuses on pig, dog, cow and other animals (Aklilu *et al*., 2010; Neela *et al*., 2009).

This study would give us a definitive idea or would recommend the most appropriate test for diagnosing methicillin resistance in *S. aureus* with utmost accuracy and speed which is very much needed to avoid false positives and false negatives, as well as for early diagnosis. This in turn helps to prevent the emergence of new antibiotic-resistant strains as well as the spread of MRSA.

The use of molecular techniques will be evaluated and compared to phenotypic methods to propose as complementary method with biochemical method for the rapid identification and characterisation of MRSA isolates.

1.2 Objectives

The main objectives of this study were:

1) To isolate and determine the level of contamination by *S. aureus* found in milk samples collected in Perak.
2) To determine antibiotic resistance profiles of *S. aureus* isolates from the milk samples .
3) To determine best DNA extraction methods for *S. aureus* from pure culture
4) To characterise MRSA isolate using duplex PCR assay.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Staphylococcus aureus*

2.1.1 Classification of *S. aureus*

S. aureus belongs to the family *Staphylococcaceae* (Table 2.1). It comprises of 41 known species and subspecies that are indigenous to humans. It grows in a grape like cluster when grown on solid media (Vos *et al*., 2009).

2.1.2 Morphology and characteristics of *S. aureus*

S. aureus is Gram-positive bacterium. *S. aureus* is an opportunistic bacteria. Which colonises a host normally harmless or cause mild infections, usually limited to the skin. However, once the host becomes severely ill the opportunities for the *S. aureus* to manifest itself as a dangerous and invasive threat to human life are greater (Van-Belkum & Melles, 2009). Pathogenic strains often promote infections by producing potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies. This facultative anaerobic bacteria resemble grape-like clusters under the microscope. However, *S. aureus* cocci can appear either as single cells or in pairs or short chains. *S. aureus* is round in shape, and the diameter of the coccus is approximately 1 µm. The bacteria form golden

Table 2.1: Summary of the classification of *S. aureus*

Domain	**Bacteria**
Kingdom	Eubacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	*Staphylococcaceae*
Genus	*Staphylococcus*
Species	*Staphylococcus aureus*

(Vos *et al*., 2009)

yellow colonies and usually produce beta-hemolysis (Samie, 2011). *S. aureus* is catalase positive bacteria and able to convert hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2). *S. aureus* also has the ability to produce the coagulase enzymes which has been used as an important tool to differentiate *S. aureus* from the most other *Staphylococcus* (Sasaki *et al*., 2010). These bacteria will cause clot formation when mixed with citrated rabbit plasma (Sasaki *et al*., 2010). There are some strains, which are unable to produce coagulase, and it is known as coagulase-negative *Staphylococcus* although majority of *S. aureus* is coagulase positive (Milanov *et al*., 2010).

S. aureus grows on non-selective medium such as blood agar. The colonies of *S. aureus* are convex, smooth, and sharply defined at room temperature (20ºC to 25ºC) when it is grown on blood agar and often with hemolysis. The colonies are gold pigmented due to carotenoid. *S. aureus* may not grow in anaerobic condition and in liquid media (Kim *et al*., 2012).

2.2 Methicillin resistant *S. aureus* (MRSA)

2.2.1 Historical perspectives

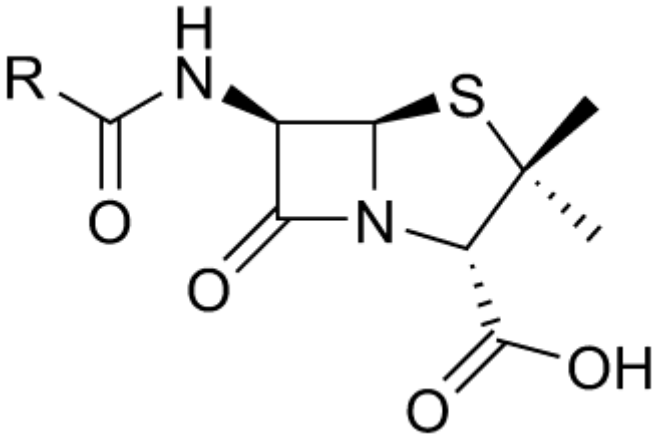
Penicillin was introduced for medical use as an antistaphyloccal in 1940. However, the resistance of staphylococci to penicillin was recognised in 1942 by

Rammelkamp. This became worse when more than 80% of both hospital and community staphylococcal isolates were reported to be resistant to penicillin in the 1960's (Stein, 2011).

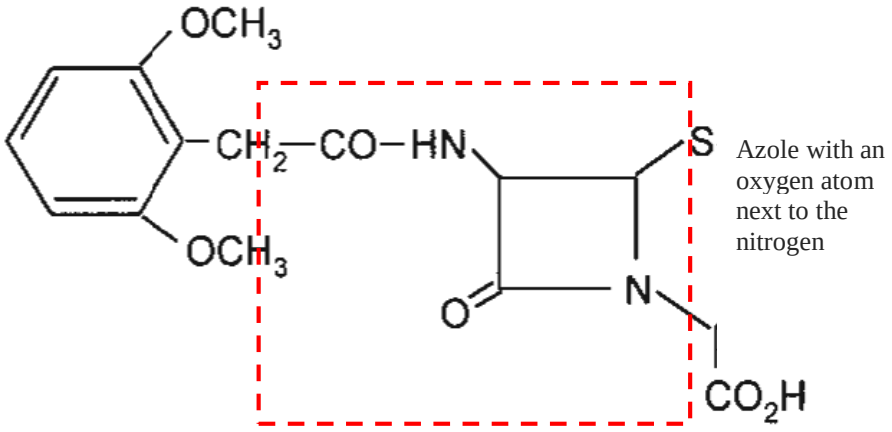
Kirby was the first scientist who successfully proved the inactivation of penicillin in *S. aureus* strains in 1944. He demonstrated the action of the enzyme penicillinase hydrolysing beta-lactam ring of penicillin (Stein, 2011). This problem was overcame by the introduction of methicillin and flucloxacillin antibiotics, which are isoxazolyl group of penicillin (Figure 2.1). The basic structure of penicillin is a nucleus consisting of a beta-lactam ring and a side chain. Isoxazole group is an azole with an oxygen atom next to the nitrogen. Isoxazolyl is the univalent radical derived from isoxazole. The beta-lactam ring present in this antibiotic is important for antibacterial activity. The side chain regulates the pharmacologic properties and the antibacterial spectrum. Unfortunately, emergence of methicillin resistance within one year has posed challenges for the healthcare industry (Van Hoek *et al*., 2011). New avenues for vaccination need to be explored, because no vaccine is currently available to treat *Staphylococcus* infection (Smith, 2015).

2.2.2 Mechanisms of antibiotic resistance in *S. aureus*

There are two mechanism of antibiotic resistance in *S. aureus.* The first involves *blaZ* gene which mediates penicillin resistance and the second involves *mecA* gene which mediates methicillin resistance. The *blaZ* gene mediates penicillin resistance by encoding for beta-lactamase. In all, there are five types of *blaZ* genes



Penicillin



Methicillin

Figure 2.3: The chemical structures of β-lactam antibiotics Penicillin and Methicillin

(Shah *et al*., 2008)

have been identified which are types A, B, C, D and E. The staphylococci produces the beta-lactamase enzyme in the presence of all the beta-lactam drugs includes penicillin, methicillin and its derivatives (Shore *et al*.,2011). The adjacent regulatory genes (repressor, anti-repressor and *blaI*) functions to regulate *blaZ* gene to produce beta lactamase when the cells are exposed to penicillin (Figure 2.2). A protein which is responsible as trans membrane sensor-transducer is cleaved when *S. aureus* is exposed to beta-lactam antibiotics. This protein acts as a protease which function in cleaving the BlaI directly or indirectly (Hisata *et al*.,2011).

The *mecA* gene mediates methicillin resistance by encoding for penicillin binding protein 2a which also known as PBP2a. It will synthesize cell wall in the presence of lactams. The SCC*mec* is a mobile genetic element of *Staphylococcus* bacterial species. The regulator gene *mecR1* is activated by beta- lactam antibiotics, which serve as a signal transducer and function to induce the expression of *mecA* and m*ecR1-mecI-mecR2* (Figure 2.3). The *mecR2* will continuously induce *mecA* because it will inactivate the *mecI* by proteolytic cleavage. *MecR1* which is found in cell membrane in the inactive form in the absence of beta-lactam antibiotic. It will be in the inactive form because stable *mecI* dimers bound to the residual copies of *mecR1* and *mecA* promoter (Arade *et al*, 2012; Shore *et al*., 2011).

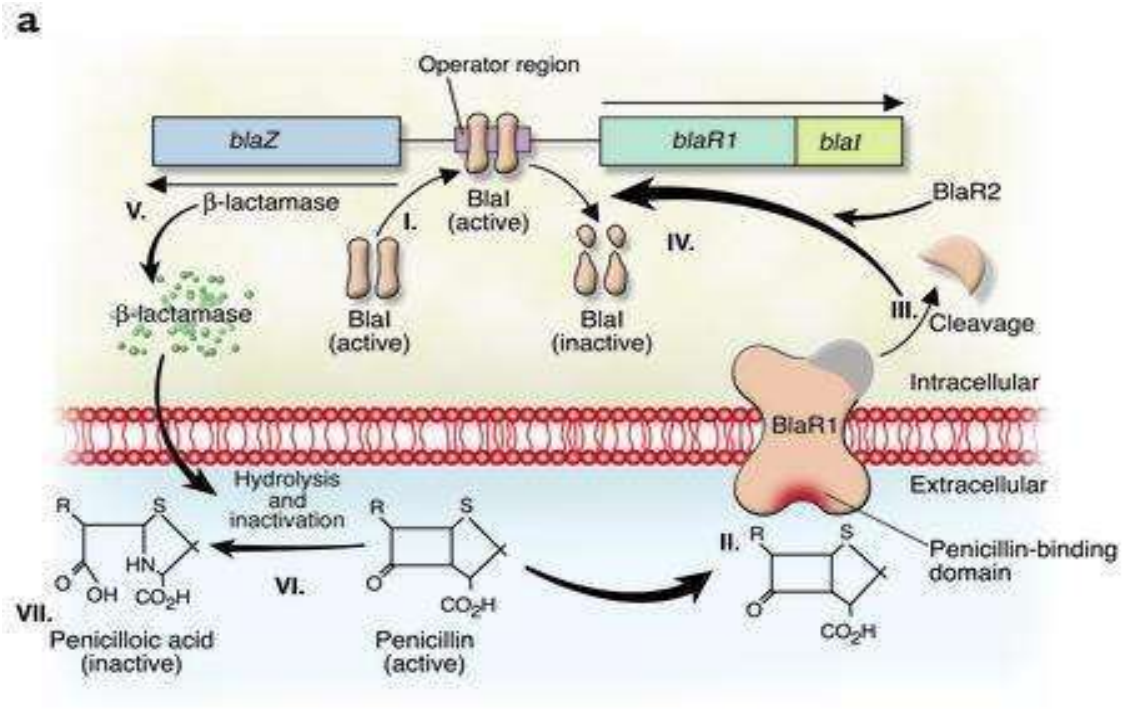


Figure 2.2:

Induction of staphylococcal β-lactamase synthesis in the presence of the β-lactam antibiotic penicillin. I) The DNA-binding protein BlaI binds to the operator region, thus repressing RNA transcription from both blaZ and blaR1- blaI. In the absence of penicillin, β-lactamase is expressed at low levels. II) Binding of penicillin to the transmembrane sensor-transducer BlaR1 stimulates BlaR1 autocatalytic activation. III–IV) Active BlaR1 either directly or indirectly (via a second protein, BlaR2) cleaves BlaI into inactive fragments, allowing transcription of both blaZ and blaR1-blaI to commence. V–VII) β-Lactamase, the extracellular enzyme encoded by blaZ (V), hydrolyzes the β-lactam ring of penicillin (VI), thereby rendering it inactive (VII).

(Boyle-Vavra & Daum, 2007)

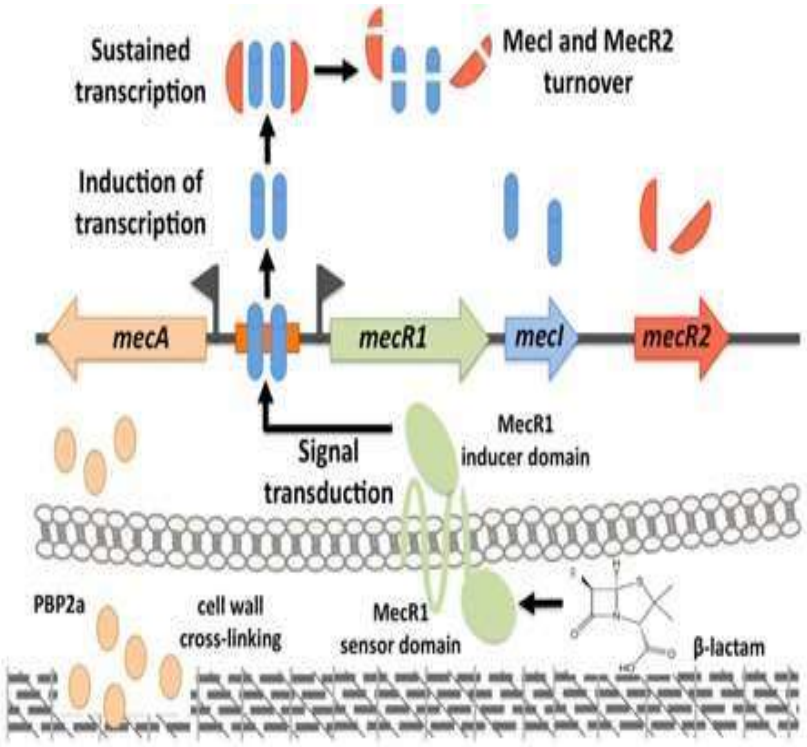


Figure 2.3: Mechanism of *S. aureus* resistance to methicillin. Synthesis of PBP2a proceeds in a fashion similar to that described for β-lactamase. Exposure of MecR1 to a β-lactam antibiotic induces MecR1 synthesis. MecR1 inactivates MecI, allowing synthesis of PBP2a. MecI and BlaI have coregulatory effects on the expression of PBP2a and β-lactamase.

(adapted from Arade *et al*, 2012)

2.3 MRSA infections in animals

MRSA became a major problem in human medicine for many years. Now this problem is increasing in veterinary medicine with various infections being reported involving animals. ST398 is the first discovered livestock-associated MRSA strain. The ST398 strain is pandemic. Researchers have found that the animal-derived strains are different than the strains from human (Smith, 2015).

MRSA is one of the major causes of mastitis in animals. The preliminary MRSA strains were isolated from cow with mastitis in 1972 (Kluytmans, 2010; Magalhaes *et al*., 2010). Mastitis cases involving cows is mainly caused by milking practices and infections have also been discovered in poultry. This infection is caused by transmission of *S. aureus* between humans and animals. *S. aureus* is mainly found in the digestive tract and mucosa of upper respiratory of birds, snakes and mammals (Kluytmans, 2010).

Studies showed that MRSA colonisations and infections are mostly concentrated on livestock animals involving swine, cattle and poultry. The first identified livestock associated MRSA strains were mainly on pigs. Infected farm workers contracted similar strains as the strain that colonizes pigs (Li *et al*., 2011). In addition to swine, MRSA was discovered in chicken meat, cattle and poultry (Persoons *et al*., 2009). The hospital acquired MRSA is different from livestock associated MRSA (Alzohairy, 2011). However, based on molecular typing studies, it has been found that all those *S. aureus* human infections were ancestors of *S. aureus* found in animals.

Leonard and Markey suggested that MRSA emerging in companion animals such as dogs, cats and some other animals such as pigeons, and guinea pigs were mainly transmitted from human (Leonard & Markey, 2008).

2.4 MRSA in cow milk

Increases in the number of MRSA in cow's poses a high risk to veterinarians, veterinary related workers and livestock animals. Studies relating to prevalence and genetic spread are very limited. Initially, very low incidence of MRSA in cow and cow milk was reported internationally (Blowey & Edmondson, 2010). The number of MRSA infection cases is increasing nowadays. Some studies show that *S. aureus* was identified in milk samples from individual cow udders. The prevalence rate of *S. aureus* increased and reached the third most reported food-borne related disease worldwide (Normanno *et al*., 2005).

S. aureus is present in cow also known as an opportunistic pathogen. It is a major source of mastitis in cows. This species spread from human to animal and even from animal to human. Animals and farmers became major source of MRSA outbreak. Over usage of antibiotics on farm animals causing cross resistance between human and animals. Generally antibiotic is used in cow to treat predominant infections such as mastitis caused by *S. aureus*. Antibiotic treatment is also practised as precautionary action during dry cow therapy (Blowey & Edmondson, 2010).

The increase in the incidence of MRSA cases in clinical infection and veterinary, causing increase awareness about MRSA infection in veterinary industries (Magalhaes *et al*., 2010).

2.5 Genes involving antimicrobial resistance in *S. aureus*

In *Staphylococcus* species, *mecA* (methicillin), *blaZ* (penicillin) *tetM/tetK* (tetracycline), *ermA/ermC* (erythromycin), *fexA* (chloramphenicol), *qnrA* (fluoroquinolone), *lnuA* (lincosamide), *aacA/aacD* (aminoglycoside) and *msrA/msrB* (macrolide) are among the antibiotic resistance genes that have been reported. The gene used to detect *S. aureus* are 16S rRNA, *nuc* gene, *eap* gene *fem* gene and IS431 gene (Giannouli *et al*., 2010).

2.6 Species specific gene and methicillin resistant gene found in *S. aureus*

The *mecA* gene allows *S. aureus* to be resistant to antibiotics such as methicillin. *femA* gene is generally found in all *S. aureus* species (Li *et al*., 2008).

2.6.1 *femA*

Factor Essential for Methicillin-resistance (*femA)* gene is the gene that is universally present in all *S. aureus*. *femA-femB* operons are chromosomally determined factors, which also play a role as regulatory gene which regulate formation of the pentaglycine interpeptide bridge that serves as the crosslink of

peptidoglycan. The *femA-femB* operon is essential to express methicillin resistance in *S. aureus* (Donovan, 2007). The *femA gene* product which is a 48-kDa protein generally present in active cells and crucial for cell wall metabolism. Interestingly, *femA* is the gene exclusively found in *S. aureus*. It is not found in other *Staphylococcus* species (Francois *et al*., 2007).

2.6.2 *mecA*

The *mecA* gene is the gene responsible in causing resistance in *S. aureus* to beta-lactam antibiotics. The *mecA* gene is found in a few *Staphylococcus* species, including *Streptococcus pneumoniae* and *S. aureus*. This gene causes *S. aureus* to be highly resistant to antibiotics such as methicillin, penicillin and other penicillin-like antibiotics. The *mecA* gene found in the mobile genetic element which also known as staphylococcal cassette chromosome *mec* (SCC*mec*) in MRSA (Tsubakishita *et al*., 2010).

The *mecA* gene will inhibit the ring-like structure of beta-lactam antibiotics from binding to the beta-lactam binding protein. This will enable the cell wall synthesis of the *S. aureus*, and the replication occurs as normal. The penicillin- binding protein 2a (PBP2a) is encoded by the *mecA* gene and has a low affinity for penicillin and penicillin derivatives. This will trigger the transpeptidase activity in the presence of beta-lactams, preventing them from inhibiting cell wall synthesis (Li *et al*., 2008).

2.6.3 SCC*mec* Elements

The mobile genetic element which is known as staphylococcal cassette chromosome *mec* (SCC*mec*) is found in *S. aureus* and other *Staphylococcus* bacteria species. The *Staphylococcus* strain will spread the gene through horizontal gene transfer. There are 11 types of SCC*mec* elements that have been identified. All the 11 types (type I-XI) of SCC*mec* elements are not identical and there is some SCC*mec* which exist without *mec* gene. The classification is based on the nucleotides of two specific regions that exist in this element. One region is the *mec* complex, which encodes resistance to methicillin, and the *ccr* complex, which encodes recombinase enzymes responsible for the mobility of the genetic element (Tulinski *et al*., 2012). The *ccr* gene complex mediates the site-specific excision and insertion of the SCC*mec* cassette either out of or into the bacterial genome and the class of *mec* complex that they bear (Ito *et al*., 2014).

The first three SCC*mec* elements were designated as types I, II, or III (Ito *et al*., 1999). This SCC*mec* types I to III were present in hospital acquired MRSA strains and were likely transferred to *S. aureus* from a commensal staphylococcal species on a few occasions (Zhang et *al*., 2008). The type II and III SCC*mec* elements include sites, associated with junkyard regions, which facilitate insertion of genes conferring non-lactam resistance phenotypes. These were followed by reports of SCC*mec* types IV-VIII (Ma *et al*., 2002). Studies shows that type IV SCC*mec* element have been transferred to a methicillin susceptible *Staphylococcus aureus* (MSSA) strain. The SCC*mec* type IV reported among both community acquired MRSA and hospital acquired MRSA (Zong *et al*., 2011). SCCmec type IV is

nonmultiresistant oxacillin-resistant *S. aureus*. SCC*mec* type V is distributed among CoNS. Further two types of SCC*mec* were identified which is type X and type XI. Limited information available on distribution of type X and XI (Figure 2.4) (Ito *et al*., 2014).

There are a few other genes present in SCC*mec* other than *mecA* which including gene such as cytolysin gene *psm-mec*. This gene functions to repress virulent properties found in Methicillin-Resistant *S. aureus* (Kaito *et al*., 2011). The SCC*mec* is integrated in a site named as *orfx* which is found in the chromosomal site of *S. aureus* (Noto *et al*., 2007).

2.6.4 *mec* complex

There are five types of *mec* gene complexes, which is known as class A, IS*431- mecA-mecR1-mecI*; class B, IS*431-mecA*-Δ*mecR1-IS1271*; class C, IS*431-mecA*-Δ*mecR1-IS431*;class D, IS*431-mecA*-Δ*mecR1* and class E *mec* complex *blaZ-mecA- mecR1-mecI* (Ito *et al*., 2014). The different types of *mec* complex form because the *mecI* gene is either completely deleted or mutated in *Staphylococcus* strains. The class A *mec* complex is the ancestral which gave this to MRSA strains, which express PBP2a only when induced by beta-lactams. The class A *mecA* gene complex form when sequence IS*431mec* is inserted. The class A *mec* complex and the class B *mec* complex, express PBP2a protein constitutively (David & Daum, 2010). The class B *mec* complex forms where the 3′ part of *mecR1 and the mecI* gene are deleted and a fragment of sequence IS*1272 is inserted (*Ito *et al*., 2014).

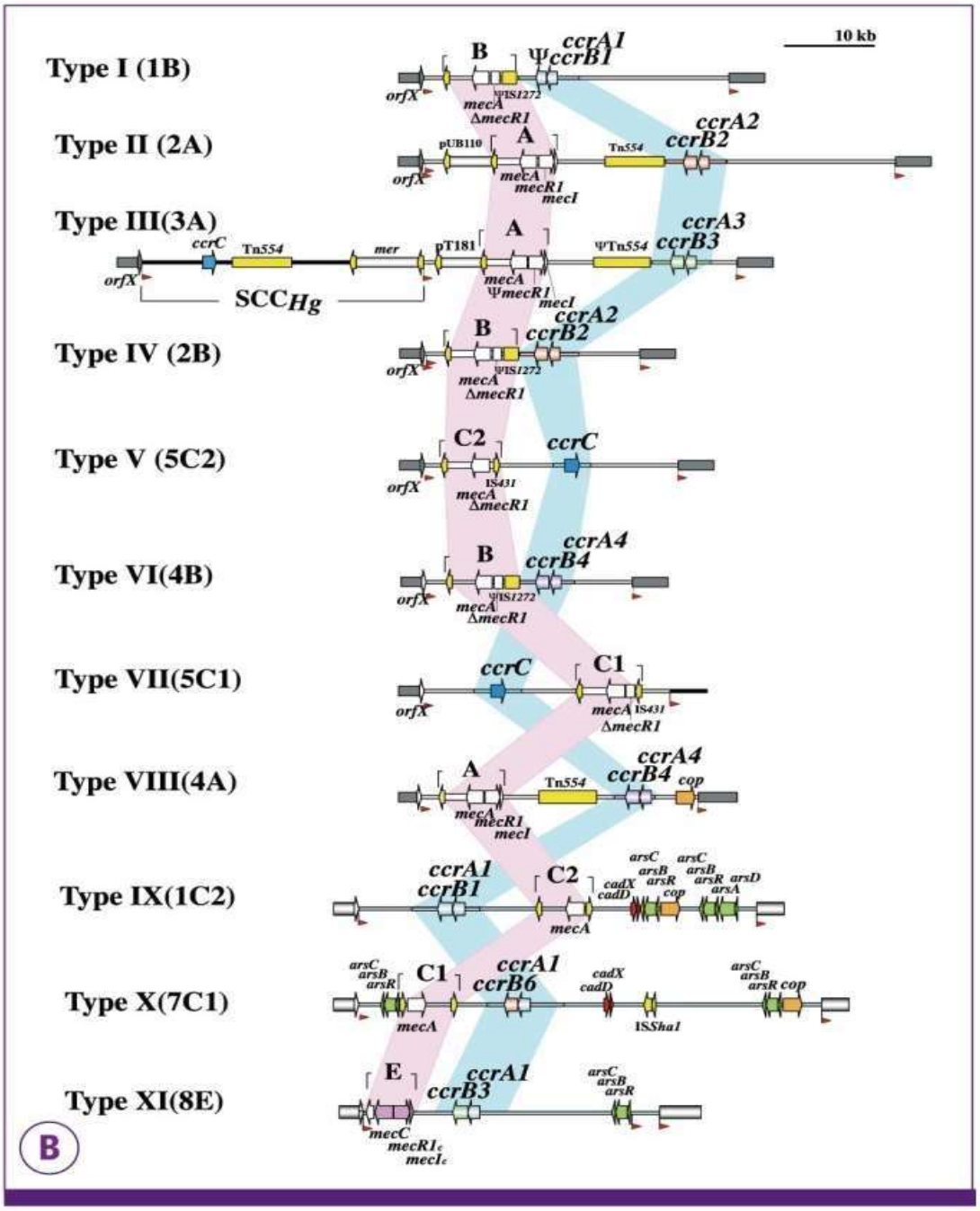


Figure 2.4 : The structures of 11 types of SCCmec are illustrated based on the nucleotide sequences. It is distinguished by the type of *ccr* gene complex that mediates the site-specific excision and insertion of the SCC*mec* cassette. Direct repeats that comprise integration site sequences of SCC are located at both extremities of SCC*mec* (the red arrowheads). The location of five (A-E) classes of mec-gene complexes is indicated by pink belt. The locations of ccr-gene complexes are indicated by blue belt. Insertion sequences and transposons are indicated in yellow.

(adapted from Hiramatsu et al., 2013)

Two more *mec* complexes have been identified, called classes C and D. Research conducted by Katayama *et al*. (2000) indicated that the methicillin-resistant level of class C strains were intermediate in level when compared to other classes in spite of carriers of *mecA* gene. The class D complex is only present in CoNS strains. The *mecI* gene is hugely found in coagulase-negative Staphylococcal species. The allelic replacement inactivates the *mecI* and will increase oxacillin resistance by several times. This shows that *mecI*-mediated repression is very important to lower antibiotic resistance. It also found that intact *mecI* in *Staphylococcus* strains cause low level beta-lactam resistance expression. The strains of *S. aureus* does not have IS431 nor the IS127 is catogorised as class D *mec* type (Hisata *et al*., 2011).

2.7 Antimicrobial resistance of *S. aureus* strains

The ability of *S. aureus* to adapt to the environmental conditions makes it as an important pathogen worldwide. Widespread use of antibiotics and drugs on *S. aureus* cause resistant strains widely dispersed around the world and cause serious infection (Chambers & DeLeo, 2009). The most commonly known resistance of *S. aureus*, is methicillin resistance, which is a major cause of *S. aureus* infection in hospitals and the community. Antibiotic resistance in *S. aureus* is triggered by conjugation and transduction in chromosomes or plasmids. Studies show that a low-level of methicillin resistance is found in *mecA* negative strains of *S. aureus*. The spread of *mecA* negative MRSA strains might possibly arise because of the hyper-production of the enzyme beta-lactamase (Garcea-Alvarez et al., 2011).

2.8 MRSA Transmissibility

MRSA has become pandemic in many countries. The prevalence rate of MRSA clonal lineage ST398, a livestock associated MRSA strains different for each country. The prevalence rate reported for personnel are 45% and for farms are 70%. The ST398 strain is not found in a few countries such as Germany and Netherlands. The prevalence of MRSA in some of the farms may be absent or even very low (Bergonier *et al*., 2014). The difference is caused by the different isolation methods used or because the location of the farm is outside prevailing area. Differences in the sampling site, isolation period, time-point of production, isolation methods used and the type of farm the samples taken gave these results. The exposed antimicrobial agent also affects the result obtained in the study (Smith, 2015).

The *mec* gene was first isolated in 1972, shows that this gene existed for long time but overlooked for a long time. This is because its dissemination was very slow during that time. The practice of manual detection became harder for detection of the gene during that time and caused it to widely spread (Caveney *et al*., 2011). The testing by using PCR is more effective. It is very hard to predict the evolution of these genes but was possible with the aid of mutation that makes, *S. aureus* become more resistant. The acquisition of extra virulence factors made it more adapted to the hosts and for the survival. Farm trading of animals became one of the transmission routes for MRSA (Fluit, 2012).

2.9 Biochemical identification of *S. aureus* and MRSA isolates

2.9.1 Gram Staining

The Gram staining method, is the initial test used in identifying bacteria in biochemical method. The primary stain that used Gram staining is crystal violet. The Gram positive bacteria have the ability to retain the crystal violet-iodine complex. The bacteria which is unable to retain the crystal violet is known as Gram negative and it appear red. The cell wall of Gram positive bacteria able to retain the stain because if its cell wall properties with higher peptidoglycan and lower lipid content than Gram negative bacteria. Future Iodine is added to form crystal violet-iodine complex. This is to ensure the dye was washed away from the cells. The next step is decolorization to dissolves the lipid layer from the Gram negative cells. The acetone also dehydrates the Gram-positive cell walls to avoid diffusion of violet-iodine complex. The decolourisation should be conducted for 1 minute. Longer exposure to decolorisation agent will remove stain from both types of bacteria. Finally conuterstain is added to give Gram negative bacteria pink in colour (Kumari & Ichhpujani, 2000).

2.9.2 Catalase Test

Catalase and coagulase tests are very useful in identifying *Staphylococcus*. *S. aureus* is catalase-positive. *S. aureus* produces catalase enzyme which neutralize bactericidal effects of hydrogen peroxide (H_2O_2). *S. aureus* produces catalase

enzyme which breakdown hydrogen peroxide into water and oxygen. Catalase test is very useful to differentiate staphylococci from enterococci and streptococci (Sasaki *et al*., 2010).

2.9.3 Coagulase Test

A small percentage of *S. aureus* can be differentiated from most other staphylococci by the coagulase test. *S. aureus* is coagulase-positive which is able to produce coagulase enzyme (Vanderhaeghen *et al*., 2010). These enzymes causes plasma to clot by converting fibrinogen to fibrin, whereas most other *Staphylococcus* species are coagulase-negative. The coagulase enzyme is tightly bound to the surface of *S. aureus* and coat the surface with fibrin when contact with plasma. However, there are some atypical strains which do not produce coagulase even though majority of *S. aureus* are coagulase-positive (Milanov *et al*., 2010).

2.9.4 Mannintol Fermentation

Mannitol salt agar and Maltose salt agar is a selective medium with 7–9% NaCl that allows *S. aureus* to grow, producing yellow-colored colonies as a result of mannitol and maltose fermentation and subsequent drop in the medium's pH. *S. aureus* ferment mannitol and maltose. *S. aureus* produces an acidic byproduct which changes pH indicator, phenol red in the agar to yellow. Most pathogenic staphylococci, such as *Staphylococcus aureus,* will ferment mannitol (Kenny *et al*., 2013).

2.9.5 Kirby Bauer disk diffusion method

Kirby-Bauer disk diffusion method is widely used in detecting methicillin resistance in *S. aureus*. *S. aureus* colonies grown on Mueller-Hinton agar plates in the presence of thin wafers (disks) containing relevant antibiotics at standardised concentrations. A clear zone appears around the disk known as the zone of inhibition showing the susceptibility to *S.aureus*. Minimum inhibitory concentration is determined according to the breakpoints guidelined by the CLSI (Martineau *et al*., 2000)

2.10 Molecular identification and characterisation assays of MRSA strains

It takes 48 hours to detect antibiotic resistance in *S. aureus* by using conventional identification. To improve detection, molecular based detection techniques, including conventional PCR have been developed for the rapid and accurate identification and characterisation of MRSA isolates (Wolk *et al*., 2009). Molecular techniques are widely used for MRSA detection together with antimicrobial susceptibility testing methods, because susceptibility testing alone is not enough to confirm MRSA presence due to lack of sensitivity of the test (Kock *et al*., 2009).

2.10.1 Application of PCR assays for identification of MRSA

Polymerase Chain Reaction (PCR) is a technique that amplifies pre- determined DNA regions using small target specific DNA primers (Van-Pelt-Verkuil *et al*., 2008).