DETECTION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN COW MILK FROM PERAK, MALAYSIA

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ABSTRACT. Staphylococci colonize a diverse range of animals and tissues causing adverse effects such as mastitis in cows. This study aims to use conventional and polymerase chain reaction (PCR) based techniques to investigate the methicillin-resistant *Staphylococcus aureus* (MRSA) in cow milk in Perak. PCR based techniques are used as an alternative method in identifying pathogenic strains of *Staphylococcus* in comparison to the current biochemical tests used in the detection of this pathogen. Cow milk samples collected from four locations in Perak were examined for *Staphylococcus aureus*. A total of 152 from a total of 225 numbers of isolates were characterized by using biochemical test and antibiotic susceptibility test. It was found that 19 (12.41 %) out of 152 *Staphylococcus aureus* isolates were found to be methicillin-resistant *S. aureus* (MRSA) with only one isolate (0.65 %) showing intermediate resistance and 133 isolates (86.93 %) as Methicillin-sensitive *S. aureus* (MSSA) based on disk diffusion test. Additionally, the 19 resistance and 1 intermediate resistance isolates were further analysed using PCR in order to confirm the presence of a 686 bp *femA* (specific for *S. aureus*) and a 310bp *mecA* (resistant to beta-lactam antibiotics) gene. From this study, the *mecA* gene was detected in 10 isolates, with seven in the methicillin-resistant *S. aureus* isolates and three in the coagulase-negative *Staphylococcus* isolates. Findings from this study indicated that there is high contamination of *S. aureus* in cow milk samples in Perak. Aside from that, it was also found that the PCR technique is more reliable for *S. aureus* detection which may complement the existing biochemical tests and will be very useful for rapid detection of pathogenic species of *S. aureus* and MRSA.

Keywords: cow milk, methicillin-resistant, PCR, *Staphylococcus aureus*

INTRODUCTION

Over the last half-decade, cow milk production has increased marginally, hovering at 500 million metric tonnes per year (Shahbandeh, 2020). Although milk is a healthy food, fresh milk enhances the growth of numerous bacteria. Some of the bacteria found in milk samples are *Escherichia coli, Streptococcus dysgalactiae, Streptococcus uberis*, *Corynebacterium bovis*, *Enterococcus*, and *Arcanobacterium pyogenes* (Taponen & Pyörälä, 2009). Milk has become one of the primary sources of infection in humans (Taponen & Pyörälä, 2009). *Staphylococcus* infection accounted for up to 5 % of all infections in European countries. *Staphylococcus aureus* is a major cause of infection in humans, causing pneumonia, septicaemia as well as mastitis in animals (Jahan *et al*., 2015).

Staphylococcus aureus is a bacterium with spherical shape, non-motile, and does not form spores with the golden colour of colonies grown on blood agar aerobically. In general, it is not harmful but there is possibility for it to become pathogenic when the growth temperature, pH, and nutrient are changed as these factors will cause it to overgrow (Hennekinne *et al*., 2010). This organism has been identified as a major concern in the genus of *Staphylococcus* as the bacterium is highly infectious which may cause mastitis cases in cows (Sawant *et al*., 2009).

Several studies identified the *S. aureus* to be most frequently isolated from infected cow mammary glands as well as their milk that is infected by mastitis (Taponen & Pyörälä, 2009).

Generally, beta-lactam antibiotics are used as treatment as well as prevention of these bacterial infections (Pamuk *et al*., 2012). The use of penicillin as an antibiotic emerged as a major problem as *S. aureus* strains developed resistance to penicillin antibiotics (Guo *et al*., 2020). This is followed by the emergence of Methicillin-resistant *S. aureus* (MRSA) (File, 2008). According to Pamuk *et al.* (2012), long-term antibiotic usage and overuse may lead to multi-resistant. The *mecA* gene has been designated as one of the genes responsible for resistance. It was found in a mobile genetic element known as SCC*mec* which is commonly present in most of the MRSA strains (Ngassam-Tchamba *et al.*, 2021).

The identification of MRSA using the biochemical method was found to be insufficient to determine the presence of MRSA strain. Conventional biochemical tests are only able to differentiate whether the suspected colonies belong to pathogenic or non-pathogenic. However, it has been reported that certain coagulase-negative *S. aureus* may also contain *mecA* gene (Iqbal *et al*., 2021). Therefore, the use of a highly sensitive tool such as PCR based technique was recommended in order to detect the strain of *S. aureus* which carries the *mecA* gene. Based on Punjabi *et al.* (2021), the duplex PCR method would be very functional to determine MRSA strains with *femA* gene and *mecA* gene. Thus, this study aims to determine the effectiveness of biochemical tests and PCR-based techniques in detecting *S. aureus* as well as to determine the contamination level of *S. aureus* in milk samples taken from several milk collection centres situated in Perak, Malaysia.

MATERIALS AND METHODS

Collection of cow milk samples

This study was conducted from March 2014 to March 2016 with milk samples obtained from four different milk collection centers situated around Perak such as Tapah, Taiping, Sungai Siput, and Parit. The milk samples were sent to the Bacteriology Laboratory, Veterinary Research Institute (VRI), Ipoh, Perak for further examination. A total of 250 milk samples were chilled upon arrival to prevent further microbial growth and processed immediately to avoid false negative results.

Isolation of *Staphylococcus*

To isolate *Staphylococci,* a loopful of milk was streaked on sheep blood agar Nataraj *et al.* (2021). There were 225 isolates obtained from 250 milk samples. The reference strains of *S. aureus* ATCC-13565 as well as ATCC-43300 from the American Type Culture Collection (ATCC) were utilized as positive and negative controls for PCR work. Positive colonies on sheep blood agar after incubation of 24 hours at 37 ^{o}C were observed. The colonies were then identified based on their morphological characteristics and haemolytic pattern on blood agar as described by Gupta *et al.* (2012).

Biochemical test

The colonies were confirmed with gram staining before proceeding with other biochemical tests based on Nataraj *et al.* (2021). The positive colonies were subcultured on new blood agar to obtain pure culture for further tests. This was followed by the catalase test (presence of bubble of oxygen) and the coagulase test (clothing) by Sarwar *et al.* (2014). All the *Staphylococcus*

colonies were further tested with mannitol and maltose to differentiate whether it is *S. aureus* or other coagulase-positive strain. The strains were incubated at 37 °C and was examined after 24 hours for growth in which yellow discolouration of medium indicated salt tolerant *Staphylococci* (Mohamed *et al*., 2020).

Oxacillin screen by disk diffusion test

Based on Lee *et al.* (2007), strains which are either oxacillin resistant or methicillin resistant were grouped as methicillin-resistant *S. aureus* (MRSA). Previous studies showed that phenotypic methods like oxacillin screen agar testing is currently accepted to detect MRSA by many reference groups which include Clinical Laboratory Standards Institute (CLSI) USA as mentioned by Anand *et al.* (2009) and Mathews *et al.* (2010). Oxacillin disk diffusion test was performed with 1 µg of oxacillin per disk placed on Mueller-Hinton agar (Thermo Scientific, United states) with 4 % NaCl supplementation. The zone of inhibition was determined after 24 hours of incubation at 35 °C. *S. aureus* ATCC-13565 were used as control in disk diffusion test. Organisms with inhibition zone equal to or less than 10 mm were considered as oxacillin resistant. However, organisms with zone equal to or greater than 12 mm were considered as susceptible. Meanwhile, the organisms with inhibition zone of 11 to 12 mm were interpreted as intermediate (Mahsa Ansari, 2012).

Polymerase chain reaction (PCR) technique

S. aureus colonies were transferred to 200 µl of sterile water. Then, cell suspensions were placed in a boiling water with temperature of 95 °C for the duration of 10 min. After that, the cells were harvested by centrifuging at 13 000 rpm for 15 minutes. By conducting this treatment, it efficiently lysed *S. aureus* cells which prevented DNase activity (Vannuffel *et al*., 1998). Based on Darwish and Asfour (2013), the purified genomic DNA was used for PCR. Published primer for detection of *femA* gene and *mecA* gene based on Vannuffel *et al.* (1998) was used in this study.

Approximately, 10 µl of DNA samples were added to 90 µl of PCR mixture consisting of 10 mM Tris HCl (pH 8.8),1.5 mM $MgCl_2$, 50 mM KCl, and 0.1 % Triton X-100, 0.25 mM (each) deoxynucleoside triphosphates Promega, 100 pmol of each primer obtained based on the work of Vennuffel *et al.* (1998), and 1.25 U of DNAzyme (Thermoscientific, United States) DNA polymerase. After an initial denaturation step (3 min at 92 °C), 30 cycles of amplification were performed as follows: denaturation at 92 °C for 1 min, annealing at 56 °C for 1 min, and DNA extension at 72 °C for 1 min with an increment of 2 seconds per cycle. The reaction ended with a final extension at 72 °C for 3 min. PCR was carried out in a GeneE thermal cycler (Techne, Cambridge, United Kingdom). After amplification, PCR reaction was loaded on a 2 % (wt/vol) agarose gel and horizontal electrophoresis performed in 1X TBE Buffer containing 0.5 mg of ethidium bromide (Promega, United States) per ml. The ethidium bromide-stained DNA fragments were then visualized on a UV transilluminator (Techne, Cambridge, United Kingdom) at 300 nm.

M1-TGGCTATCGTGTCACAATCG and M2-CTGGAACTTGTTGAGCAGAG and F1-CTTACTTACTGGCTGTACCTG and F2-ATGTCGCTTGTTATGTGC primers were used to amplify a 310-bp fragment of the *mecA* gene and a 686-bp fragment of the *femA* gene, respectively. *Staphylococcus aureus* ATCC 43300 was used as the positive control as it contained the *mecA* gene and *S. aureus* ATCC 13565 was used as the negative control.

RESULTS

Out of four milk collection centres, 225 bacteria isolates were successfully isolated from 250 milk samples, with the highest isolates number reported from Sungai Siput milk collection centre (Table 1).

Based on National Committee for Clinical Laboratory Standards (NCCLs) for Kirby-Bauer Test guidelines, 152 isolates were confirmed as *S. aureus* based on catalse, coagulase, mannitol and maltose biochemical tests (Wolfensberger *et al*., 2013) (Table 2). From oxacillin screening by disk diffusion test, it was found that 19 *S. aureus* isolates (12.41 %) were methicillin resistant (MRSA), only one isolate (0.65 %) showed the intermediate resistant and 132 isolates (86.93 %) were methicillin susceptible *S. aureus* (MSSA).

Table 1. Number of bacterial isolates from milk samples in Perak, Malaysia

Milk collection centers	No. of isolates
Taiping	48
Parit	44
Sungai Siput	85
Tapah	48
Total	225

Table 2. Number of positive isolates for biochemical and antibiotic susceptibility tests

Milk collection centre	No of. positive isolates			
	Biochemical test	Oxacillin screen by disk diffusion test		
		Susceptible	**Intermediate**	**Resistant**
Taiping	38	31	0	7
Parit	30	25	1	4
Sungai Siput	62	58	0	4
Tapah	22	18	0	4
Total	152	132	1	19

Resistant - the inhibition zone is ≤10 mm
Intermediate - the inhibition zone is between 11 to 12 mm
Susceptible - the inhibition zone is >12 mm

Table 3. List of *S. aureus* shows resistant and intermediate resistance to oxacillin screen by disk diffusion test and their biochemical characteristic

Isolates	Haemolysis	Gram staining	Catalase test	Coagulase test	Maltose and mannitol sugar test	Oxacillin screen by disk diffusion test
S78b	+	+	+	+	+	Resistant
S65c	+	+	+	+	+	Resistant
P1	+	+	+	+	+	Resistant
P4b	+	+	+	+	+	Resistant
P6	+	+	+	+	+	Susceptible
P12	+	+	+	+	+	Resistant
T13	+	+	+	+	+	Resistant
M18b	+	+	+	-	+	Resistant
P27b	+	+	+	-	+	Resistant
S2a	+	+	-	-	+	Resistant
S28	+	+	+	-	-	Resistant
S3	+	+	+	-	-	Resistant
S19	+	+	+	-	-	Resistant
S52	+	+	+	-	-	Resistant
T22a	+	+	+	-	-	Resistant
T78	+	+	+	-	-	Resistant
T55	+	+	+	-	-	Resistant
M12	+	+	+	-	-	Resistant
M34	+	+	+	-	-	Resistant
M45b	+	+	+	-	-	Resistant

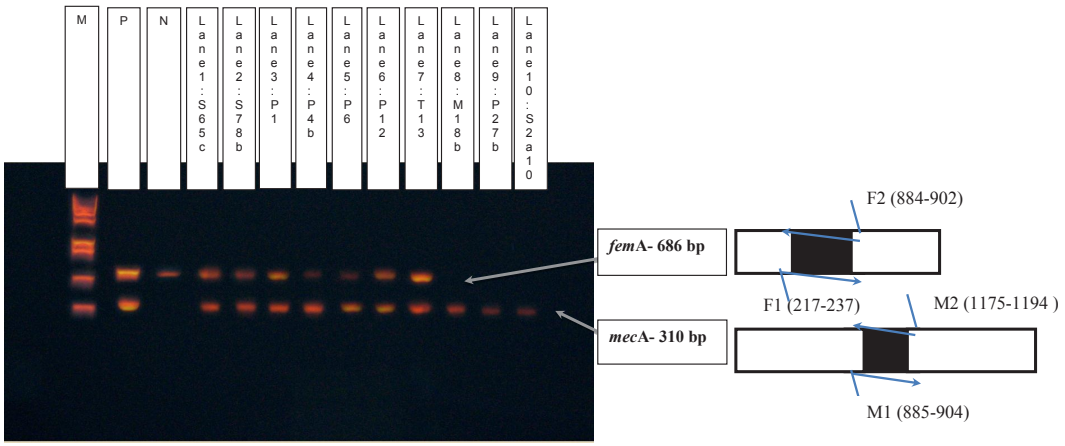
(+): Positive; (-): Negative

PCR analysis after the biochemical test (Table 3) was conducted for 19 resistant strains and one intermediate resistant strain obtained by oxacillin disk diffusion test. Ten isolates were found to be the carriers of the *mecA*. The *mecA* was detected in seven (36.8 %) MRSA isolates, and three Coagulase-negative MRSA as shown in Table 4 and Figure 1.

Table 4. Overall result obtained from *Staphylococcus* isolates by biochemical method and duplex PCR technique.

Isolates	Haemolysis	Gram staining	Catalase test	Coagulase test	Maltose and mannitol sugar test	Oxacillin screen by disk diffusion test	*femA*	*mecA*	Results	Collection centre
S78b	+	+	+	+	+	Resistant	+	+	MRSA	Sungai Siput
S65c	+	+	+	+	+	Resistant	+	+	MRSA	Sungai Siput
P1	+	+	+	+	+	Resistant	+	+	MRSA	Parit
P4b	+	+	+	+	+	Resistant	+	+	MRSA	Parit
P6	+	+	+	+	+	Susceptible	+	+	MRSA	Parit
P12	+	+	+	+	+	Resistant	+	+	MRSA	Parit
T13	+	+	+	+	+	Resistant	+	+	MRSA	Taiping
M18b	+	+	+	-	+	Resistant	-	+	CoNS	Tapah
P27b	+	+	+	-	+	Resistant	-	+	CoNS	Parit
S2a	+	+	-	-	+	Resistant	-	+	Non *S. aureus*	Sungai Siput
S28	+	+	+	+	-	Resistant	-	-	Non *S. aureus*	Sungai Siput
S3	+	+	+	+	-	Resistant	-	-	Non *S. aureus*	Sungai Siput
S19	+	+	+	+	-	Resistant	-	-	Non *S. aureus*	Sungai Siput
S52	+	+	+	+	-	Resistant	-	-	Non *S. aureus*	Sungai Siput
T22a	+	+	+		-	Resistant	-	-	Non *S. aureus*	Taiping
T78	+	+	+	+	-	Resistant	-	-	Non *S. aureus*	Taiping
T55	+	+	+	+	-	Resistant	-	-	Non *S. aureus*	Taiping
M12	+	+	+	+	-	Resistant	-	-	Non *S. aureus*	Tapah
M34	+	+	+	+	-	Resistant	-	-	Non *S. aureus*	Tapah
M45b	+	+	+	+	-	Resistant	-	-	Non *S. aureus*	Tapah

(+): Positive; (-): Negative



Notes: *mecA* gene present in a mobile genetic element known as SCC*mec*, the gene responsible for MRSA resistance. *femA* gene specific for *Staphylococcus aureus.*

Figure 1. PCR products obtained with *femA* gene (686 bp), *mecA* gene (310 bp), showing *S. aureus mecA* positive and *femA* positive as controls. Lane N (*S. aureus* ATCC-43300) shows negative control, Lane P (*S. aureus* ATCC-13565) shows positive control and Lane 1 to 7: amplification of 686 bp of *femA* gene S65c, S78b, P1, P4b, P6, P12, T13 respectively; Lane 8,9,10: *femA* negative and amplification of 310 bp of *mecA* positive CoNS isolate M18b, P27b respectively. S2a (ii) is non-*Staphylococcus* since it shows negative coagulase.

DISCUSSION

This study reported on the occurrence of *Staphylococcus aureus* in several milk samples collected from milk collection centres (MCCs) situated in Perak. There were nine (4 %) incidences of MRSA found around Perak based on PCR which showed the presence of *femA* in this research. Few incidences of *S. aureus* in raw cow milk have been reported in several studies with the incidence rate of 17.39 % in Ahmednagar district, India by Tambekar and Bhutda (2010) whereas 18.80 % by Zakary *et al*. (2011) in Malaysia. Aside from that, negligence in hygienic conditions such as dirty udder, improper cleaning of the bulk tank as well as contaminated milking equipment were also the reasons for the occurrence of *Staphylococcus* contamination (Sawant *et al*., 2009).

It was found that ten isolates (S28, S3, S19, S52, T22a, T78, T55, M12, M34, M45b) showed positive result for the oxacillin test but negative in mannitol and maltose sugar. This is due to the reason that S. *epidermidis* and *S. saprophyticus* showed positive to coagulase test, but they could only be differentiated by using mannitol fermentation as well as maltose fermentation. Both of the *S. epidermidis* and *S. saprophyticus* are non-mannitol fermenters. These two species can be easily differentiated using mannitol fermentation compared to the coagulase test. This is because *S.epidermidis* appears as small pink colonies while *S. saprophyticus* looks like large deep orange colonies (Chikere *et al*., 2008).

According to Emelda and Vijayalakhsmi (2012), only coagulase-negative colonies can grow on either mannitol or maltose media. The same results appeared in M18b and P27b which were found to be positive for mannitol sugar but negative for coagulase test. This research

includes *Staphylococcus* which gives a negative result in the coagulase test since there are possibilities that coagulase-negative *S. aureus* is present. All of these strains can be identified only by mannitol and maltose sugar tests.

Other coagulase-negative *Staphylococcus* sp. were resistant to oxacillin but did not harbour the *mecA* gene or *femA* gene. This suggests that the resistant phenotype in other *Staphylococcus* sp. might be due to extrinsic factors and not due to the presence of genes (Martineau *et al*., 2000).

Meanwhile, the PCR technique resulted in the S2a strain did not have *femA* gene but *mecA* gene. The *mecA* gene which was detected by conventional method was complicated because non-*S. aureus* species or coagulase-negative staphylococci also carried *mecA* gene (Hagen *et al*., 2005; File, 2008). The S2a strain from conventional test result contradicted the genotypic test result. S2a also showed negative results for coagulase and catalase tests. Such isolates in this research were identified by using biochemical testing as *Staphylococcus* spp. This is because the *mecA* gene has been found in non-staphylococcal genera such as *Morganella morganii, Proteus vulgaris*, *Enterococcus faecalis* (Kassem *et al*., 2007) which implies that PCR screening needs to be done with biochemical tests to avoid false-positive data generated for MRSA (Velasco *et al*., 2014).

The present PCR result confirmed that not all MRSA strains positive by oxacillin screen by disk diffusion test are carriers of the *mecA* gene (S28, S3, S19, S52, T22a, T78, T55, M12, M34, M45b). The *mecA* gene fragment was detected in 10 (4 %) isolates. High levels of methicillin resistance were expressed in the analysis of the *femA* product, which indicated that this protein is associated without affecting PBP2a production. Demonstration that Coagulase negative *Staphylococcus* (CoNS) methicillin-resistant strain with negative *femA* gene (M18b and P27b) lost its methicillin resistance trait by the significance of the *femA* genes in the mechanism of methicillin resistance, but transduction of *femA* genes restored the methicillin resistance (Al-Talib *et al*., 2009; Giannouli *et al*., 2010).

The P6 strain showed heterogeneous properties. This is because the strain was susceptible to oxacillin disk diffusion test but positive when tested by PCR. Even though genotypically the *mecA* gene was confirmed by PCR, there are possibilities that the *mecA* gene was non-functional which could not produce PBP2a or the strains might lack the regulatory genes (Barski *et al*., 1996). There is no expression phenotypically or caused by the presence of pseudogene also known as borderline resistance of *S. aureus*. This condition might have happened to P6 due to hyperproduction of beta-lactamase (Martineau *et al.,* 2000). S2a strain is resistant to oxacillin and contains *mecA* gene but it is not *Staphylococcus* sp. which was not positive in catalase test. Only seven isolates (S65c, S78b, P1, P4b, P6, P12 and T13) were homogeneous, in which all the cells expressed resistance when tested by phenotypical method and PCR technique.

Simultaneous detection of *femA* as well as *mecA* for MRSA is important in the identification of *S. aureus.* The *femA* gene is an essential gene in *S. aureus* being used as a molecular target in detecting the polymorphisms of *S. aureus.* From different point of view, methicillin resistance is not only determined by the *mecA* gene alone. There were several studies which showed that the auxiliary genes such as *fem A/B/X* or *fem* need to be present together with *mecA* in order to trigger the expression of methicillin resistance. The formation of the pentaglycine bridge which is encoded by *fem ABX* operon is essential for the cell wall synthesis of the *S. aureus* (Sogut *et al*., 2020).

The false negative results in which the *mecA* gene persisted but showed susceptibility when tested by disk diffusion test acquired in this research showed that the agar screen method and oxacillin diffusion have lower efficiency than the PCR technique. The disk diffusion test has limitations. It also frequently shows variations in the results (Mirzapour *et al*., 2018). However, the disk diffusion method is the gold standard method and needs to run with positive and negative control strains. Phenotypic expression of methicillin resistance can be heterogeneous. As such, this factor complicates the methicillin resistance detection in the strains which shows low levels of resistance. The PCR techniques are highly sensitive and specific. Besides, it is an independent method which does not depend on the culture's chemical or physical conditions (Mirzapour *et al*., 2018; Ramandinianto *et al*., 2020).

CONCLUSION

There are several risk factors that are associated with the MRSA contamination. Healthy human being can become carrier of persistent or intermittent MRSA which could possibly spread to the community, and among the animals in the dairy environment. The contamination rate of 3.11 % *S. aureus* in fresh cow milk in the state of Perak as shown in this study indicated that the level of contamination is low in Perak. In the future, the authors give recommendation for the use of *femA* and *mecA* as a marker for molecular study of *S. aureus* strain for the communities. The manipulation of these regions such as in cloning and mutation studies of the gene could be very beneficial for the determination of its pathogenic status and the identification of *S. aureus*.

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