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RESEARCH ARTICLE

Prevalence and Antibiotics Resistance of *Staphylococcus aureus* Isolates Isolated from Raw Milk Obtained from Small-Scale Dairy Farms in Penang, Malaysia

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ABSTRACT

The study was carried out to determine the prevalence and antibiotics resistance of *S. aureus* in raw milk samples obtained from dairy farms in Penang, Malaysia. A total of 60 samples were examined and all the samples examined were positive for *S. aureus* with counts ranging from 2.88 to 3.41 log cfu/mL. Milk samples obtained from different farms had similar *S. aureus* counts (P>0.05). All the isolates examined were susceptible to gentamycin, kanamycin, chloramphenicol and ciprofloxacin. *S. aureus* isolates were resistant to penicillin (23.3%), ampicillin (23.3%), trimethoprim (18.3%), cefoxitin (15.0%), linezolid (11.7%), clindamycin (10.0%), erythromycin (8.3%) and tetracycline (5.0%). 28.3% of the isolates were resistant to at least one antibiotic with MAR index ranging from 0.08 to 0.67. The following genes, blaZ, ermA and tetK were detected in 9, 5 and 1 isolate/s of *S. aureus* respectively. Presence of high *S. aureus* counts and antibiotic resistant strains of *S. aureus* might pose a health hazard if milk is not pasteurized adequately and prolonged storage of milk after milking at ambient temperature might further aggravate the problem.

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INTRODUCTION

Staphylococcus aureus is a common microorganism found in raw milk and has been associated with food poisoning due to consumption of raw milk and contaminated dairy products Presence of *S. aureus* in milk can be due to poor hygiene practices of milk handlers because *S. aureus* is naturally present on the hands, nasal cavity and skin of human (Jorgensen *et al*., 2005; Popov *et al*., 2014). Other than milk handlers and farm workers, the cow itself can be a source of *S. aureus*, especially if it is suffering from clinical or subclinical mastitis or having skin lesions (Lammers *et al*., 2001; Bradley, 2002). In Malaysia, most dairy farms are small where milking is done by hand and this might increase the risk of direct human contact with the raw milk. When cow is sick, suffering from clinical or subclinical mastitis or having skin lesions such as boils the cow should be isolated to prevent it from infecting other cows in the farm and also to avoid the milk from being mixed together with milk from healthy cows (Bradley, 2002). However, this is not routinely practice in Malaysia, especially on small scale dairy farms, since animal health is not given priority and the cattle are not regularly check by veterinarian. Moreover the dairy farmers are ignorant of good farm practices.

Antimicrobial agents are normally administered to livestock animals such as cattle to treat microbial infections (Jamali *et al*., 2015). Antimicrobial agents such as penicillin, tetracycline, oxacillin, erythromycin, cefazolin, clindamycin and tobramycin are used for treatment of bovine mastitis (Lammers *et al*., 2001; Goa *et al*., 2012; Jamali *et al*., 2014). Prolonged use of antimicrobial agents may lead to the emergence of antimicrobial resistant bacterial strains which is a serious concern not only in animal health but also more importantly to human health. Presence of antimicrobial resistant genes in *Staphylococcus* species is also of great concern since resistance genes can be transferred between staphylococcal species through lateral transfer and these pathogens harboring resistant genes can be transferred to humans from animals (Walther and Perreten, 2007). In *Staphylococcus* species, mecA (methacillim), 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 (Lina *et al*., 1999; Ardıc *et al*., 2005; Haveri *et al*., 2005; Wang *et al*., 2008; Argudín *et al*., 2011; Kamal *et al*., 2013; Jamali *et al*., 2014). The aim of this study was to investigate the prevalence and antimicrobial resistance among *S. aureus* isolates isolated from raw milk obtained from dairy farms in Penang, Malaysia.

MATERIALS AND METHODS

Sampling: Sixty raw milk samples were obtained directly from five small scale dairy farms within Penang, Malaysia. Milk samples were collected on 12 different occasions from the morning milking sessions. Raw milk from different cows on the same farm was pooled and 500 mL of milk sample was obtained and brought back to the laboratory under aseptic condition in an ice box and analyzed immediately upon arrival.

Enumeration and isolation of *S. aureus:* Enumeration of *S. aureus* was performed according to ISO 6888-1 method. Ten-fold serial dilution with sterile buffered peptone water (BPW) (Merck, Darmstadt, Germany) was carried out and 0.1 mL of appropriate dilution was spread-plated in duplicate onto Baird Parker Agar (Merck, Germany) which were then incubated at 37±1°C for 48±2 h. Plates having 15 to 150, typical black shiny colonies with clearing zone around them were considered as *S. aureus* and counted. Well isolated colonies were purified on nutrient agar (Merck, Germany) and subjected to the following biochemical tests: gram staining (+ and coccus), catalase (+), oxidase (-), and coagulase (+).

Antibiotic susceptibility testing: Antibiotic susceptibility of *S. aureus* isolates was determined using the Kirby-Bauer disc diffusion assay method on Muller Hinton agar (MHA) (Oxoid, Basingstoke, UK). A total of 12 antimicrobial agent which are penicillin (P) 10IU, ampicillin (Amp) 10µg, cefoxitin(Fox) 30µg, tetracycline (Te) 30µg, gentamycin (Cn) 10µg, kanamycin (K) 30µg, erythromycin (E) 15µg, clindamycin (DA) 2µg, trimethoprim (W) 5µg, chloramphenicol (C) 30µg, linezolid (Lzd) 30µg and ciprofloxacin (Cip) 5µg (Oxoid, UK) were tested. The surface of MHA plates were inoculated by swabbing 3 times in different directions using overnight broth cultures of *S. aureus* with turbidity adjusted to 0.5 McFarland Standard .Antibiotic discs were placed on MHA (4 discs per agar plate) and incubated at 37 ± 1 °C for 16 to 18 h. The inhibition zone was measured and results were interpreted according to CLSI guidelines (CLSI, 2013). *S. aureus* ATCC 25293 was used as control. Multiple antibiotic resistance (MAR) index was determined according to the method describe by Krumperman (1983).

Molecular detection of antimicrobial resistance gene: DNA extraction of overnight *S. aureus* cultures was carried out using Wizard Genomic DNA Purification Kits (Promega, Wisconsin, USA) according to manufacturer's instruction. Briefly the pellet cell was first suspended in EDTA and lytic enzymes (lysozyme and lysostaphin) followed by addition of nuclei lysis solution for cells lysis. Next, protein precipitation was carried out using protein precipitation solution. DNA was than precipitated using isopropanol and finally rehydration solution was added to rehydrate the DNA pellet.

Detection blaZ, mecA, ermA, ermC, tetK and tetM genes: The above mentioned genes were detected using primers, PCR assay and PCR protocols described by various researchers (Table 1). All PCR products were visualized under UV transilluminator gel doc system (Bio-Rad, California, USA), after gel electrophoresis (Bio-Rad, USA) for 60 min at 90 V on 1.0% agarose gel (Vivantis, Selangor, Malaysia) with Ez-Vision DNA dye (Amresco, Ohio, USA).

Statistical analysis: The difference in *S. aureus* counts among dairy farms was analyzed by the analysis of variance (ANOVA) using *SPSS* predictive analytics software (Version 22.0, IBM, New York, USA) at significant level of $P<0.05$.

RESULTS

Prevalence of *S. aureus* in raw milk: *S. aureus* count of 60 raw milk samples obtained from the five different dairy farms in Penang, Malaysia ranged from 2.88 to 3.41 log cfu/mL. There was no difference ($P<0.05$) observed between the *S. aureus* counts from the different farms (data not shown).

Antibiotics resistance of *S. aureus isolates* isolated from raw milk: The antibiotics resistance among *S. aureus* isolates isolated from raw milk samples obtained from small scale dairy farms is presented in Table 2. All the isolates were susceptible to gentamycin, kanamycin, chloramphenicol and ciprofloxacin. Resistance towards penicillin, ampicillin, trimethoprim, cefoxitin, linezolid, clindamycin, erythromycin and tetracycline were detected in 23.3, 23.3, 18.3, 15.0, 11.7, 10.0, 8.3 and 5.0% of the isolates respectively (Table 2). 23.3% of the isolates were resistant to 3 to 8 antibiotics and among these isolates, 1.7% were resistant to seven and eight antibiotics respectively, while 5.0% of the isolates were resistant to six antibiotics (Table 3). Twelve different antibiotic resistant patterns (antibiogram) were observed among the seventeen antibiotic resistant isolates The most common antibiogram among *S. aureus* isolates isolated from raw milk were P-Amp-Fox-W (n=4) and P-Amp-W (n=3). Among the 17 antibiotic resistant isolates, nine and five isolates harbored the blaZ and ermA genes respectively, while tetK gene was detected in one isolate (Table 3). The MAR index was in the range of 0.08 to 0.67 for the 17 *S. aureus* isolates which were resistant to at least one antibiotic. An organism is considered to have multiple-antibiotic resistance when it is resistant to at least 2 different antibiotics (Magiorakos *et al*., 2011). In raw milk 26.7% of the *S. aureus* isolates were resistant to more than 2 antibiotics.

S. aureus isolates isolated from raw milk obtained from farm A had higher MAR index compared to isolates isolated from the other farms, with each isolate resistant to

Table 1: Target antibiotics resistance gene and primers used in this study

Resistance gene	Primers	Size of target region (bp)	References
blaZ (penicillin)	BlaZ 1: AAGAGATTTGCCTATGCTTC BlaZ 2: GCTTGACCACTTTTATCAGC	517	Haveri *et al.* (2005)
mecA (methacillin)	mecA For: AAGCAATAGAATCATCAGAT mecA Rev: AGTTCTGCAGTACCGGATTTGC	451	Kamal *et al.* (2013)
tetK (tetracycline)	tetK For: GTAGCGACAATA GGTAATAGT tetK Rev: GTAGTGACAATAAACCTCCTA	360	Ardıc *et al.* (2005)
tetM (tetracycline)	tetM For: AGTGGAGCGATTACAGAA tetM Rev: CATATGTCCTGGCGTGTCTA	158	
ermA (erythromycin)	ermA For: AAGCGGTAAACCCCTCTGA ermA Rev: TTCGCAAATCCCTTCTCAAC	190	
ermC (erythromycin)	ermC For: AATCGTCAATTCCTGCATGT ermC Rev: TAATCGTGGAATACGGGTTTG	299	

Table 2: Number and percentage of *S. aureus* isolates isolated from raw milk obtained from different farms resistant to different antibiotics.

Antibiotics	Number of isolates / total number of isolates (percentage) of resistance *S. aureus* isolates					
	Farm A	Farm B	Farm C	Farm D	Farm E	Total
Penicillin	4/12 (33.3)	3/12 (25.0)	2/12 (16.7)	2/12 (16.7)	3/12 (25.0)	14/60 (23.30)
Ampicillin	4/12 (33.3)	3/12 (25.0)	2/12 (16.7)	2/12 (16.7)	3/12 (25.0)	14/60 (23.30)
Cefoxitin	4/12 (33.3)	1/12 (8.3)	2/12 (16.7)	2/12 (16.7)	0/12 (0.0)	9/60 (15.0)
Tetracycline	1/12 (8.3)	2/12 (16.7)	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	3/60 (50.0)
Gentamycin	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	0/60 (0.0)
Kanamycin	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	0/60 (0.0)
Erythromycin	3/12 (25.0)	1/12 (8.3)	0/12 (0.0)	1/12 (8.3)	0/12 (0.0)	5/60 (8.3)
Clindamycin	3/12 (25.0)	2/12 (16.7)	0/12 (0.0)	1/12 (8.3)	0/12 (0.0)	6/60 (10.0)
Trimethoprim	3/12 (25.0)	2/12 (16.7)	2/12 (16.7)	2/12 (16.7)	2/12 (16.7)	11/60 (18.3)
Chloramphenicol	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	0/60 (0.0)
Linezolid	3/12 (25.0)	3/12 (25.0)	0/12 (0.0)	1/12 (8.3)	0/12 (0.0)	7/60 (11.7)
Ciprofloxacin	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	0/12 (0.0)	0/60 (0.0)

Table 3: Antibiogram and presence of antibiotic resistant genes in *S. aureus* isolated from raw milk

Antibiogram	No of isolates	Type of gene/s detected	Farm/s	MAR index
P-Amp-Fox-W	3	blaZ	A, C,C	0.33
P-Amp-Fox-W	1	nd	D	0.33
P-Amp-W	3	blaZ	D, E,E	0.25
P-Amp-Lzd	1	nd	B	0.25
E-Da-Lzd	1	ermA	D	0.25
P-Amp-Fox-E-Da-Lzd	1	ermA	A	0.50
P-Amp-Te-Da-W-Lzd	1	blaZ	B	0.50
P-Amp-Fox-Da-W-Lzd	1	nd	B	0.50
P-Amp-Fox-E-Da-W-Lzd	1	blaZ, ermA	A	0.58
P-Amp-Fox-Te-E-Da-W-Lzd	1	blaZ, ermA	A	0.67
Te-E	1	ermA, tetK	B	0.17
P-Amp	1	nd	E	0.17
Fox	1	nd*	D	0.08

Antibiotics: Penicillin (P) 10IU; Ampicillin (Amp) 10µg; Cefoxitin (Fox) 30µg; Tetracycline (Te) 30µg; Erythromycin (E) 15µg; Clindamycin (DA) 2µg; Trimethoprim (W) 5µg; Linezolid (Lzd) 30µg; Resistance gene: ermA (erythromycin); blaZ (penicillin); tetK (tetracycline); *nd –none detected

at least 4 different antibiotics. Resistance towards penicillin, ampicillin and trimethoprim was observed among isolates isolated from all five dairy farms. Resistance to erythromycin, clindamycin and linezolid were observed in *S. aureus* isolates isolated from farm A, B and D, while resistance to tetracycline was only observed in isolates from farm A and B. *S. aureus* isolates from farm E was not resistant to cefoxitin.

DISCUSSION

S. aureus counts in raw milk obtained in this study were similar to those reported by Sim *et al*. (2012). They reported that *S. aureus* counts of raw milk samples obtained from dairy farms in Sabah, Malaysia, ranged from 2.73 to 3.55 log cfu/mL. On the contrary, Chye *et al*. (2004) reported that *S. aureus* counts of 930 raw milk samples obtained from four different regional milk collecting centers in Malaysia has an average count of 4.08 log cfu/mL. Gundogan *et al*. (2006) also reported that all 60 raw milk samples examined were positive for *S. aureus*.

S. aureus counts of all raw milk samples tested exceeded the limit set by European Union Council Directive (92/46/EEC) for direct human consumption in which the count should be less than 5×10^2 cfu/mL. However, only 18.3% of the raw milk samples exceeded the European Union Council Directive (92/46/EEC) *S. aureus* limit for raw milk intended for processing in which the count should be less than 2×10^3 cfu/mL (Pelesa *et al*., 2007). The high prevalence and count of *S. aureus* obtained in this study can be attributed to unhygienic conditions on the farm, improper handling and lack of refrigeration facilities on the farm and storage at ambient temperature which leads to contamination and proliferation of *S. aureus*. Another reason that contributes to high *S. aureus* count of raw milk might be due to the cattle having subclinical mastitis caused by *S. aureus*.

Similar to this study, Frey *et al*. (2013) reported that *Staphylococcus* isolates isolated from raw milk and dairy products were resistant to clindamycin, erythromycin, linezolid and trimethoprim. However Frey *et al*. (2013) also reported that *Staphylococcus* isolates were resistance to chloramphenicol, gentamycin and kanamycin which differ to findings in this study in which, *S. aureus* isolates were susceptible to chloramphenicol, gentamycin and kanamycin. Prior studies have reported very high prevalence of penicillin and tetracycline resistant *S. aureus* isolates isolated from raw milk. Jamali *et al.* (2015) reported that 44.4 and 56.2% of *S. aureus* isolates isolated from bovine raw milk in Iran were resistant to penicillin and tetracycline. Similarly, Goa *et al*. (2012) reported that 96.2 and 98.1% of *S. aureus* isolates from raw milk in China were resistant to penicillin and tetracycline respectively. Both these studies reported

much higher prevalence of penicillin and tetracycline resistant isolates as compared to current study.

In most countries, penicillin and tetracycline are routinely used to treat *S. aureus* infection in cattle (Chambers, 2001). Widespread and continuous use of penicillin and tetracycline leads to increase in resistance towards these antimicrobial agents (Chambers, 2001; Jamali *et al.*, 2015). However in Malaysia, the National Pharmaceutical Control Bureau (NPCB) of the Ministry of Health reported that drugs or antimicrobial agents are mostly used in poultry and pig farms and less in cattle and goat farms (FAO, 2012). This might be the reason that contributes to the lower frequency of antimicrobial resistance observed among *S. aureus* isolates in this study. In Malaysia, most dairy cows are not heavy milk producers as most of them produced about 5 liters of milk per milking session, this could be attributed to breed, feed and weather. It is a known fact that cows which produce little milk are not prone to acute mastitis but might suffer from sub clinical mastitis.

Resistance gene ermA was detected in all *S. aureus* isolates which were resistant to erythromycin but ermC resistance gene was not detected in any of the isolates. The result are not in agreement with research by Gao *et al*. (2012) which reported that ermC is more common than ermA among *S. aureus* isolates isolated from cow with mastitis in which all isolates that are phenotypically resistance to erythromycin shows the presence of ermC while ermA was not detected at all. Tetracycline resistance gene tetM was not detected; however tetK gene was detected in only 33.3% of the phenotypically resistance tetracycline *S. aureus* isolate. Cengiz *et al*. (2015) also reported that phenotypically resistance tetracycline strains were more prevalent as compared to genotypically resistance strains in which only 33.3% out of the phenotypically resistance *S. aureus* strains shows the presence of tetracycline (tetK/tetM) resistance gene. Resistance gene blaZ was detected in 64.3% of *S. aureus* isolates which shows phenotypic resistance to penicillin. Haveri *et al*. (2005) also reported that not all strains that exhibit phenotypic resistance to penicillin harbor blaZ resistance gene in which there was isolates which were phenotypically resistance to penicillin but did not show the presence of blaZ gene. MecA gene was not detected among the *S. aureus* isolates which exhibited phenotypic resistance to cefoxitin. Kamal *et al*. (2013) reported a low prevalence of mecA gene (5.3%) among *S. aureus* isolates from raw milk and dairy products. CLSI guideline suggests the use of cefoxitin or oxacillin disk diffusion or minimum inhibitory concentration (MIC) as an alternative method for detection of methacillin resistant *S. aureus* (MRSA). Due to the difference between the test methods and resistance mechanisms, which mediate methicillin resistance in *S. aureus*, detection of MRSA cannot be based on either phenotypic or genotypic methods but both methods should be used in combination (Araj *et al*., 1999).

Conclusions: The result of this study shows prevalence of high *S. aureus* counts in raw milk produced by small-scale dairy farms. This indicates that there is a need to implement proper hygiene and sanitation of milk handling practices on the dairy farms and along the food chain in order to reduce contamination and improve the microbiological quality of raw milk. Presence of *S. aureus* isolates with multiple- antimicrobial resistance was also observed in this study. It is necessary that relevant authorities need to assist the dairy farmers on issues concerning animal health and also control and monitor the use of antibiotics to prevent widespread emergence of multiple drug resistance pathogens. Both high counts and presence of multiple- antimicrobial resistance *S. aureus* are issues that have negative implication on human health and economics thus it should not be taken lightly, immediate action should be carried out to ensure safety and quality of raw milk.

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Author's contribution: GR supervised and guide the study. WNWA co-supervised the study. SSAK carried out sampling and laboratory analysis. CLO assist with the molecular analysis. All authors wrote, revised and approved the manuscript.

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