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Prevalence of Methicillin Resistant *Staphylococcus aureus* in Raw Goat Milks from Selected Farms in Terengganu, Malaysia

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ABSTRACT

The emergence of antimicrobial drug resistant bacteria has been a concern worldwide. One of bacteria that has been reported to develop resistance is Methicillin Resistant *Staphylococcus aureus* (MRSA). Recent studies showed that Livestock Associated MRSA (LA-MRSA) was found in domestic food animals and their handlers. The aim of this study was to investigate the prevalence of MRSA in goat milk of goat farms located in Terengganu. A total of 664 udder milks were taken from 332 goats at 40 selected farms within Terengganu state. Then, screening of bacteria and isolation of suspected *S. aureus* isolates in the milk samples was done using selective agar, Gram staining and biochemical tests. The identity of the bacteria isolated was further confirmed using PCR where specific designed primers were used to detect the presence of *nuc* gene of *S. aureus* (278bp) and *mecA* gene (533bp) of MRSA. Both *S. aureus* and MRSA isolates were also tested for their susceptibilities toward the antimicrobial drugs. Fifty milk samples were found to contain *S. aureus* and one of the *S. aureus* isolates were MRSA. The bacteria isolates were found to have higher tendency to be resistance toward Penicillin (26.0%) and Oxacillin (12.0%). This study provides useful data on the current status of MRSA prevalence in small ruminant's milk, which can be used to prevent transmission of LA-MRSA to human and other animals.

Keywords: goat milk; antibiotic susceptibility; MRSA; nuc gene and mecA gene

INTRODUCTION

Recently, the emergence of antimicrobial resistant bacteria has been a concern worldwide and one of the bacteria that has been well known to develop resistance toward antibiotics is Methicillin Resistant *Staphylococcus aureus* (MRSA). In animals, MRSA also causes an array of infections in economically important livestock animals (Fitzgerald, 2012). Treatment of MRSA infection can be difficult due to the ability of the bacteria to inhibit the reaction of antibiotics, thus limiting the effectiveness of antibiotic treatment. It was found that MRSA is capable of making a penicillin binding protein (PBP2a) which is encoded by the *mec*A gene (Cuny *et al*., 2015). The PBP2a prevents beta-lactam antibiotics from binding to the cell wall protein and thus mediate the inhibitory effect of those antibiotics (Cuny *et al*., 2015).

MRSA was first found in 1961 after methicillin was introduced (Chamber & DeLeo, 2009). In the late 1990s, new MRSA clones emerged in the community, where healthy individuals without history of hospitalization had been found to carry this strain (Guardabassi *et al*., 2013). Then, MRSA was found to be present in livestock animals. In the early 21st century, a livestock associated MRSA strain with a sequence type 398 (ST398) was first found in pig, which suggested the emergence of Livestock Associated MRSA (LA-MRSA). Recent studies have shown that LA-MRSA was frequently found in various livestock and food animal products around the globe (Lozano *et al*., 2016; Tegegne *et al*., 2017). In early studies, the antimicrobial resistance and genetic background of MRSA were thought to be different according to their specific hosts. However, this perspective changed when MRSA from different sources were found to have low host specificity and similar genomic sequence (Cuny *et al*., 2015). Hence, this suggests that MRSA can be transmitted across species, such as from animal to human and vice versa. This raise health and food hygiene concerns as LA-MRSAs that present in live animals and their products, such as raw milk, egg and meat are capable of transmitting, colonizing and infecting the human.

In Malaysia, MRSA isolates were found in pigs. A study conducted by Neela *et al*. (2009) reported that 10 (1.96%) LA-MRSA (ST9 and ST1) were isolated from pigs in 9 different pig farms, showing that LA-MRSA already present in Malaysian farming industry. However, the data on the prevalence of *S. aureus* and MRSA in

raw milk as well their antibiotic resistance profiles in Malaysian goat are still limited. As MRSA had been successfully isolated from both healthy and mastitis milk in dairy animals around the globe (Holmes & Zadoks, 2011; Nam *et al*., 2011; Gopal *et al*., 2017; Tegegne *et al*., 2017), it is a possibility that MRSA may present among dairy goats in Malaysia. This study aims to study the prevalence of MRSA in Terengganu's raw goat milks (subclinical and clinical mastitis as well healthy milks) and the antibiotic resistance profile of *S. aureus* isolates. The prevalence data in this study will provide information that help to prevent further transmission of MRSA to the other animal farms and human community as well as raise the awareness of applying good farm biosecurity practices. Besides, the antibiotic resistance profile will help the Terengganu farmers and veterinary officers to have a better understanding on the current AMR status in goat and provides insight on the kind of drugs that can be used to treat *S. aureus* infection, particularly mastitis in goats.

MATERIALS AND METHODS

Milk Samples Collection

A total of 664 udder (both left and right sides) raw milk samples from 332 lactating goats were collected at 40 small ruminant farms located in Terengganu, Peninsular Malaysia. The sample size was determined using GraphPad StatMate© software for cross sectional and random sampling. A 5% absolute precision and 95% level of confidence was used for determining the sample size. An expected prevalence of 30% was used to determine the maximum sample size. The prevalence of MRSA was determined as the percentage (%) of raw milk samples which positive for *mecA* gene. The milk was collected using a sterile hand milking method and approximately 10 mL of milk sample was collected in a sterile falcon tube. The subclinical mastitis was pre-examined by Californian Mastitis Test (CMT) and clinical mastitis was determined based on the clinical signs such as udder pain, swelling, redness, warmth and gross changes in the milk. The samples were stored in a cooler box with approximately at 4°C and transported from the sampling sites to the Microbiology Laboratory, Universiti Sultan Zainal Abidin, Besut Campus within one day period for further analysis.

Phenotypic Identification of *S. aureus*

A 10 µL of milk samples were plated on Mannitol Salt agar (Sigma-Aldrich, USA) and incubated at 37°C up to 48 hours. The appearance of the bacterial colonies was observed and recorded on 24 and 48 hours. Yellow bacterial colonies with yellow zones on the Mannitol Salt Agar were picked and cultured on the Nutrient Agar supplemented with 7.5% of Sodium chloride. The suspected bacterial colonies were further examined using Gram staining and biochemical tests (haemolysis test, catalase test, oxidase test and coagulase test). Bacterial isolates that were gram positive, cocci shaped and showed biochemical characteristics identical to *S. aureus* were kept and cultured on the enrichment agar for further testing.

Genotypic Identification of *S. aureus* and MRSA

Genotypic identification of *S. aureus* and MRSA started with DNA extraction of suspected *S. aureus* described by Suhaili *et al.* (2018). DNA amplification was then performed using two different sets of primers to detect the presence of *nuc* gene in *S. aureus* and *mecA* gene in MRSA (Suhaili *et al.,* 2018). Amplification products were separated using gel based electrophoresis in a 2.0% (w/v) agarose gel (Promega, USA) in a 1X tris-borate-EDTA (TBE) running buffer (Bioline, UK). The gels were visualized and using Fujifilm LAS-4000 gel documentation system. Bacteria isolates showing DNA bands at the size of 278 bp (*nuc* gene) and 533 bp (*mecA* gene) were considered as *S. aureus* and MRSA respectively.

Antibiotic Susceptibility Tests

The antibiotic susceptibility tests were conducted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Antibiotic discs were placed on Muller Hinton Agar plates inoculated with 0.5 McFarland Standardized cultures. Three duplicates were done for each isolate. The plates were then incubated at 35°C for 18-24 hours. The diameter of the inhibition zones were measured and compared to the CLSI Disc Diffusion breakpoints (CLSI, 2018).

RESULTS

Prevalence of MRSA in Raw Goat Milks

Out of 664 milk samples, 50 (7.5%) were from clinical mastitis and 67 (10.1%) were from subclinical mastitis animals (Table 1). Bacteriological examination revealed that 198/664 (29.8%) of raw milk samples were positive for *S. aureus*. Out of the 198 presumptive *S. aureus*, 50 (25.3%) isolates were found to carry *nuc* gene (Figure 1) that unique to *S. aureus*. All of the 50 isolates that contained *nuc* gene were later tested using gel-based PCR to investigate the presence of *mecA* gene.

Table 1. Number of samples and the results of bacteriological examination and PCR assay

Mastitis status	No. of samples	No. of samples positive for *S. aureus* isolation	No. of samples positive for *nuc* gene	No. of samples positive for *mec*A gene
Clinical mastitis	50	18	10	0
Subclinical mastitis	67	25	11	0
No mastitis	547	155	29	1

One (1/50; 2%) of the *S. aureus* isolate from normal milk sample was found to carry *mecA* gene (Figure 2) and thus this isolate is considered to be MRSA.

Antibiotic Susceptibility Profiles

Antibiotic susceptibility profiles of the isolated *S. aureus* are shown in Table 2. Of the 50 *S. aureus* isolates, 26% (13/50) were resistant to penicillin and 10% (5/50) were resistant to oxacillin, respectively. In addition, about 6% (3/50) of the *S. aureus* were resistant to tetracycline. Of a total of 50 isolates of *S. aureus*, 74% (37/50) showed full susceptibility to vancomycin, cefotaxime, doxycycline, amikacin, norfloxacin, clindamycin, kanamycin, cephalothin and chloramphenicol. The MRSA isolate showed a similar antibiotic susceptibility pattern as the *S. aureus* isolates (Table 2).

DISCUSSION

Recently, livestock-associated MRSA (LA-MRSA) has become an emerging problem in the field of veterinary medicine due to their multiple antibiotic resistance characteristics. The livestock-associated MRSA (LA-MRSA) was first isolated from a dairy cow with mastitis (Fitzgerald, 2012). Later on, MRSA has been reported globally in cattle, pigs, horses and poultry (Petersen *et al*., 2013).

In the present study, the prevalence of MRSA in raw goat milks was 2% (1/50). This result was lower than that of Tegegne *et al.* (2017) who recorded 25.7% (9/35) prevalence rates of MRSA in raw goat milk. Previous study by Aras *et al*. (2012) reported MRSA was found in caprine mastitis only with a low prevalence (4.8%; 2/42). Meanwhile, study conducted by Virdis *et al*. (2010) reported none of the *S. aureus* isolates obtained were MRSA. *MecA* gene encodes for the penicillin binding protein (PBP-2a) which allows the bacteria to be resistance towards beta-lactam antibiotics. This chromosomally derived gene can be frequently found in MRSA but absent in methicillin susceptible Staphylococci isolates. Detection of *mecA* gene using PCR amplification technique is considered to be a reliable method to identify MRSA (Tsubakishita e*t al*., 2010; Bastidas *et al*., 2019).

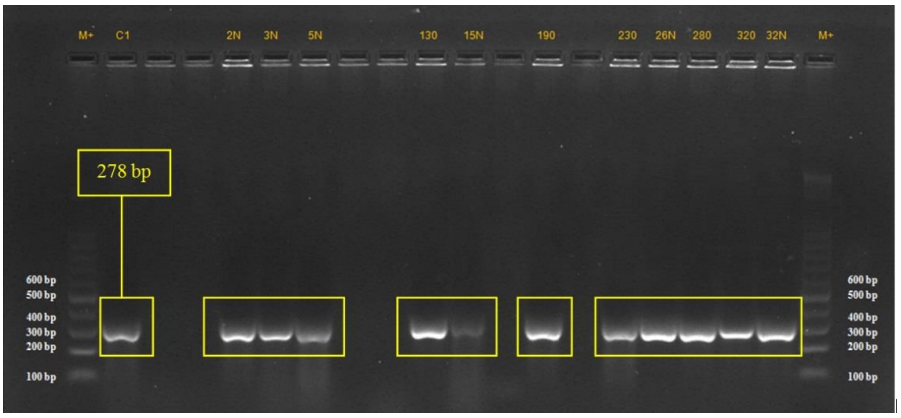

Figure 1. Agarose gel electrophoresis image of the *nuc* gene (278 bp) from representative isolates (lane 2N, 3N, 5N, 130, 15N, 190,230, 26N, 280, 320 & 32N); lane C1 is a positive control (ATCC 700699) and lane M+ is a 1000 bp ladder.

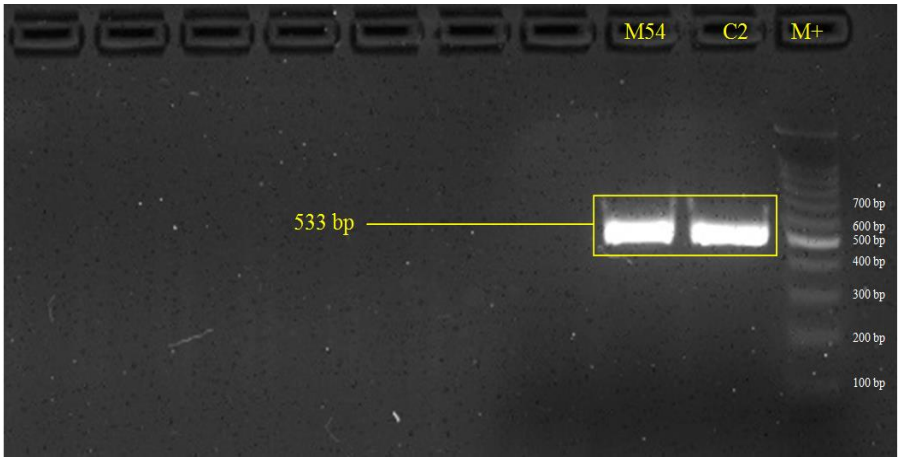

Figure 2. Agarose gel electrophoresis image of the *mec*A gene (533 bp) from representative isolates lane; lane C2 is a positive control (ATCC 700699) and lane M+ is a 1000 bp ladder.

Table 2. Antibiotic susceptibility profiles of *S. aureus* and MRSA isolates

Isolates	Antimicrobials	Symbol	Disk potency (μg)	Number of isolates (%)		
				Resistant	Intermediate	Sensitive
S. aureus	Penicillin	P	10	13 (26)	0 (0)	37 (74)
	Oxacillin	OX	1	6 (12)	0 (0)	44 (88)
	Tetracycline	TE	30	3 (6)	1 (2)	46 (92)
	Amoxycillin	AMC	10	2 (4)	0 (0)	48 (96)
	Cefoxitin	FOX	30	1 (2)	0 (0)	49 (98)
	Vancomycin	VA	30	0 (0)	0 (0)	50 (100)
	Cefotaxime	CTX	30	0 (0)	0 (0)	50 (100)
	Doxycycline	DO	30	0 (0)	0 (0)	50 (100)
	Amikacin	AK	30	0 (0)	0 (0)	50 (100)
	Norfloxacin	NOR	10	0 (0)	0 (0)	50 (100)
	Clindamycin	DA	2	0 (0)	0 (0)	50 (100)
	Kanamycin	K	30	0 (0)	0 (0)	50 (100)
	Cephalothin	KF	30	0 (0)	0 (0)	50 (100)
	Chloramphenicol	C	30	0 (0)	0 (0)	50 (100)
MRSA	Penicillin	P	10	1 (100)	0 (0)	0 (0)
	Oxacillin	OX	1	1 (100)	0 (0)	0 (0)
	Tetracycline	TE	30	1 (100)	0 (0)	0 (0)
	Amoxycillin	AMC	10	0 (0)	0 (0)	1 (100)
	Cefoxitin	FOX	30	0 (0)	0 (0)	1 (100)
	Vancomycin	VA	30	0 (0)	0 (0)	1 (100)
	Cefotaxime	CTX	30	0 (0)	0 (0)	1 (100)
	Doxycycline	DO	30	0 (0)	0 (0)	1 (100)
	Amikacin	AK	30	0 (0)	0 (0)	1 (100)
	Norfloxacin	NOR	10	0 (0)	0 (0)	1 (100)
	Clindamycin	DA	2	0 (0)	0 (0)	1 (100)
	Kanamycin	K	30	0 (0)	0 (0)	1 (100)
	Cephalothin	KF	30	0 (0)	0 (0)	1 (100)

Apart from mecA gene, amplification of *nuc* gene is important in order to study the prevalence of *S. aureus* in individual goat milks in Terengganu. Many researches had indicated that detection of S. *aureus* by using PCR amplification of *nuc* gene has a great potential to be used for rapid diagnosis of *S. aureus* (Gao *et al*., 2011; Kateete *et al*., 2010; Banada, *et al*., 2012). In the present study, 50 isolates were confirmed of carrying *nuc* gene (Figure 1), indicating the presence of *S. aureus* in single individual raw goat milk. The number of confirmed *S. aureus* isolates through PCR is lower than that of confirmed by phenotypic screening methods. This is not surprising, as these phenotypic screening methods often lead to ambiguous results due to the large amount of field isolates, inconsistent test performances and different biochemical characteristics of bacteria isolates that differ from the patterns of a known genus and species (Kateete *et al*., 2010). The present study showed a slightly higher *S. aureus* prevalence rate (25.3%) compared with Qian *et al.* (2019) who recorded the prevalence rate of 23.5% (68/289). It is important to note that the presence of *S. aureus* in raw goat milks as the bacteria are often related to Staphylococcal food poisoning and raise public health concern among raw milk consumer (Qian *et al*., 2019).

In this study, the *S. aureus* isolates showed different degrees of resistances toward various antibiotics. *S. aureus* isolates were found to have higher tendency to be resistance toward beta-lactams antibiotics such as oxacillin (12.0%) and penicillin (26.0%). This may due to the frequent usage of penicillin related drugs for mastitis treatment by the local farmers. However, the percentages of antibiotic resistance to penicillin were still low compared to the previous studies conducted by Ganai *et al*. (2016) and Massawe *et al*. (2019) that reported the resistance rates at 76.47% and 57.2% respectively. Resistance towards beta-lactams sensitive antibiotics and related antibiotics among *S. aureus* is frequently observed and may be aids by defensive mechanisms that existed within the *S. aureus* population. Since Staphylococci are capable of expressing the penicillin-binding protein 2a (PBP 2a), it can be speculated that the *S. aureus* isolated produce the defensive enzyme to protect themselves from beta-lactam related antibiotics.

In the present study, 3/50 of the *S. aureus* isolates were resistant to tetracycline. The findings of this study are consistent with previous study by Rubin *et al*. (2011) reported that *S. aureus* isolated from various animals showed resistance towards tetracycline. In another study conducted by Massawe *et al*. (2019) reported 19% of *S. aureus* isolates were found to be resistant towards tetracycline. According to Foster (2017), the presence of Tet efflux pumps (TetA(K) and TetA(L)) and TetO/M determinants within the Staphylococci assists the sur-

vival of Staphylococci towards tetracycline. The present study showed only a low number of *S. aureus* isolates were resistance to amoxicillin (2/48; 4%) and cefoxitin (1/49; 2%). However, our study showed 100% sensitivity toward vancomycin, cefotaxime, doxycycline, amikacin, norfloxacin, clindamycin, kanamycin, cephalothin and chloramphenicol in *S. aureus* isolates, implying those antibiotics can be used to treat *S. aureus*-related subclinical and clinical mastitis infection of goats in Terengganu.

In general, the antibiotic resistance profile of *S. aureus* in this study is almost similar to that reported by Suhaili *et al.* (2018) where most of *S. aureus* isolates from human in Malaysia are susceptible to most of antibiotics, with the exception of penicillin (49/49; 100%) erythromycin (8/49; 16%), cefoxitin (4/49; 8%), oxacillin (4/49; 8%) and clindamycin (2/49; 4%). However, the resistance rates of *S. aureus* towards antibiotic in the present study are lower when compared to a similar study carried by Qian *et al*. (2019). Qian *et al.* (2019) reported 97.06% (66/68) *S. aureus* from raw goat milk in China have resistance at least towards one tested antibiotics where the isolates showed resistance towards important antibiotics such as penicillin (79.41%, 54/68), oxacillin (60.29%, 41/68) piperacillin (41.18%, 28/68), trimethoprim-sulfamethoxazole (33.82%, 23/68), ciprofloxacin (29.41%, 20/68), gentamicin (27.94%, 19/68), clindamycin (20.59%, 14/68), cefazolin (19.12%, 13/68) and linezolid (14.71%, 10/68). Besides, *S. aureus* isolated from caprine mastitis were found to be resistance toward kanamycin (7/22; 28%), oxytetracycline (4/22; 16%) and ampicillin (3/22; 12%) (Virdis *et al*., 2010).

CONCLUSION

This study demonstrates that the presence of MRSA (2%; 1/50) and *S. aureus* (25.3% 50/198) in raw goat milks in Terengganu. The isolated *S. aureus* showed resistances towards penicillin, oxacillin, tetracyclines, amoxycillin and cefoxitin, suggesting the emergence of antibiotic resistance in goat's milk. Therefore, continued efforts of antimicrobial stewardship programme are needed to promote good hygiene practices and responsible usage of antimicrobials in goat farms in Terengganu, Malaysia.

CONFLICT OF INTEREST

None of the authors have any potential conflict of interest to declare.

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