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Molecular identification of multidrug-resistant *Staphylococcus aureus* (MDRSA) carriage and pattern of antibiotic resistance from rabbit, rabbit handler and rabbitry in the east coast region of Malaysia

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Infections caused by *Staphylococcus aureus* (*S. aureus*) with antimicrobial resistance traits have been a growing problem in humans and animals seen throughout the world. Nevertheless, information regarding *S. aureus,* multidrug-resistant *S. aureus* (MDRSA) and methicillin-resistant *S. aureus* (MRSA) in rabbits is still limited. The objectives of this study are to determine the prevalence rate and assay of the antibiogram profiles of *S. aureus*, MDRSA and MRSA isolated from rabbits, rabbit handlers and rabbitry environment in east coast regions of Malaysia. Swabs samples from 183 rabbits, 45 rabbit handlers and rabbitry environment were collected from 16 rabbit farms. Screening and isolation of *S. aureus* in the swabs were done using routine microbiological methods. PCR screening of *nuc* and *mecA* genes were carried out to detect the presence of *S. aureus* and MRSA. The antibiogram profile of the *S. aureus* was determined using Kirby-Bauer method. *S. aureus* were detected in 19% of rabbits, 26.7% of rabbit handlers and 8.8% of swabs from the rabbitry environment. However, MRSA (0%) was not detected. Antibiotic susceptibility test revealed that *S. aureus* from rabbit showed low level of resistance (<20%) against 15 different antibiotics while fully susceptible to four antibiotics. Meanwhile, *S. aureus* from rabbit handlers were highly resistant against penicillin (86%), oxacillin (64%) and amoxicillin (50%). This study suggests the emergence of MDRSA in the rabbit farms settings in east coast regions of Malaysia.

Keywords: Rabbit, *Staphylococcus aureus*, MDRSA, MRSA, Antibiogram

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a zoonotic, opportunistic Gram-positive, commensal bacterium colonising both humans and animals

(Suhaili et al. 2018b). *S. aureus* is capable of causing several diseases in humans, ranging from minor skin infection to life-threatening illness (Suhaili et al. 2018b). In rabbits, *S. aureus* can be commonly found on skin, but it is also one of the main pathogens related to dermatitis, mastitis and metritis infections, causing losses in rabbits farming industry (Agnoletti et al. 2014; Attili et al. 2020). Both asymptomatic carriers and infected individuals are capable of spreading the *S. aureus* directly or indirectly to others (Smith et al. 2013). Due to the zoonotic nature of *S. aureus*, warm-blood animals are now considered to be a significant vehicle for the spread and transmission of *S. aureus*.

Antimicrobials are commonly used to treat bacterial infections and to prevent further spread of the infections in rabbit and other livestock production settings. Recently, the emergence of bacteria with antimicrobial resistance (AMR) traits such as *S. aureus* is a significant public health concern, with MRSA being one of the major widespread nosocomial pathogens seen worldwide. *S. aureus* that showed multidrug resistant traits such as MRSA and MDR *S. aureus* (MDRSA) are considered to be a serious pathogenic risk to human and animal health throughout the world. The MRSA strains have become endemic in hospitals, including those in Asia (Chen and Huang, 2014). Multiple reports on the presence of MRSA in animals have been documented worldwide and the apparent animal-to-human transmissions have increased concerns on the risks of animal populations as potential reservoirs for this zoonotic infection (Smith, 2015). Apart from human and animal transmission, there has been an increasing attention to the possible role of the environment as a potential reservoir for MRSA infection. Previous studies had reported the detection of MRSA from various environmental samples associated with animals such as dust, farm rats and environmental wipes (Friese et al. 2013).

Similar to other animals, rabbits are also prone to various bacterial infections (Attili et al. 2020). In fact, the medication level in rabbit farming is the highest among food producing animals (Agnoletti et al. 2018). Thus, this creates an optimum environment for the emergence of AMR bacterium. Recently, the occurrence of rabbits carrying *S. aureus* with antimicrobial resistance traits has been reported (Attili et al. 2020). Moreover, livestock associated MRSA ST398 has been described to be present in both farm and pet rabbits (Loncaric et al. 2013; Agnoletti et al. 2014). In Malaysia, although most of the rabbit farms are considered to be small scale, they still have the potential risk for the spread of AMR. Thus, it is advisable to implement surveillance plans to regulate the usage of antibiotics and prevent the further emergence of multidrug-resistance bacteria. However, there is no study documented regarding the prevalence as well the antibiotic susceptibility pattern of *S. aureus* and MRSA from rabbit farms in Malaysia. The objectives of the present study are to determine the prevalence and antibiogram profiles of *S. aureus*, MDRSA and MRSA isolated from rabbits, rabbit handlers and rabbit farm settings within the east coast regions of Malaysia.

MATERIALS AND METHODS

Ethics approval

The method of sampling and experimental design of this study was approved by the UniSZA Animal and Plant Research Ethics Committee (Protocol code: UAPREC/04/18/006.) and UniSZA Human Research Ethics Committee (Protocol code: UniSZA/UHREC/2019/85). Written informed consent was obtained from each rabbit handler before obtaining swabs samples.

Swabs samples collection

Swab samples (183 oral and 183 ear swabs) from 183 randomly selected adult rabbits from 16 different rabbit farms located in the east regions of Malaysia were obtained using sterilized cotton swabs. 90 swab samples (45 oral and 45 nasal swabs) were collected from 45 human handlers that have close contact with rabbits. Ear swab samples were collected by swabbing both the external ear canals of each rabbit. Nasal swabs were collected by inserting the swab into nostrils (2 cm from the opening) and rotated against the nostril wall for a few seconds. Meanwhile, oral swabs were taken by swapping the back of the throat and allowing sterile cotton swab to be covered with some mucous. For environmental swabs, swab samples were taken from feeders, drinkers, door lock, wall and floor of randomly selected rabbits' cages as well from the boots of the rabbit handlers. Triplicate environmental swabs (n= 180) were collected at different places in each of the rabbit farms. The swab samples were kept in modified transport media containing Nutrient Broth (HiMedia, India) with 6.5% sodium chloride (NaCl) and were transported to the laboratory within the same day for further analysis.

Bacteriological examination

The samples were inoculated onto Mannitol Salt Agar (MSA) plates (Sigma-Aldrich, USA) and incubated at 37°C for 48 hours. Bacteria colonies with the yellow and round appearance (yellow-coloured as a result of mannitol fermentation) were picked and cultured on the nutrient agar plates (HiMedia, India) followed by incubation for 24 hours at 37°C. The suspected *S. aureus* colonies were further examined using Gram staining and biochemical tests (coagulase, catalase and oxidase test test). Bacteria colonies that showed phenotypic characteristics similar to *S. aureus* were kept under 4°C prior to PCR testing.

Genotypic identification of bacterial isolates

Genomic DNA of the bacterial isolates was extracted using simple boiling method (Suhaili et al. 2018a). The DNA templates were stored at -20˚C prior to genotypic identification analysis. DNA amplification of specific genes was carried out using *nuc* and *mecA* gene primers to determine the prevalence of *S. aureus* and MRSA respectively (Saiful et al. 2006). Amplified PCR products were separated using gel-electrophoresis where the products were loaded in 2.0% (w/v) agarose gel (Promega, USA) and ran at 80V for 2 hours. The gels were viewed and documented using the Fujifilm LAS-4000 gel documentation system. Bacteria that showed DNA fragments at 278 bp (*nuc* gene) and 533 bp (*mecA* gene) were categorised MRSA. Bacterial isolates that only harboured *nuc* genes were referred to as methicillin-susceptible *S. aureus* (MSSA).

Antibiotic susceptibility test

The antibiogram profile of *S. aureus* isolated from rabbit and rabbit handlers were determined using Kirby-Bauer test. The test was conducted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. 19 different antibiotic disks were used in this study include penicillin (10 units), oxacillin (1 µg), amoxicillin/clavulanate (10 µg), tetracycline (30 µg), erythromycin (15 µg), clindamycin (2 µg), cefotaxime (30 µg), trimethoprim-sulfamethoxazole (25 µg), amikacin (30 µg), cefoxitin (30 µg), chloramphenicol (30 µg), cephalothin (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), gentamicin (30 µg), kanamycin (30 µg), linezolid (30 µg), norfloxacin (10 µg) and quinupristin-dalfopristin (15 µg). The plates were incubated at 37°C for up to 24 hours. The size of inhibition zones (in diameter) displayed by the bacteria against each antibiotic were measured and interpreted according to CLSI Disc Diffusion breakpoints (CLSI, 2018). *S. aureus* isolates that were resistant against three and more classes of antibiotics were categorized as multidrug-resistance *S. aureus* isolates (Jaja et al. 2020).

Data analysis

The number of *S. aureus* isolates that showed resistance against each antibiotic were counted and presented in percentages. Categorical data was compared and analysed using Pearson Chi-square test or Fisher's exact test (Minitab® 16.1.1, 2010), with 95% confidence interval ($p< 0.05$) was set to indicate the significance difference. Prevalence of antibiotic resistance was presented as the proportion of isolates tested with an inhibition zone (diameter) below the respective antibiotic breakpoint. The relationships between antibiotic exposure and overall antibiotic resistance in *S. aureus* isolates were assessed using multiple antimicrobial resistance index (MARI). The MARI was calculated as the proportion of antibiotics tested to which the isolate was phenotypically resistant. A dendrogram based on the phenotypic antibiotic resistance profile was generated using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method in BioNumerics 8.0 software (Applied Maths, Texas) to visualize the relatedness of the *S. aureus* isolated from rabbit and rabbit handlers.

RESULTS

Screening of *nuc* gene (Figure 1) has yielded a total of 70 *S. aureus i*solates. 40 *S. aureus* were from 35 different rabbits (19.1%; 35/183). 14 *S. aureus* isolates were from 12 (26.7%; 12/45) animal handlers while 8.8% (16/180) of the environmental swabs were *S. aureus*-positive (Table 1). However no MRSA was detected in this study. The antibiogram profile of *S. aureus* from rabbit handlers and rabbits were summarized in Table 2 and Table 3 respectively. MARI assessment of the *S. aureus* isolates was summarized in Table 4. A dendrogram generated using UPGMA to illustrate the relatedness of *S. aureus* based on their phenotypic antibiotic resistance pattern was shown in Figure 2.

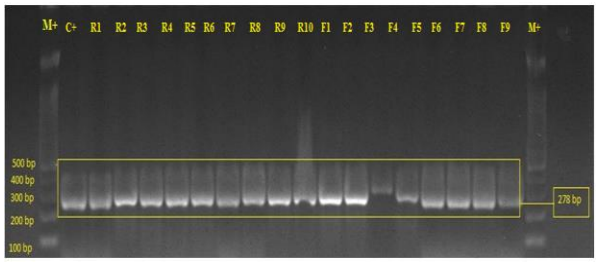

Figure 1: Agarose gel electrophoresis image of *nuc* gene (278bp) from representative *S. aureus* isolates. R represents rabbit while F represents rabbit handlers. Lanes labelled M+ are DNA ladder while lane labelled C+ is Control (ATCC700699).

Table 1: Occurrence rates of *S. aureus* according to the sampling sites in the rabbit farm environments.

Sampling sites	No. of samples	No. of *nuc* gene positive samples
Feeder	30	1
Drinker	30	5
Door lock	30	3
Wall of cage	30	2
Floor of cage	30	1
Boots	30	4

Table 2: Antibiogram of *S. aureus* isolates from rabbits (n=40).

	Number of isolates (%)		
Antibiotics	**S**	**I**	**R**
Chloramphenicol	34 (85)	0 (0)	6 (15)
Amoxicillin/clavulanate	36 (90)	0 (0)	4 (10)
Clindamycin	36 (90)	0 (0)	4 (10)
Linezolid	36 (90)	0 (0)	4 (10)
Norfloxacin	36 (90)	0 (0)	4 (10)
Oxacillin	30 (75)	6 (15)	4 (10)
Penicillin	36 (90)	0 (0)	4 (10)
Quinupristin-Dalfopristin	36 (90)	0 (0)	4 (10)
Tetracycline	36 (90)	0 (0)	4 (10)
Amikacin	36 (90)	2 (5)	2 (5)
Cefotaxime	36 (90)	2 (5)	2 (5)
Ciprofloxacin	36 (90)	2 (5)	2 (5)
Doxycycline	36 (90)	2 (5)	2 (5)
Erythromycin	38 (95)	0 (0)	2 (5)
Gentamicin	35 (87.5)	4 (10)	1 (2.5)
Trimethoprim-Sulfamethoxazole	36 (90)	4 (10)	0 (0)
Cefoxitin	40 (100)	0 (0)	0 (0)
Cephalothin	40 (100)	0 (0)	0 (0)
Kanamycin	40 (100)	0 (0)	0 (0)

S represents susceptible, I represents intermediate resistant while R represents resistant.

Table 3: Antibiogram of *S. aureus* isolates from rabbit handlers (n=14).

	Number of isolates (%)		
Antibiotics	**S**	**I**	**R**
Penicillin	1 (7.6)	0 (0)	13 (92.8)
Oxacillin	5 (36)	0 (0)	9 (64)
Amoxicillin/clavulanate	3(21)	4 (29)	7 (50)
Tetracycline	12 (86)	0 (0)	2 (14)
Erythromycin	13 (93)	0 (0)	1 (7)
Clindamycin	10 (71)	4 (29)	0 (0)
Cefotaxime	13 (93)	1 (7)	0 (0)
Trimethoprim-Sulfamethoxazole	13 (93)	1 (7)	0 (0)
Amikacin	14 (100)	0 (0)	0 (0)
Cefoxitin	14 (100)	0 (0)	0 (0)
Chloramphenicol	14 (100)	0 (0)	0 (0)
Cephalothin	14 (100)	0 (0)	0 (0)
Ciprofloxacin	14 (100)	0 (0)	0 (0)
Doxycycline	14 (100)	0 (0)	0 (0)
Gentamicin	14 (100)	0 (0)	0 (0)
Kanamycin	14 (100)	0 (0)	0 (0)
Linezolid	14 (100)	0 (0)	0 (0)
Norfloxacin	14 (100)	0 (0)	0 (0)
Quinupristin-Dalfopristin	14 (100)	0 (0)	0 (0)

S represents susceptible, I represents intermediate resistant while R represents resistant.

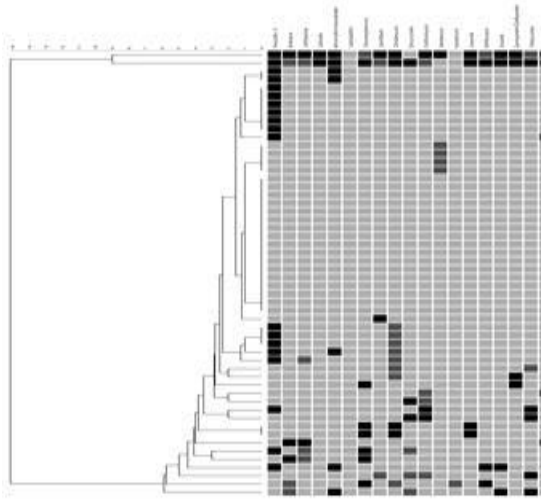

Figure 2: Dendrogram illustrating the relatedness of *S. aureus* based on their phenotypic antibiotic resistance pattern. Black colour columns = resistance, dark grey columns = intermediate resistance and light grey columns = susceptible

Table 4: MARI assessment of *S. aureus* isolates from rabbit and handlers (n=54).

Resistance to number of antibiotics	MAR index	Number of isolates (%)
0	0	32 (59.3)
1	0.05	13 (24.1)
2	0.11	6 (11.1)
3	0.16	1 (1.9)
4	0.21	0 (0)
5 and above	0.26	2 (3.7)

DISCUSSION

In this study, 19.1% of the rabbits sampled were found to be *S. aureus* carriers. This result is lower than a previous study that reported 41% to 63% of *S. aureus* occurrence rate in lesion swab samples of rabbits obtained from commercial rabbit farms in Spain, Portugal and China (Moreno-Grúa et al. 2018; Wang et al. 2019). Genotypic analysis revealed that 26.7% (12/45) of animal handlers were carrying *S. aureus* where most of the *S. aureus* isolates were from oral. This finding is in agreement with the statement given by Kozajda et al. in 2019 which reported that 20% to 40% of the general human population are *S. aureus* carriers. However, another report by Agnoletti et al. (2014) revealed the presence of *S. aureus* in nasal among 58.3% of individuals associated with rabbit farms in Italy after. This difference in *S. aureus* prevalence rates of *S. aureus* in both rabbit and handlers may be due to geographical factors, different sampling sites and sampling size.

This study showed almost 9% (16/180) of the environmental swabs were *S. aureus*-positive, indicating low *S. aureus* contamination rate at surface of feeder, drinker, door lock, wall and floor of cages and boots of farmers and their families (Table 2). Further data analysis revealed the *S. aureus* are mostly found in drinkers (17%; 5/30), suggesting that drinkers and drinking water play a significant role in the transmission and dispersion of *S. aureus*. Other previous studies conducted in rabbit farm environment also reported the presence of *S. aureus* on various farm surface, suggesting that various environmental surface in rabbit farms may as a vehicle of transmission for *S. aureus* (Friese et al. 2012; Friese et al. 2013; Agnoletti et al. 2014).

PCR detection of the *mecA* gene was applied to detect the presence of MRSA. Briefly, the *mecA* gene is responsible for the production of penicillin binding protein (PBP-2a) (Chai et al. 2020). PBP-2a allows the bacteria to develop resistance against antibiotics from beta-lactams class (Suhaili et al. 2018b). *MecA* gene is common in MRSA but absent in MSSA (Suhaili et al. 2018b; Chai et al. 2020). Today, PCR detection of the *mecA* gene is considered to be the "gold-standard" in identifying MRSA (Chai et al. 2020). The absence of *mecA* gene in the *S. aureus* isolates in this study indicated the absence of MRSA. The finding is in agreement with the study by Attili et al. (2020) that reported 0% MRSA prevalence rate among live rabbits from Italy. However, another previous study reported the presence of MRSA among rabbit handlers and rabbits in Italy commercial rabbit farms with the prevalence rate recorded at 32% and 3% respectively (Agnoletti et al. 2014). Furthermore, the presence of MRSA in the rabbit holdings was also reported (Agnoletti et al., 2014).

The emergence of antimicrobial resistance in important pathogens of humans and animals is a great concern. In the present study, *S. aureus* from rabbit and rabbit handlers show different degrees of resistance towards 18 selected antibiotics. However, the antibiogram of *S. aureus* from rabbit handlers and rabbits were different from each other. In rabbits, *S. aureus* showed a relatively low level of resistance (below 20%) against 15 different antibiotics, with the highest resistance rate against chloramphenicol (15%; 6/40). This finding is different from the result reported by another study where the highest level of resistance of *S. aureus* isolated from rabbits in Italy was observed against antibiotics from tetracyclines (96%) and macrolides (94%) (Attili et al. 2020). Meanwhile, *S. aureus* isolated from rabbit handlers only showed resistance rate against five different antibiotics, including penicillin (86%; 12/14), oxacillin (64%; 9/14), amoxicillin/clavulanate (50%; 7/14), tetracycline (14%; 2/14) and erythromycin (7%; 1/14). This finding is not surprising as beta-lactam antibiotics such as penicillin are often prescribed to treat bacterial infections in humans. A report by Che Hamzah et al. (2019) reported that 84.4% of MSSA isolated in patients from Hospital Sultanah Nur Zahirah, Malaysia were resistant to penicillin. However, it is noteworthy to point out that the oxacillin resistance rate displayed by *S. aureus* from rabbit handlers is higher than the 5.5% resistance prevalence rate given by the previous study (Che Hamzah et al. 2019). The findings from previous and present study suggest that the usage of beta-lactam antibiotics, especially penicillin to treat *S. aureus* infections in humans

may no longer be a viable choice in the near future. Nonetheless, all of the *S. aureus* isolated from rabbit and their handlers were fully susceptible to cephalothin, cefoxitin and kanamycin, suggesting that these antibiotics can be used to treat persistent *S. aureus* treatment for both rabbit and rabbit handlers in the selected rabbit farms.

Further analysis of antibiogram data have revealed that three of *S. aureus* isolates from human (1/14; 7.1%) and rabbit (2/40; 5%) can be categorised as MDRSA according to the definition given by Magiorakos et al. (2012). This result is lower than the reported 93% MDRSA prevalence rate obtained in rabbits from Italy (Attili et al. 2020). Nonetheless, the presence of MDRSA in rabbits should be a concern as AMR carrying bacteria may spread further to other animals and humans. The emergence of antimicrobial resistance bacteria strain is often the result of intensive and unregulated usage of antibiotics in human medicine and animal farming industry (Ariffin et al. 2019; Ariffin et al. 2020). Thus, it is possible that these MDRSA from both humans and rabbits may come from an environment with high antibiotic usage. To assess this possibility, MARI assessment was carried out and the data (Table 5) showed that only two of *S. aureus* from rabbits have the MARI value of 0.2 and above, indicating the isolates originate from environments with frequent antibiotic usage (Riaz et al. 2011). In addition, a dendrogram (Figure 2) was generated based on the antibiogram profile of the *S. aureus* showing high diversity among the isolates, indicating that these *S. aureus* may have great differences in antibiotic exposure and genetic background.

CONCLUSION

This study showed that the *S. aureus* have been successfully isolated from rabbit, rabbit handlers and environment. No MRSA were identified, but the presence of MDRSA was detected in both rabbits and rabbit handlers. *S. aureus* from rabbit handlers showed resistance against penicillin, oxacillin, amoxicillin/clavulanate, tetracycline and erythromycin. Meanwhile, *S. aureus* from rabbit showed resistance against 15 different antibiotics. These finding suggest the emergence of antibiotic resistant among *S. aureus* that presence in the rabbit farms settings. The rabbit farming in Malaysia is rapidly blooming with bright prospect. Therefore, antibiotic usage in rabbit health management needed to be regulated to prevent further emergence of AMR. Antimicrobial stewardship program need to be conducted to educate the rabbit farm owners and workers regarding the risk of AMR. More surveillance programmes that investigate the AMR status in rabbits from other regions in Malaysia should be conducted to have a better insight on the AMR status in Malaysia rabbit farming industry.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

CMH involved in the study design and data collection as well drafted the manuscript. MEAM, MZS, NMN, NAM, NANMA and NMM were involved in the sampling, data collection and contributed to the manuscript preparation. SMZA and MFG took part in the study design, preparation and critical checking of this manuscript.

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