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Phenotypic and genotypic characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from dogs and cats at University Veterinary Hospital, Universiti Putra Malaysia

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Abstract. Methicillin-resistant *Staphylococcus aureus* (MRSA) is known to cause nosocomial infections and is now becoming an emerging problem in veterinary medicine. The objective of the study was to determine the presence of MRSA in 100 cats and dogs sampled between November 2007 and April 2008 at the University Veterinary Hospital, Universiti Putra Malaysia. MRSA was detected in 8% of pets sampled. Ten percent (5/50) and 6% (3/50) of the isolates were from dogs and cats, respectively. All MRSA isolates possessed the *mec*A gene and were found to be resistant to at least three antimicrobials with a minimum of Oxacillin MIC of 8 µg/mL. One isolate (CT04) had an extremely high MIC of >256 µg/mL. The MLST type ST59 found in this study have been reported earlier from Singapore and other countries as a strain from animal and community-associated MRSA respectively. Pulsed-field gel electrophoresis revealed five pulsotypes. Two isolates from cats (CT27 and CT33) and three isolates from dogs (DG16, DG20, and DG49) were respectively assigned to pulsotypes B and D. The study suggests that cats and dogs in Malaysia are potential reservoirs for MRSA.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) emerged as an important nosocomial pathogen in the early 1960s. Since then, MRSA has been causing hospital-associated infections throughout the world; however, recent epidemiological shift has been observed as MRSA started to prevail in the community and causing associated diseases (Ochoa *et al.*, 2005; Zetola *et al.*, 2005). Moreover several recent reports showed that MRSA is becoming an emerging problem in veterinary medicine, particularly in small animal and equine practices (Leonard & Markey, 2008). The first case of MRSA in veterinary species was identified in the milk of a cow with mastitis in 1972 (Duquette & Nuttall, 2004; O'Mahony *et al.*, 2005). Methicillin resistance in *S. aureus* is mediated by the penicillin-binding protein PBP2a, which has a low binding affinity to β-lactam antibiotics. It is encoded by the *mec*A gene located on one of the six types of staphylococcal cassette chromosome mec (SCCmec) (Hiramatsu *et al.*, 2001; Morgan, 2008).

Initially, reports of MRSA colonization in small animals were infrequent, however, marked increment of case reports have been appearing in recent years (Boag *et al.*, 2004; van Duijkeren *et al.*, 2004; Rich & Roberts, 2004). Inter-transmission of MRSA between animals and humans have been reported (Manian, 2003; Weese *et al.*, 2005) and companion animals have been indicated as a potential reservoirs of MRSA

(Cefai *et al.*, 1994; O'Mahony *et al.*, 2005). This is becoming a public health concern because companion animals often are in close contact with their owners, risking them to the transmission of pathogenic bacteria (Gurdabassi *et al.*, 2004).

Studies conducted on MRSA in Malaysia so far have been conducted on humans, mainly focusing on major hospitals in the country (Neela *et al.*, 2008; Norazah *et al.*, 2009). However, data related to MRSA in animals are scarce. To date, there is only a single report of MRSA study in cats and dogs conducted by Jacklyn (2006), and there is no data on the molecular epidemiology of MRSA isolated from cats and dogs. Therefore, the objectives of this study were to detect MRSA in cats and dogs referred to the University Veterinary Hospital, Universiti Putra Malaysia and to determine the molecular typing of the isolates using Pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Isolation and Identification

Fifty cats and 50 dogs were included in this study. The animals were residents, inpatients, and visiting cases at the University Veterinary Hospital (UVH), Universiti Putra Malaysia. Samples were collected between November 2007 and April 2008. Two separate sterile swabs with Stuart Transport Medium (Oxoid, UK) were used to hygienically collect samples from each nostrils and the perianal region of the animals. The swabs were placed into 10 mL Tryptone Soya Broth (Oxoid, UK) for 48 h for enrichment and later streaked onto 7% blood agar and incubated at 37ºC overnight. Catalase and oxidase positive colonies were subcultured onto mannitol salt agar and further confirmed by Staphytect Plus® (Oxoid, UK). The selection of MRSA were made on Oxacillin-resistant agar base media (ORSAB; Oxoid, UK) supplemented with ORSAB selective supplement (Oxoid, UK), consisting of oxacillin and polymyxin B. Inoculated plates were aerobically incubated at 37ºC for 24-48 h.

Antimicrobial Susceptibility Test

Inocula were prepared from overnight MRSA cultures on blood agar supplemented with 2% sodium chloride (NaCl). Pure colonies were emulsified into normal saline (0.85% NaCl) and turbidity of the suspension was equilibrated to 0.5 MacFarland and spread onto Mueller-Hinton agar (MHA; Oxoid, UK). Fifteen antimicrobial discs were used in this study. The interpretative criteria for each antimicrobial tested were made according to the recommendations published by the Clinical and Laboratory Standards Institute (CLSI, 2006). Oxacillin MIC was determined by using E test (AB Biodisk, Solana, Sweden) on MHA with 2% NaCl.

Detection of *mec*A gene by Polymerase Chain Reaction (PCR)

Extraction of genomic DNA from MRSA culture was performed using DNeasy Blood and Tissue DNA purification kit (Qiagen) per procedures recommended by the manufacturer. The PCR amplification was done as described earlier by Murakami *et al.* (1991), with the following sequences: mecA1, 5'-AAA ATC GAT GGT AAA GGT TGG C-3' and mecA2, 5'-AGT TCT GCA GTA CCG GAT TTG C-3'. The PCR amplification mixture consisted of 200 µM of dNTPs, 1 IU of Taq polymerase, 1.5 mM $MgCl_2$ (PROMEGA Corporations, USA), 2 µM of each primer, and 50 to 100 ng of template. The amplification was carried using the following parameters: 94ºC for 45 s, 30 cycles of (94ºC for 45 s, 55ºC for 1 min, 72ºC for 2 min) and 72ºC for 2 min. PCR products were resolved by electrophoresis with 1% agarose gel, stained with ethidium bromide and visualized using UV-Gel Doc system (Bio-Rad). *Staphylococcus aureus* ATCC 29253 (negative *mec*A) and *S. aureus* ATCC 43300 (positive *mec*A) were used as controls.

spa Typing

Staphylococcal protein A (*spa*) and MLST typing were conducted on three selected isolates, two dog isolates (DG16, DG36), and one cat isolate (CT04). Accordingly

amplification of the spa repeat region was performed using primers *spa*-1113f (5'-AAAGACGATCCTTCGGTGAGC-3') and *spa*-1514r (5'-AGCAGTAGTGCCGTTT GCTT-3') as described previously by Harmsen *et al.* (2003). A PCR mixture of 50 µl final volume was constituted by 1.25 IU Taq polymerase, 1.mM MgCl2, 200 µM of dNTP mix, 0.2 µM of each primer (*spa*-1113f, and *spa*-1514r), 5 µl of 10x PCR buffer and 3 µl of genomic DNA. The cycling condition was done for 5 min at 80ºC, followed by 35 cycles of 45s denaturation at 94ºC, 45 s of annealing at 60ºC, 90s of extension at 72ºC, and a final extension step of 10 min at 72ºC. Purification of the PCR product was done by using QIAquick PCR Purification Kit (QIAGEN) and sent for sequencing. The sequences were aligned and the analysis and subsequent typing were done by feeding sequence repeats into online spa serve (http://spaserver.ridom.de/)

MLST Typing

Multilocus sequence typing of the same selected isolates has been conducted by amplifications of internal fragments of the seven housekeeping genes of *S. aureus* according to Enright *et al.* (2000) with minor modifications. The master mix for each pair of primer consisting 3 µl of DNA template, 200 µM dNTP mix, 1.25U Taq polymerase, 0.25 µM of each primer, 5 µl of 10x PCR buffer, sterile deionised distilled water was added to make 50 µl of reaction volume. The PCR was conducted with an initial 5-min denaturation at 95ºC, followed by 30 cycles of annealing at 55ºC for 1 min, extension at 72ºC for 1 min, and denaturation at 95ºC for 1 min, followed by a final extension step of 72ºC for 5 min The annealing temperature was raised to 60ºC for amplification of gmk and pta genes. The PCR products were purified by QIAquick PCR Purification Kit (QIAGEN) and sent for sequencing. The sequenced products were aligned and assignment of the allele and sequence type was made using online database (http://saureus.mlst.net).

PFGE Typing

Pulsed-field gel electrophoresis of the isolates was conducted according to Harmony protocol (Murchan *et al.*, 2003). *S. aureus* colonies from overnight cultures were embedded into agarose plugs. After bacterial lysis with lysozyme (100 mg/L), lysostaphin (10 000 U/mL) and RNase (50 mg/L) for 2 h at 37ºC, genomic DNA was digested using *Sma*I (40 U/mL) at room temperature, overnight. Separation of the digested fragments (PFGE) was done using a CHEF DRII (Bio-Rad). The parameters of electrophoresis were 6v/cm with an angle of 120º and initial switch time of 5s to 15s for 10 hrs and final switch time of 15s to 60s at 14ºC. Gel pictures were obtained by using Alpha Imager (Bio-Rad).

RESULTS

Isolation of MRSA

MRSA were detected in 5 dogs (10%) and 3 cats (6%) of the 100 pets sampled. Among the MRSA positive dogs, 8% (4/50) and 2% (1/50) were inpatient and outpatient dogs respectively. In cats, MRSA was isolated from inpatients only.

Antimicrobial Susceptibility

All isolates (100%) were resistant to at least 3 antimicrobials including oxacillin, methicillin and amoxicillin. Whereas 75% (6/8) of the isolates have shown resistance to amikacin and cefoxitin. All isolates were susceptible to impenem (10 µg/mL), vancomycin (30 µg/mL), and rifampicin (15 µg/mL) (Figure 1). All isolates had MICs of at least 8 µg/mL towards oxacillin, confirming that that the isolates are oxacillin-resistant. It was found that isolate CT04 had a high level of resistance with an MIC of 256 µg/mL (Table 1).

PCR Amplification of the *mec*A

All the isolates were positive for *mec*A (533 bp), the gene mostly responsible for methicillin (oxacillin) resistance (Figure 2).

MLST and *spa* Typing

The results from MLST assigned the two isolates from dogs (DG 16 and DG 20) to ST59 whereas the isolate from a cat (CT04) was typed as ST55. On the other hand, all the three isolates tested were typed to be different by *spa* typing (Table 1).

PFGE Results

Using the criteria proposed by Tenover *et al*. (1995), visual analysis of the PFGE patterns revealed five pulsotypes. Two cat isolates (CT27 and CT33) and three dog isolates (DG16, DG20, and DG49) shared indistinguishable PFGE patterns. Isolate

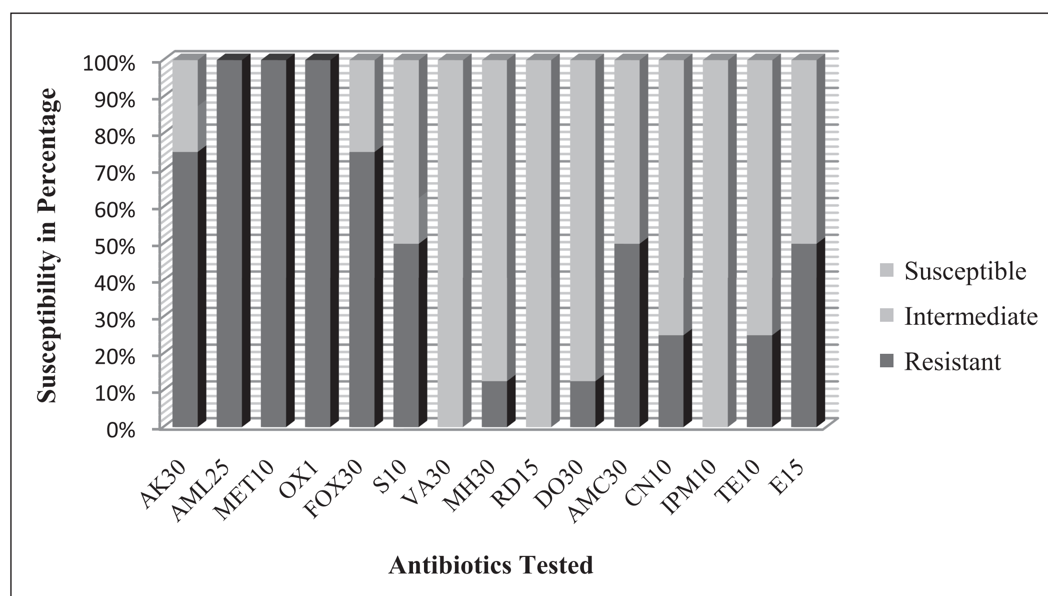

Figure 1. Antibiotic susceptibility profile of MRSA isolated from cats and dogs. AK30 (Amikacin 30 µg), AML25 (Amoxicillin 25 µg), MET10 (Methicillin 10 µg), OX1 (Oxacillin 1 µg), FOX30 (Cefoxitin 30 µg), S10 (Streptomycin 10 µg), VA30 (Vancomycin 30 µg), MH30 (Minocycline 30 µg), RD15 (Rifampicin 15 µg), DO30 (Doxycycline Hydrochloride 30 µg), AMC30 (Amoxycillin-Clauvulanic acid 30 µg), CN10 (Gentamicin 10 µg), IPM10 (Impenem 10 µg), TE10 (Tetracycline 10 µg), E15 (Erythromycin 15 µg)

Table 1. MIC, *mecA*, *spa* typing and MLST results of MRSA isolates from dogs and cats. *Spa* typing and MLST were conducted for three selected isolates

Isolate ID	Source	MIC in µg/mL	*mec*A	*spa* Type	MLST(ST)
DG13	Dog	12 µg/ml	Positive	ND*	ND
DG16	Dog	16 µg/ml	Positive	t3590	ST59
DG20	Dog	8 µg/ml	Positive	t267	ST59
DG36	Dog	16 µg/ml	Positive	ND	ND
DG49	Dog	12 µg/ml	Positive	ND	ND
CT04	Cat	>256 µg/ml	Positive	t346	ST55
CT27	Cat	32 µg/ml	Positive	ND	ND
CT33	Cat	32 µg/ml	Positive	ND	ND

*ND: Not determined

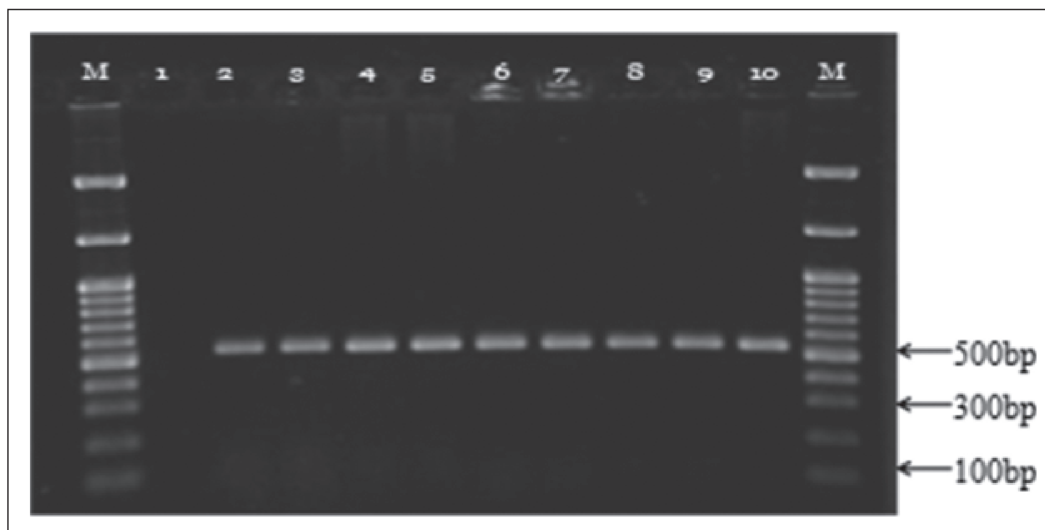

Figure 2. Agarose gel electrophoresis of amplified 533bp segment of the *mec*A gene. (Lanes: M, 100bp size markers; 1, ATCC29253; 2, DG13; 3, DG16; 4, DG20; 5, DG36; 6, DG49; 7, CT04; 8, CT27; 9, CT33; 10, ATCC43300)

CT04 and DG13 had band difference of ≥ 6 with respect to all other isolates, hence they were assigned to distinct pulsotypes (Figure 3).

DISCUSSION

MRSA, emerging soon after methicillin was used in treating infections had caused nosocomial infections worldwide. The organism has since been isolated from livestock and companion animals. Cats and dogs represent potential sources of the spread of MRSA due to the extensive use of the antimicrobial agents and their close contact with humans (Gurdabasi *et al.*, 2004).

The current study revealed that 8% (8/100) of pets sampled at the UVH, UPM was positive for MRSA. Of this, 10% (5/50) and 6% (3/50) were from dogs and cats, respectively. Other studies have also reported more MRSA in dogs (Rich & Roberts, 2004; O'Mahony *et al.*, 2005; Morgan, 2008); compared to cats. However, the prevalence rates of MRSA found in several studies were much lower compared to that of the current study. Kown *et al.* (2006) reported MRSA in 1.9% (3/157) of hospitalized dogs in Korea, Malik *et al.* (2006) reported MRSA in 0.8% (2/252) of dogs and cats in Australia and Lilenbaum *et al.* (1998) found MRSA in 2% of healthy cats in Brazil. In Malaysia, a previous study by Jacklyn (2006) reported MRSA in 1.93% (7/362) of cats and dogs, a rate that is much lower that reported in the current study. The current finding is consistent with the recent surveillance of MRSA in small animals in the United States which indicated that MRSA has been detected at increasingly higher rates (Morris *et al.*, 2006; Jones *et al.*, 2007).

All MRSA isolates in the current study were resistant to three or more of the antimicrobials tested including Oxacillin and Methicillin. Although vancomycin resistance (Tiwari & Sen, 2006) and imipenem resistances (Goff & Dozicky, 2007) were reported in previous studies, all the isolates in the current study were susceptible to both antibiotics. In our study, the MIC for all isolates were more than 8 µg/mL and one isolate from a cat had an extremely high MIC of more than 256 µg/mL. This suggests that a highly oxacillin and multidrug resistant *S. aureus* is also a problem in companion animals. It is most probably that these infections were

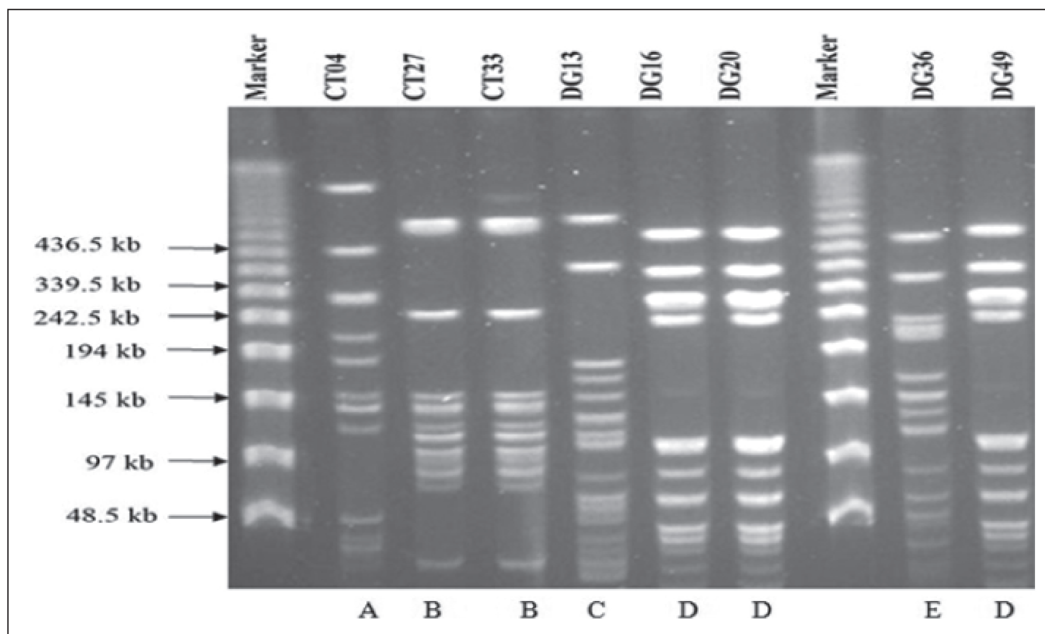

Figure 3. PFGE patterns of MRSA isolated from cats and dogs. A, a pair of indistinguishable feline isolates (CT27 and CT33); B. Canine isolates (DG16, DG20, DG49) sharing the same PFGE patterns

acquired by the animals from MRSA of human origin. Several epidemiological studies and case reports have suggested that MRSA transmission from human to companion animals and vice versa is possible (van Duijkeren *et al.*, 2004; Baptiste *et al.*, 2005; Weese *et al.*, 2005; Hanselman *et al.*, 2008).

Referring to the MLST database, the MLST type ST59 found in this study was also reported from neighboring countries Singapore, Australia, Taiwan, Japan, UK, and US. Most of the data referring to ST59 revealed that these MRSA isolates were community-associated and possessed PVL genes (Vandenesch *et al.*, 2003; Takano *et al.*, 2008). While ST55 was reported from UK only, in which it was grouped as an intermediate between major MRSA clones ST36 and ST45, and CC8 clones, it was the first MRSA lineage to be described (Cooper & Feil, 2006). This finding may imply that an epidemic community-associated MRSA is also colonizing pets in Malaysia and this entails that the MRSA strains could have been acquired by the pets from human sources. It has been reported that dogs and other pets living in close contact with human MRSA carriers can become colonized with MRSA of human origin (Duqquette & Nuttal, 2004; van Duijkeren *et al.*, 2004). On the other hand the increasing reports of MRSA isolation from companion animals has also raised public health concern as these animals can potentially pose risks to humans by serving as reservoirs (Cefai *et al.*, 1994; Moore & Lindsay, 2002; Manian, 2003; van Duijkeren *et al.*, 2004). In addition to MLST typing, a single *spa* typing has proven to be the method of choice for its rapidity, low cost and reproducibility in such a way that it can predict clonal lineages of *S. aureus* (Koreen *et al.*, 2004). The *spa* type t3590 isolated from a dog in the current study have previously been reported from China. While t267 and t346 were reported from at least five European countries (http://spaserver2.ridom.de/spatypes.shtml). The fact that these *spa* types are found in pets in this study might indicate the possible dissemination of the strains into Malaysia most likely through human carriers with subsequent transmission of the strains to pet animals. The PFGE patterns of the isolates suggest that there are two main

clonal types of MRSA, characterized by pulsotype B and D that are colonizing cats and dogs presented to the university small animal hospital. This finding may indicate that the two MRSA types here (B and D) have been prevailing and circulating in the small animal hospital and colonizing animals admitted. Furthermore, these MRSA clones can serve as potential contaminants to the environment and source of infection for humans through direct or indirect contact to the pets.

Although the sample size of our study was small, we believe that it gives a picture of the overall occurrence of MRSA in cats and dogs in Malaysia at present. It is a common practice for antimicrobial prescription for the clinical cases to be instituted before confirmatory diagnosis. Such practice invites potential microbes like *S. aureus* to become resistant to a wide range of antimicrobials. Irrespective of the mode of transmission, whether from human-to-animals or vice versa, the high number of positive cases suggests high number of individuals serving as reservoir hosts for the MRSA, who may then become the source of infections or colonization. With the increase in pet-ownership, the inter-transmission of antimicrobial resistant bacteria will persist unless the transmission is interrupted. Therefore, it is important that studies on MRSA in Malaysia be conducted on a larger scale to monitor the MRSA occurrence in the country. This ought to be done by employing more discriminatory identification approaches such as the molecular tools to reveal the epidemiological patterns of the organism. Implementation of appropriate control and prevention methods could help to contain the colonization and/or infection. However, other approaches such as public education for a more stringent personal and environmental hygiene, test and decolonization of carrier individuals, prudent use of antimicrobial agents are among strategies that may also be employed.

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