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A new *spa* type t5696 MRSA Isolated from Veterinary Medicine Student at Universiti Putra Malaysia

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been spreading worldwide since its emergence. Swab samples were collected from the nostrils and oral cavity of veterinary medicine student at Universiti Putra Malaysia. Phenotypic identification of MRSA was made by employing broth enrichment, catalase and cogulase tests, mannitol fermentation test on mannitol salt agar (MSA), slide latex agglutination test, growth on selective media, Oxacillin-resistant screening agar base (ORSAB). Intense blue colonies on ORSAB presumptively confirmed MRSA. Antibiotic sensitivity tests using 15 antibiotics revealed that the isolate was resistant against Amikacin 30 µg (AK30), Amoxicillin 25 µg (AML25), Methicillin 10 µg (MET10), Oxacillin 1 µg (OX1) Streptomycin 10 µg (S10), and Tetracycline 10 µg (TE10) while being intermediately resistant Oxacillin (OX1) and susceptible to other antibiotics tested. The minimum inhibitory concentration (MIC) of the isolate was 8 µg/mL. However, detection of the resistance gene *mec*A was proved negative. This discrepancy between phenotypic and mecA detection methods is attributed to the fact that there are other non-*mec*A dependent Oxacillin resistance factors in *S. aureus*. Amplification of the polymorphic X region of the staphylococcal protein A gene was done by PCR. Purified PCR product was sequenced, aligned and repeat numbers were assigned. Repeat sequence pattern was assigned a unique *spa* type, t5696. The finding hints the importance of further studies by focusing on the occurrence of MRSA in veterinary professionals and students with effective molecular typing techniques such as *spa* typing. This will in turn helps in understanding the similarities and uniqueness of local isolates in reference to MRSA isolates reported anywhere in the world

Keywords: MRSA, antibiotic sensitivity test, MIC, mecA gene, spa typing

Introduction

Staphylococcus aureus features incredibly diverse pathogenic and resistance mechanisms, and MRSA has emerged as the major bacterial pathogen of the 21st century (Bartlett and Stenger, 2008). Since its emergence in 1961 in UK, the incidence of MRSA infections has been increasing steadily (Moodley *et al*., 2006). Methicillin resistance in *S. aureus* is mainly attributed to the presence of *mec*A gene which encodes the production of an additional protein-binding protein (PBP) named PBP2'/PBP2a. The PBP2a has been shown to be present in 90% of MRSA isolates (Ohwada *et al*., 1999). There are numerous techiniques available to differentiate S. aureus and specifically MRSA. Phenotypical methods such as antibiogram and phage typing were previously being used as the sole typing methods. However, DNA-based techniques such as *spa* typing, multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) became popular and more reliable tools (Shopsin *et al*., 1999).

The staphylococcal protein A gene (*spa*) contains a polymorphic region X of the *spa* gene consists of a variable number of tandem repeats, generally 24 bp but repeats of 21 and 27 bp in length have been identified (Moodley *et al*., 2006). The technique combines a number of technical advantages, such as rapidity, reproducibility, and portability. Moreover, due to its repeat structure, the *spa* locus simultaneously indexes genetic variations that accumulate both rapidly and slowly (micro-and macro-variations), enabling the use of *spa* typing in both local and global epidemiological studies (Hallin *et al*., 2009).

In Malaysia, there were molecular typing studies conducted to assess epidemiology of MRSA in major hospitals in the country by using PFGE, MLST, and SCC*mec* typing as the main tools (Sam *et al*., 2008; Norazah *et al*., 2003). However, there was no data as to the occurrence and molecular typing of MRSA isolated from veterinary professionals, particularly veterinary medicine students. In this study we report a new spa sequence type for an MRSA isolated from veterinary medicine student.

Methodology

Isolation and Identification of MRSA

Nasal and oral swab samples were obtained from the student (PJ404) on voluntary basis in which the student signed a consent form following briefing about the objectives and procedures of the study. Swabs were placed into 10 mL Tryptone Soya Broth (TSB) for 48 hours to enrich the growth after which streaking was done onto blood agar with 7% horse blood. The inoculated plates were incubated aerobically at 37^{o}C for 18-24 h. Gram-staining, catalase and coagulase tests, mannitol fermentation test on MSA and slide latex agglutination were carried out to identify S. aureus colonies. The presumptive *S. aureus* colonies (yellow colonies) were then streaked onto ORSAB (Oxoid, UK) supplemented with ORSAB selective supplement consisting of 1 mg oxacillin and 25,000 IU polymyxin B incorporated into 500 mL of the agar solution.

Antibiotic Sensitivity Test

Antibiotic sensitivity tests were carried out for MRSA isolates according to CLSI standards (2006). The antimicrobials tested were: Amikacin 30 µg (AK30), Amoxicillin 25 µg (AML25), Methicillin 10 µg (MET10), Oxacillin 1 µg (OX1), Cefoxitin 30 µg (FOX30), Streptomycin 10 µg (S10), Vancomycin 30 µg (VA30), Minocycline 30 µg (MH30), Rifampicin 15 µg (RD15), Doxycycline Hydrochloride 30 µg (DO30), Amoxycillin-Clauvulanic acid 30 µg (AMC30), Gentamicin 10 µg (CN10), Impenem 10 µg (IPM 10), Tetracycline 10 µg (TE10), Erythromycin 15 µg (E15).

Minimum Inhibitory Concentration (MIC) By Etest

Determination of MIC was done by using Oxacillin Etest (AB Biodisc, Solana, Sweden) strips according the manufacturer's recommendations. According to the break points defined (CLSI, 2006), isolates with MIC $\geq$ 4 µg/mL were considered oxacillin resistant.

PCR Amplification of mecA Gene

The PCR amplification was done by using *mec*A primers (Bio Basic Inc., Canada) which were previously designed by Murakami *et al.*(1991), which amplifies the 533 bp segment of the *mec*A

gene and with the following sequences: *mec*A1, 5'-AAA ATC gAT ggT AAA ggT Tgg C-3' and *mec*A2, 5'-AgT TCT gCA gTA CCg gAT TTg C-3'.

spa typing

The polymorphic x region of *S. aureus* protein A was amplified using forward and reverse primers, *spa*-1113f (5'-AAAGACGATCCTTCGGTGAGC-3') and *spa*-1514r (5'-CAGCAGTAGTGCCGTTTGCTT-3') respectively as described previously by Harmsen *et al.* (2003). The PCR products were purified by QIAquick PCR Purification Kit (Qiagen) and the purified products were sent to MACROGEN (S. Korea) for sequencing.

Results

Identification of MRSA

Typical intense blue colonies of the isolate on ORSAB showed that the isolate was MRSA. However, PCR amplification for detection of the *mec*A gene proved negative result.

Antibiotic Sensitivity Test and MIC determination by Etest

The isolate was resistant against AK30, AML25, MET10, S10, and TE10 while being intermediately resistant Oxacillin (OX1). Whereas the isolate was detected to be susceptible to all the remaining antibiotics tested including cefoxitine (FOX30) and vancomycin (VA30) and Impenem (IMP10). The MIC value for the isolate was 8 µg/mL, which is above the 4µg/mL breakpoint recommended for MRSA according to the CLSI standards.

spa Typing

Amplification of the polymorphic X region of the *spa* gene gave a 240 bp product. Upon sequence alignment and analysis, the repeat sequences were identified, assigned repeat numbers and *spa* type by the help of the online database (http://www.ridom.de). Accordingly the repeat pattern was 07-16-12-23-02-02-02-02-02-34. The repeat patterns along with the chromatogram

were sent to the *spa* curator to be recognized and assigned a new *spa* type. Hence, the repeat pattern was assigned a new *spa* type, (t5696), GeneBank accession number: GU229647.

Discussions

Methicillin-resistant *S. aureus* has recently been considered as important pathogen in animals and animal health practitioners as it has been increasingly reported in veterinary medicine (Moodley *et al*., 2008). Previously we reported the occurrence of MRSA among veterinary medicine students at Universiti Putra Malaysia in which 23.3% (24/103) were MRSA culture positive (Aklilu *et al*., 2008). A study conducted by Wulf *et al*. (2006) also revealed MRSA carriage among veterinary doctors and students in the Netherlands. On the other hand, a screening survey conducted on pre-clinical medical students at Melaka Manipal Medical College in Malaysia did not find any MRSA (Santhosh *et al*., 2007).

In the current study, discrepant results in phenotypic and molecular identification have been observed. This can be ascribed to the absence of the resistant gene, *mec*A. Absence of the *mec*A gene can be attributed to several factors since there are other mechanisms that are non-*mec*-dependent that contribute individually or in combination towards antibiotic resistance in staphylococci strains (Berger-Bachi and Tschierske, 1998; Berger-Bachi, 1995). Overproduction of normal PBP with an altered binding capacity or other unidentified factors potentially contribute to the rise of methicillin resistance in *mec*A negative *S. aureus* strains (Chambers, 1997). Such features imply that these *mec*A negative isolates might be those MRSA strains showing border-line resistance towards oxacillin/methicillin (BORSAs). Strains with borderline or low-level methicillin resistance have MIC values of 1.5-12 mg/L for the majority of the cell population, but clinical strains without *mec*A and with methicillin MICs in the 4-16 mg/L range have also been reported (Bignardi *et al*., 1996). Borderline methicillin-resistant *S. aureus* may or may not carry *mec*A and are extremely heterogeneous or produce PBP2a or be penicillinase hyperproducers (Hall, 2003; Brown, 2001). Moreover, the MRSA selective agar (ORSAB) used in this study does not exclude growth of *S. aureus* that are hyperproducers of penicillase (Nahimana *et al.* 2006).

Among the various sequence-based MRSA typing methods, *spa* typing has been shown to be an effective and rapid method for typing MRSA (Shopsin *et al*., 1999). In the current study, the MRSA isolate was designated as a new *spa* type. The differences in *spa* types among *S. aureus* isolates is attributed to the diversity of short sequence repeat(SSR) region arise from deletion and duplication of the repetitive units and also by point mutation (Shopsin *et al*., 1999; Brigido *et al*., 1991). Previous studies by Wulf *et al*. (2006) in the Netherlands and Moodley *et al*. (2006) in UK and Irelands also used *spa* typing for MRSA isolated from veterinary medicine students and staff. The discovery of a new *spa* type in the current study confers a unique identity to the isolate in question. This can further be used as a reference for future studies to be done on spa typing of *S. aureus* isolates. Moreover, the finding pin points the importance of further studies focusing on the occurrence of MRSA in veterinary professionals and students with effective molecular typing techniques such as *spa* typing. This will in turn helps in understanding the similarities and uniqueness of local isolates in reference to MRSA isolates reported anywhere in the world.

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