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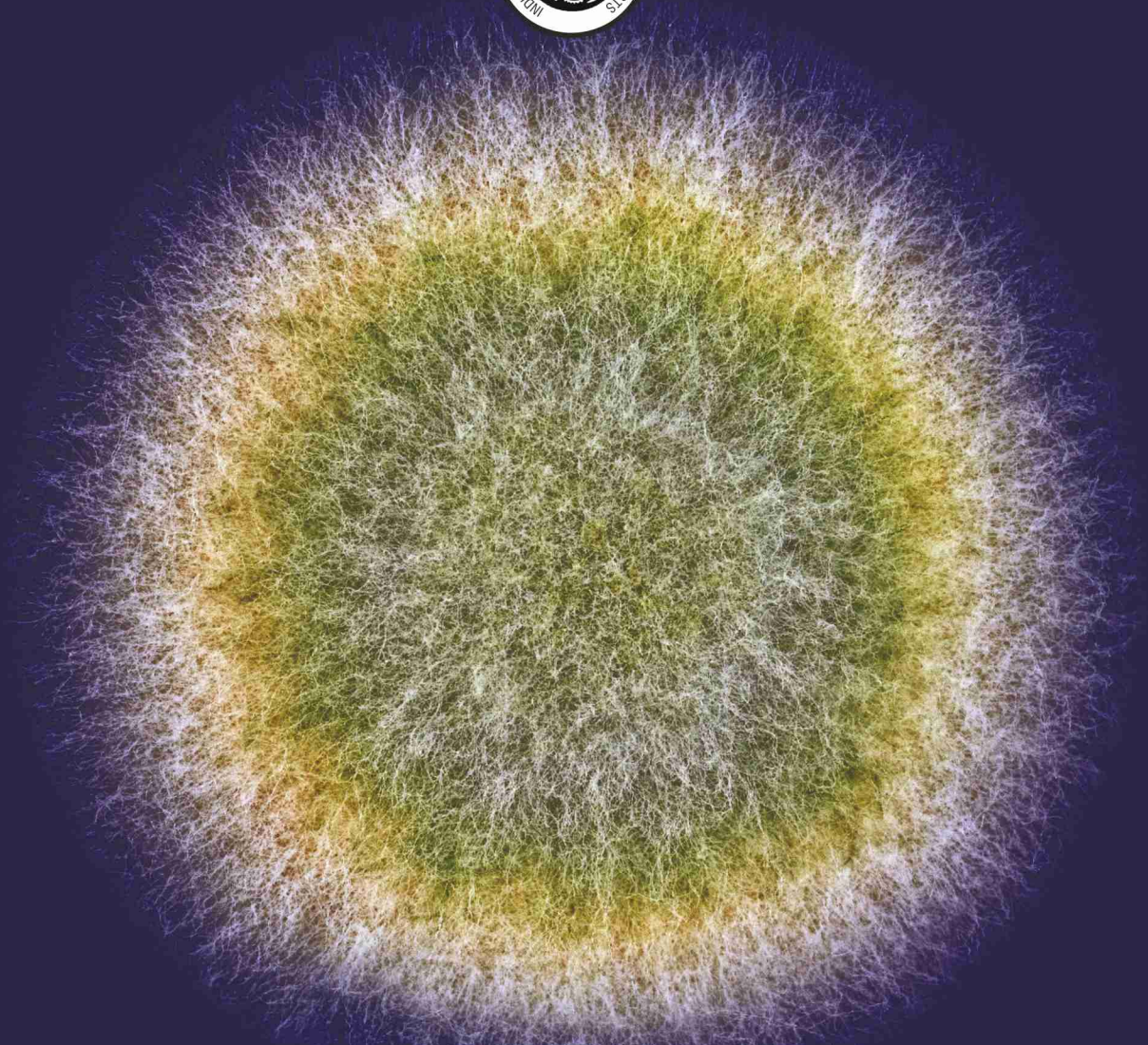

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Brief Communication

ermA, ermC, tetM and tetK are essential for erythromycin and tetracycline resistance among methicillin-resistant *Staphylococcus aureus* strains isolated from a tertiary hospital in Malaysia

KT Lim, YA Hanifah, MYM Yusof, *KL Thong

Abstract

The objective of this study was to determine the expression and transferability of tetracycline and erythromycin resistance among 188 MRSA strains from a Malaysian tertiary hospital. The minimum inhibitory concentrations (MICs) for oxacillin, erythromycin, tetracycline and ciprofloxacin ranged from 4 to 512 µg/ml, 0.25 to 256 µg/ml, 0.5 to 256 µg/ml and 0.5 to 512 µg/ml, respectively. Tetracycline-resistant strains showed co-resistance towards ciprofloxacin and erythromycin. There was a significant increase (P<0.05) of high-level tetracycline (≥MIC 256 µg/ml) and erythromycin (≥MIC 128 µg/ml) resistant strains in between the years 2003 and 2008. All erythromycin-resistant strains harboured *ermA* or *ermC* gene and all tetracycline-resistant strains harboured *tetM* or *tetK* gene. The *blaZ* was detected in all MRSA strains, whereas *ermA, tetM, ermC, tetK* and *msrA* genes were detected in 157 (84%), 92 (49%), 40 (21%), 39 (21%) and 4 (2%) MRSA strains, respectively. The *blaZ, tetM, ermC and tetK* genes were plasmid-encoded, with *ermC* gene being easily transmissible. Tn5801-like transposon was present in 78 *tetM*-positive strains. *ermA* and *tetM* genes were the most prevalent erythromycin and tetracycline resistance determinants, respectively, in MRSA strains. The association of resistance genes with mobile genetic elements possibly enhances the spread of resistant traits in MRSA.

Key words: *Ciprofloxacin, erythromycin, Methicillin-resistant Staphylococcus aureus, tetracycline, transformation*

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important bacterial pathogen associated with community (CA) and health-care (HA) infections in Malaysia and worldwide. A local study reported that the MRSA strains in Malaysia are often resistant to erythromycin, gentamicin and ciprofloxacin.[1]

In many cases, erythromycin resistance is also associated with the resistance to other macrolides, lincosamides and type B streptogramin (MLS_B). There are three mechanisms involved in erythromycin resistance: (i) the use of an energy-dependent efflux, (ii) production of inactivating enzymes and (iii) alteration of 23S rRNA methylases.[2] On the other hand, tetracycline resistance is mediated by enzyme inactivation, ribosomal protection proteins and efflux proteins.[3]

Currently, there is no report on the distribution of erythromycin and tetracycline-resistance genes among MRSA strains in Malaysia. Thus, this study was aimed at determining the expression and transferability of erythromycin and tetracycline resistance of MRSA strains isolated from a local teaching hospital in Malaysia.

Materials and Methods

In this study, 188 MRSA strains including 162 from our previous study, which were resistant to erythromycin, ciprofloxacin or tetracycline, were further analysed. The remaining new strains were obtained from the year 2004 (n=9), 2007 (n=16) and 2008 (n=1).

Polymerase chain reaction (PCR) for the detection of resistance genes for erythromycin (*ermA, ermB, ermC* and *msrA*), tetracycline (*tetK, tetL, tetM, tetO* and *tetS*), β-lactams (*blaZ*) and tetracycline transposon-associated genes (*Tn916* and *Tn5801*) were carried out as previously described[4-7] using both genomic and plasmid DNA as PCR templates.

Plasmid DNA from 30 selected MRSA strains were electroporated into *S. aureus* ATCC29213 as previously described by Lannergard *et al*.[8] with minor modifications.

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Briefly, 40 µl of electrocompetent cells were mixed with 2–5 µg of plasmid DNA, transferred to a 1-mm electroporation cuvette and electroporated using a Bio-Rad gene pulser (Bio-Rad Laboratories, Hercules CA, USA). The gene pulser was set at 200 Ω resistance, 25 µF capacitance and 2.5 kV. Transformants were selected on Typtic Soy Agar plates supplemented with either erythromycin (50 µg/ml) or tetracycline (50 µg/ml).

The antimicrobial susceptibility of transformants to three antimicrobial agents [oxacillin (1 µg), tetracycline (30 µg) and erythromycin (15 µg) (Oxoid Ltd., Basingstoke, Hampshire, UK)] was determined by disc diffusion method.[9] The presence of erythromycin-resistance gene in transformants was detected by PCR as described earlier. Size determination of the plasmids extracted from transformants was carried out by digestion of plasmid DNA with *Eco*RI (Promega, Madison, WI, USA), and the products were separated in 0.8% agarose gel for 6 h at 90 V.

Statistica software (version 8.0, StatSoft, Inc., Tulsa OK, USA) was used for data analysis. Comparisons of certain variables were done by Fisher's exact test. The association between different resistance genes and their minimum inhibitory concentration (MIC) values were determined by Spearman's rank order correlation test and Kruskal–Wallis test. The letter "H" refers to Kruskal–Wallis test. P value <0.05 (two-tailed) was taken as the level of significance for Fisher' exact test and Kruskal–Wallis test. The breakpoints for association of resistance factors are defined as follows: Perfect association with R=1, no association with R=0 and inverse correlation with R=−1.

Results

All strains were resistant to oxacillin (MIC 4–512 µg/ml). The MIC for erythromycin, ciprofloxacin and tetracycline ranged from 0.25 to 256 µg/ml, 0.5 to 512 µg/ml and 0.5 to 256 µg/ml, respectively. The temporal changes in the MIC of four antimicrobials for MRSA are summarised in Table 1. There was a significant increase (P<0.05) of high-level erythromycin (128 and 256 µg/ml), medium- (16 µg/ml) to high-level tetracycline (256 µg/ml) and medium-level ciprofloxacin (64 µg/ml) resistant strains between the years 2003 and 2008.

Based on Spearman's rank correlation coefficient test, the correlation between erythromycin and ciprofloxacin resistance was observed (R=0.607, P<0.05). Similarly, correlations between erythromycin and tetracycline (R=0.1922, P<0.05), and ciprofloxacin and tetracycline (R=0.0795, P<0.05) were also observed. The values indicate that tetracycline-resistant strains most likely to show co-resistance towards ciprofloxacin and erythromycin. Similarly, these values also indicate that erythromycin-resistant strains were most likely to show co-resistance towards ciprofloxacin and tetracycline.

Established primers for detection of erythromycin-, tetracycline- and β-lactam-resistance genes were carried out on genomic DNA of 188 MRSA strains. The results showed all erythromycin-resistant strains harboured either *ermA, ermC* or *msrA* gene and tetracycline-resistant strains harboured either *tetK* or t*etM* gene. Specifically, *blaZ* gene was detected in all strains, whereas *ermA*, *tetM, ermC*, *tetK* and *msrA* specific amplicons were detected in 157 (84%), 92 (49%), 40 (21%), 39 (21%) and 4 (2%) strains, respectively. However, no amplicon was obtained with primers that were specific for the *ermB, tetL, tetO* and *tetS* genes.

Majority (78%) of tetracycline-resistant strains harboured *tetK* and *tetM* genes with MIC ranging from 16 to 64 µg/ml. Based on the Kruskal–Wallis test, no significant difference was found between the level of erythromycin resistance for 2008 strains and the presence of different types of erythromycin-resistance genes (H=5.29, df=2, P=0.071).

PCR amplifications using plasmid DNA as templates were carried out in parallel with additional 16S rRNA primers to preclude chromosomal DNA contamination. Results showed *ermC, tetK, tetM* and *blaZ* amplicons were detected with absence of 16S rRNA specific amplicon. This indicates that *ermC, tetK, tetM* and *blaZ* genes were plasmid-borne.

On the other hand, Tn5801-like transposon was detected in 78 *tetM*-positive strains, and no Tn916-like transposon was detected. Based on Spearman's rank correlation test, correlation between *tetM* and *Tn5801* was observed (R=0.8647, P<0.05).

Plasmid analyses showed that 176 (94%) MRSA strains harboured 1–12 plasmids with sizes ranging from 1.9 to 169 kb. Transformation studies were performed on 30 randomly selected MRSA strains that harboured the plasmids. Plasmid transfers for erythromycin and tetracycline resistance were done by electroporation with the recipient *S. aureus* ATCC29213. However, the transfer only yielded success in 6 out of 30 strains for erythromycin resistance and no transfer of tetracycline resistance was observed. Further analysis showed that all resultant transformants were resistant to erythromycin (MIC 50–256 µg/ml). All transformants were sensitive to tetracycline since no tetracycline-resistance phenotype was transferred (based on the disc diffusion test).

Only *ermC* gene in the parental MRSA strains was successfully transferred into recipient *S. aureus* ATCC29213, suggesting that this resistant determinant was likely plasmid encoded. Plasmids of sizes ranging from 2.5 to 89 kb were detected in six transformants. Identical *Eco*RI restriction profiles were obtained from two plasmids extracted from donor MRSA (MRSA0812-1

Table 1: MIC values of oxacillin-, erythromycin-, ciprofloxacin- and tetracycline-resistant MRSA strains from a tertiary teaching hospital at Kuala Lumpur, Malaysia

Year	MIC	2003–2004 *n* (%)	2007–2008 *n* (%)	Total (%)	*P* value	Resistance genes				
						Erythromycin			Tetracycline	
		n=61	*n*=127	*n*=188		*ermA*	*ermC*	*msrA*	*tetM*	*tetK*
Antibiotic						-	-	-	-	-
Oxacillin	4	6	11	17	0.79	-	-	-	-	-
	8	5	12	17	1	-	-	-	-	-
	16	3	13	16	0.27	-	-	-	-	-
	32	2	2	4	0.59	-	-	-	-	-
	64	5	10	15	1	-	-	-	-	-
	128	10	24	34	0.83	-	-	-	-	-
	256	29	50	79	0.34	-	-	-	-	-
	512	1	5	6	0.67	-	-	-	-	-
Total resistance		61 (100)	127 (100)	188 (100)	1	-	-	-	-	-
Ciprofloxacin	0.5	0	2	2	1	-	-	-	-	-
	1	2	7	9	0.75	-	-	-	-	-
	2	0	5	5	0.17	-	-	-	-	-
	8	8	12	20	0.45	-	-	-	-	-
	16	3	10	13	0.55	-	-	-	-	-
	32	4	8	12	1	-	-	-	-	-
	64	18	18	36	0.02*	-	-	-	-	-
	128	5	12	17	1	-	-	-	-	-
	256	21	46	67	0.87	-	-	-	-	-
	512	-	7	7	0.09	-	-	-	-	-
Total resistance		59 (97)	113 (89)	172 (91)	0.09	-	-	-	-	-
Erythromycin	0.25	0	1	1	1	0	0	0	0	0
	0.5	2	6	8	1	0	0	0	0	0
	4	0	1	1	1	0	0	0	0	0
	8	4	2	6	0.08	7	1	0	0	0
	16	0	5	5	0.17	5	0	0	0	0
	32	4	9	13	1	13	1	0	0	0
	64	13	19	32	0.30	29	3	1	0	0
	128	14	9	23	0.003*	20	5	0	0	0
	256	24	75	99	0.01*	83	30	3	0	0
Total resistance		59 (97)	119 (94)	178 (95)	0.50	-	-	-	-	-
Tetracycline	0.25	1	0	0	0.33	0	0	0	0	0
	0.5	4	0	4	0.10	0	0	0	0	0
	1	6	11	17	1	0	0	0	0	0
	2	11	14	25	0.25	0	0	0	0	0
	4	19	14	33	0.002*	0	0	0	0	0
	8	3	9	12	0.75	0	0	0	0	0
	16	6	30	36	0.01*	0	0	0	34	17
	32	4	17	21	0.43	0	0	0	21	8
	64	4	14	17	0.43	0	0	0	17	6
	128	3	7	10	1	0	0	0	10	4
	256	0	11	11	0.01*	0	0	0	10	4
	Total	17 (28)	78 (61)	95 (51)	0	-	-	-	-	-

*Indicative of significantly different ($P<0.05$); MIC: Minimum inhibitory concentration; MRSA: Methicillin-resistant *Staphylococcus aureus*

and MRSA0804-1) and their respective transformants. The other plasmids extracted from transformants were smaller than plasmids from the donors. All the transformants which carried *ermC* contained plasmids with sizes of~2.5 to 2.7 kb.

Discussion

In our previous report, high oxacillin-, erythromycin- and ciprofloxacin-resistance rates were observed in

162 MRSA strains based on the disc diffusion test only. Significant increase in tetracycline-resistance rate was also observed. Thus, we further extended the study to determine the association between MIC values and presence of resistance genes of erythromycin and tetracycline-resistant strains and its transferability.

The significant increase of high-level tetracycline- (MIC≥256 µg/ml) and erythromycin (MIC≥128 µg/ml)-resistant strains between 2003 and 2008 along with the co-resistance between erythromycin, ciprofloxacin and tetracycline is worrisome because choices of antimicrobial agents for treatment of life-threatening cases will be limited. Furthermore, use of tetracycline, ciprofloxacin and erythromycin is still very common in Malaysian hospitals for treatment of respiratory tract and other nosocomial infections.[10]

Erythromycin and tetracycline resistance is always attributed to the presence of resistance genes. In this study, the predominant resistance gene reported in erythromycin-resistant strains was *ermA*. This differs from the report by Spiliopoulou *et al*.[11] where most of their erythromycin-resistant strains harboured *ermC* gene. In addition, our results also showed that presence of different erythromycin-resistance genes (*ermA, ermC* and *msrA*) was not directly related to higher MIC values (based on Kruskal–Wallis test). For example, 31% of erythromycin-resistant strains that harboured *ermA* or *ermC* gene was associated with an MIC value of 8–64 µg/ml. However, this differs from the report by El-Madhy *et al*.[12] as all their *ermC*-positive strains displayed higher MIC values (MIC>1024 µg/ml). A majority of tetracycline-resistant strains harboured *tetM* (97%) followed by *tetK* (41%) gene. This is in contrast to reports by Jones *et al*.[13] and El-Madhy *et al*.[12] where *tetK* gene was the predominant gene in tetracycline-resistant strains. Spearman's rank correlation tests showed that a strain which harboured *tetK* gene also harboured *tetM* gene. This is in agreement with the report of Schmitz *et al*.,[14] as their MRSA strains also harbour both *tetK* and *tetM*. Schmitz *et al*.[14] also reported that strains with both *tetK* and *tetM* genes often display higher MIC values than strains containing a single gene. In contrast, our study showed that the combination of *tetK* and *tetM* genes did not display higher MIC values as over 78% of the strains that harboured both *tetK* and *tetM* together were associated with an MIC value of 16–64 µg/ml. De Vries *et al*.[8] reported that *tetM* gene is located on both Tn5801-like and Tn916-like transposons. Their finding coincided with our data as majority (85%) of the *tetM* gene reported here was located on Tn5801-like conjugative transposon. Moreover, result of Spearman's rank correlation test also established the correlation between *tetM* and *Tn5801*.

Transformation experiments showed that only *ermC* was transmissible. Although Monecke *et al*.[15] reported that *tetK* gene is plasmid-borne and transmissible, none of the *tetK* genes was transferable in this study. Similarly, no *tetM* gene was transferable. Discrepancy observed might be due to the inherent property of recipient *S. aureus* ATCC29213 as Schenk and Laddaga[16] reported that this strain ATCC29213 has lower transformation efficiency when compared to another recipient strain, RN4220.

Size of the plasmid carrying *ermC* gene reported in this study was similar to the plasmid size (2.5 kb) reported by Westh *et al*.[17] Based on the *Eco*R1 restriction profiles obtained, the size of plasmids that were isolated from the transformants (~2.5 to 81 kb) was slightly smaller than its donor (~2.5 to 89 kb). This was probably because the donor strains harboured more than a single type of plasmids.

In conclusion, tetracycline-resistant strains often showed co-resistance towards ciprofloxacin and erythromycin. The *ermA* and *tetM* were the predominant genes detected in erythromycin- and tetracycline-resistant strains, respectively. The presence of *ermA, ermC, tetM* and *tetK* genes was responsible for erythromycin and tetracycline resistance among Malaysian MRSA strains. Association of resistance genes (*ermC, tetM* and *tetK*) with mobile genetic elements possibly enhances the spread of resistant traits in MRSA. The persistence of erythromycin, ciprofloxacin and tetracycline resistance remains a problem in UMMC, and therefore good infection control procedures should be applied. This data set may act as a reference for monitoring erythromycin, tetracycline resistance among MRSA strains in Malaysia.

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